Oral Microbiology Immunology 2009: 24: 236–242
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Role of *Aggregatibacter actinomycetemcomitans* in glutathione catabolism

L. Chu[1], X. Xu[2], J. Su[1,*], L. Song[1], Y. Lai[1], Z. Dong[3], D. Cappelli[4]

Departments of [1]Orthodontics and [2]Periodontics, University of Texas Health Science Center, San Antonio, TX, [3]Department of Cellular Biology and Anatomy, Medical College of Georgia and Charlie Norwood VA Medical Center, Augusta, GA, [4]Department of Community Dentistry, University of Texas Health Science Center, San Antonio, TX, USA

*Present address: Capital Medical University, Beijing, China.

Chu L, Xu X, Su J, Song L, Lai Y, Dong Z, Cappelli D. Role of Aggregatibacter actinomycetemcomitans *in glutathione catabolism.*
Oral Microbiol Immunol 2009: 24: 236–242. © 2009 John Wiley & Sons A/S.

Introduction: Our previous studies demonstrated that three enzymes, γ-glutamyltransferase (GGT), cysteinylglycinase (CGase) and cystalysin, are required for the catabolism of glutathione to produce hydrogen sulfide (H_2S) in *Treponema denticola*. In this study, we examined glutathione catabolism in *Aggregatibacter actinomycetemcomitans*.
Methods: The GGT and CGase of *A. actinomycetemcomitans* were determined by biological methods and GGT was characterized using a molecular biological approach.
Results: *A. actinomycetemcomitans* showed GGT and CGase activity, but could not produce H_2S from glutathione. The addition of recombinant *T. denticola* cystalysin, an L-cysteine desulfhydrase, to whole cells of *A. actinomycetemcomitans* resulted in the production of H_2S from glutathione. Subsequently, we cloned *A. actinomycetemcomitans* GGT gene (*ggt*) and overexpressed the 63 kDa GGT protein. The recombinant *A. actinomycetemcomitans* GGT was purified and identified. The K_{cat}/K_m of the recombinant GGT from *N*-γ-L-glutamyl-4-nitroaniline as substrate was 31/μM/min. The activity of GGT was optimum at pH 6.9–7.1 and enhanced by thiol-containing compounds.
Conclusion: The results demonstrated that *A. actinomycetemcomitans* had GGT and CGase activities and that the GGT was characterized. The possible role of *A. actinomycetemcomitans* in glutathione metabolism and H_2S production from oral bacteria was discussed.

Key words: *Aggregatibacter actinomycetemcomitans*; glutathione catabolism; hydrogen sulfide production; oral bacteria; γ-glutamyltransferase

Lianrui Chu, Department of Orthodontics, University of Texas Health Science Center, San Antonio, TX 78229, USA
Tel.: +1 210 567 6269;
fax: +1 210 567 2614;
e-mail: chul@uthscsa.edu

Accepted for publication November 16, 2008

Hydrogen sulfide (H_2S), a malodorous and highly toxic compound (2, 10, 39), can be found in relatively high levels in periodontal pockets (28, 30) and may play a role in the initiation and progression of periodontitis. A number of reports from Carlsson's group described the production of this volatile sulfur compound from human serum proteins, L-cysteine, as well as glutathione by numerous oral bacteria (4, 5, 7, 29). Glutathione is present in host cells, especially white blood cells (3), and in gram-negative bacteria, including oral bacteria (14). Therefore, glutathione is considered to be a potential source for H_2S production in periodontal pockets.

Only a few species of oral bacteria (e.g. *Peptostreptococcus micros* and *Treponema denticola*) have the metabolic capabilities to produce H_2S from glutathione (4, 5, 8). Our laboratory delineated a three-step pathway for glutathione metabolism in *T. denticola* (8). Initially, glutathione is cleaved into cysteinylglycine (Cys-Gly) and glutamate by γ-glutamyltransferase (GGT), which is located on the membrane of *T. denticola* (9, 23). In the second step, Cys-Gly is degraded into glycine and L-cysteine by cysteinylglycinase (CGase) (13). Finally, L-cysteine is hydrolysed by cystalysin, an L-cysteine desulfhydrase releasing ammonia, pyruvate and H_2S (6, 11, 12, 21).

In this study, we determined the GGT and CGase activities of *A. actinomycetemcomitans*. We further identified, expressed and characterized the GGT of *A. actinomycetemcomitans*.

Materials and methods

Materials, bacterial strains and cultural conditions

Unless otherwise indicated, all chemicals and reagents were purchased from the

Sigma Chemical Company, St. Louis, MO. *Aggregatibacter actinomycetemcomitans* strains were either from the American Type Culture Collection (ATCC; Rockville, MD; HK1651, 33384 and 29523) or from clinical isolates JP-2 and C031. Other bacteria used in this study were *Porphyromonas gingivalis* strain W83, *Tannerella forsythia* (formerly *Bacteroides forsythus*) ATCC 43037 and *Eubacterium nodatum* ATCC 33099. All strains were cultured anaerobically in a Coy anaerobic chamber (5% CO_2, 10% H_2 and 85% N_2) on the surface of enriched Trypticase soy agar (ETSA) blood agar plates (40) supplemented with 5% rabbit serum. For all studies, *A. actinomycetemcomitans* strains were grown in a modified thioglycolate broth (33). *T. denticola* strain ATCC 35405 was grown in GM-1 medium. Plasmids pUC18 and pRSETA were used as vectors for cloning. *Escherichia coli* TB-1 and BL21 (DE3) were used as host strains for plasmids and routinely grown in Luria–Bertani (LB) broth or on LB agar plates supplemented with ampicillin (50 μg/ml) when appropriate.

Analysis of GGT activity

Preparation of bacterial cells

The bacterial cells were prepared by using the method described by Carlsson's group (7) with modifications. The colonies grown on the blood agar plate for 2–5 days were scraped off the surface and resuspended in 10 ml buffer solution containing 20 mM phosphate buffered normal saline (0.15 M NaCl, pH 7.2; PBS). The cells were washed once using PBS and the pellet cells were resuspended in the reaction mixture. The concentrations of bacterial proteins or purified protein were determined by BCA Protein Assay Reagent (Pierce, Rockford, IL) (20). The cells were diluted to the final concentration at 0.5 mg/ml PBS buffer.

Analysis of GGT activity from N-γ-L-*glutamyl-4-nitroaniline*

GGT activity was determined using a method described by Mäkinen and Mäkinen (23). Bacterial cells were suspended in a standard reaction buffer containing PBS buffer with 2 mM β_2-mercaptoethanol (2-ME). *N*-γ-L-glutamyl-4-nitroaniline (GNA), a substrate for GGT at concentrations as designed, was used in this analysis. After incubation of the mixture at 37°C for 60 min, the reaction was monitored in a cuvette at 25°C with a DU65-spectrophotometer (Beckman, Corona, CA) and the value of optical density was recorded. The increase in absorbance at 405 nm represented GGT activity. GGT activity was measured from the optical density value and calculated using the standard curve from completely hydrolysed GNA product by *T. denticola* GGT (9).

Analysis of Cys-Gly, glutamic acid/glutamine and glycine generated from glutathione degradation by A. actinomycetemcomitans *GGT or whole cells*

A concentration of 0.5 mg/ml whole cells or 2 μg/ml purified recombinant GGT was mixed with 1 mM glutathione (final concentration) in 20 mM Tris buffer (pH 7.2) containing 2 mM 2-ME. After 2 h of incubation at 37°C, the mixture was subjected to ultrafiltration using a 10-kDa cut-off Centricon concentrator. The resultant < 10 kDa fraction was collected and concentrated using a SpeedVac 100 concentrator for high-performance liquid chromatography (HPLC) analysis. HPLC analysis of amino acids was performed as described previously (9) using a 2690 Separations Module (Waters Co., Milford, MA). Sigma amino acid standards, water or experimental samples in 5 μl were analysed. Cys-Gly and glutamate were also used as standards to calculate levels of Cys-Gly and glutamate released from glutathione.

Analysis of L-cysteine as a CGase enzyme product

The amount of L-cysteine was measured as described by Gaitonde (15, 22). The standard reaction buffer for CGase assays was 50 mM Tris–HCl (pH 7.3) with 0.2 mM $MnCl_2$. *A. actinomycetemcomitans* whole cells were incubated in the reaction buffer with 2 mM Cys-Gly, unless another concentration is indicated, for 60 min at 37°C and reactions were stopped by the addition of 5% trichloroacetic acid. The absorbance at 560 nm was measured. L-cysteine concentrations were calculated from a standard curve with known amounts of L-cysteine, after subtracting a blank.

Analysis of H_2S, ammonia and pyruvate

H_2S production was determined using a method described by Siegel (32), except that the reaction mixture was prepared in a 1-ml volume by using 1.5-ml microcentrifuge tubes sealed with parafilm-wrapped caps as stoppers. The reaction was measured by adding 0.1 ml of 0.02 M *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.1 ml of 0.03 M $FeCl_3$ in 1.2 M HCl into the tubes. The absorbance at 630 nm was determined after developing the color for 20 min and removing the precipitate by centrifugation. The sulfide concentration was calculated based on a Na_2S standard curve. Pyruvate was analysed using the method described by Zheng et al. (44). Ammonia concentrations were determined using a method described by Bauer et al. (1). All analyses were carried out in triplicate unless otherwise indicated.

Cloning and sequencing of the *ggt* gene of *A. actinomycetemcomitans*

Gene amplification by polymerase chain reaction

We searched glutamyltranspeptidase against the *A. actinomycetemcomitans* HK1651 genome sequenced by Fores Z. Najar in University of Oklahoma (ftp://ftp.genome.ou.edu/pub/act/act_annotated_genome.txt) using the key word 'gamma-glutamyltransferase'. The entire genomic DNA sequence encoding GGT of *A. actinomycetemcomitans* HK1651 was found from the database (open reading frame AA01845 of the *A. actinomycetemcomitans* HK1651). A pair of primers (forward 5′-CCGCTCGAGATGAAGA AATTCACAACAAAATG and reverse 5′-GGGGTACCGAAAAAATAACCGCA TTTTAATG) were designed to amplify the full length of the DNA fragment of the open reading frame of *A. actinomycetemcomitans* GGT, and to put a *Xho*I site (forward) and *Kpn*I site (reverse) at the ends of the polymerase chain reaction (PCR) product. *A. actinomycetemcomitans* HK1651 or JP-2 genomic DNA was used separately as template to clone and sequence the *ggt* genes. An initial denaturing step (5 min at 94°C) was followed by 40 cycles of amplification (1 min at 94°C, 1 min at 48°C and 3 min at 72°C) in an Eppendorf Mastercycler (Eppendorf AG 22331, Hamburg, Germany).

Cloning and sequencing of the ggt *gene*

To clone the *ggt* gene of *A. actinomycetemcomitans* HK1651 or JP-2, the 1.8-kb PCR product was ligated into vector pRSETA (Invitrogen, San Diego, CA) via *Xho*I–*Kpn*1 digestion sites and the constructed plasmid was transformed into *E. coli* TB-1. The successfully constructed plasmid was then transferred into *E. coli* DL21 (DE3) and a colony was selected for expression of the *A. actinomycetemcomitans* GGT. The

insert containing the *ggt* gene of *A. actinomycetemcomitans* HK1651 or JP-2 was sequenced, independently from both strands, in the Center for Advanced DNA Technology at the University of Texas Health Science Center at San Antonio using a 3130 xl Genetic Analyzer (Applied Systems, Foster, CA).

Expression and purification of recombinant *A. actinomycetemcomitans* GGT

To overexpress the recombinant GGT from *A. actinomycetemcomitans*, a concentration (final) of 400 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the *E. coli* cells harboring the expression plasmid from *A. actinomycetemcomitans* HK1651 as described in a previous study (9). One liter of the bacterial culture was centrifuged at 6000 ***g*** for 5 min and the cells were washed once using 20 mM PBS (pH 7.4). The cells were then resuspended in 60 ml of PBS buffer and disrupted in a sonicator (Branson Sonifier 450; VWR Scientific, Danbury, CT) for 5 min on ice. Soluble fractions from the cell sonicate were obtained by centrifugation at 16,500 ***g*** for 30 min and adjusted to pH 8.0 using 20 mM imidazole in 0.5 M NaCl. Since the recombinant CGase has a 6-histidine tag, it was purified on a 2.5 ml Ni-NTA gel column according to the supplier's instructions (Qiagen, Chatsworth, CA). After loading 45 ml of the soluble cell solution onto the column, a step-gradient elution with imidazole (20–250 mM) was used to collect the purified recombinant GGT. The concentration of protein was determined by BCA protein assay.

Identification of the recombinant *A. actinomycetemcomitans* GGT

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis

A mini sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was used to identify the purified protein. The resolving gel consisted of 12% acrylamide in 0.125 M Tris–HCl (pH 8.8), and the stacking gel contained 4% acrylamide in 0.125 M (pH 6.8). The gel was stained using 0.025% Coomassie Brilliant Blue R-250.

Mass spectrometry identification of the purified recombinant protein with GGT activity

The purified recombinant *A. actinomycetemcomitans* GGT band on SDS gel stained by Coomassie Brilliant Blue was excised from the SDS gel. The recombinant GGT protein was identified, after digestion with trypsin, by capillary HPLC-electrospray ionization tandem mass spectrometry (37) in the University of Texas Health Science Center San Antonio Institutional Mass Spectrometry Laboratory. The resulting survey scan followed by acquisition of data-dependent collision-induced dissociation spectra were searched against the database (ftp://ftp.genome.ou.edu/pub/act/act_annotated_genome.txt).

Properties of the recombinant *A. actinomycetemcomitans* GGT

Determination of optimal pH

The effect of pH on CGase activity was determined in the standard reaction buffer, except the pH was varied from pH 5.0 to pH 9.5 in approximately 0.2–0.3 pH units.

Kinetic analysis of recombinant A. actinomycetemcomitans *GGT enzyme activity*

The kinetic analysis was performed by the addition of 5 μg/ml (final concentration) of the purified GGT to the reaction mixture containing 2 mM 2-ME and 20 mM Tris–HCl buffer (pH 7.2) with 0.01–4 mM GNA (final concentration). After 20 min of incubation at 37°C, the samples were cooled on ice and the increase of absorbance at 405 nm was monitored at room temperature (25°C) in a cuvette with a DU65-spectrophotometer (Beckman) (9). Enzyme activity was calculated based on the standard curve for 4-nitroaniline. The rate of enzyme reaction was expressed as mole per milligram protein per minute. V_{max}, K_m and K_{cat} were calculated as described previously (21).

Effects of various treatments on A. actinomycetemcomitans *GGT activity*

Glutathione (reduced form), Cys-Gly and L-cysteine and other selected compounds were used as potential substrates for the purified recombinant GGT. The GGT (2 μg/ml final concentration) was used to determine the effects of chemical and physical treatment by using GNA as the substrate. The activity of untreated GGT was used as a control (100%). Effects on protein of thiol compounds, proteinase inhibitors and selected treatments for GGT activity were examined for the evaluation of the enzyme activities.

Effects of thiol compounds

Six millimoles of each thiol compound, 2-ME, dithiothreitol and L-cysteine, was incubated with 4 μg/ml GTT at 37°C for 30 min. GNA at a concentration of 1 mM was added for another 60 min. GTT enzyme activity was determined using the protocol described previously in this section.

Effects of selected proteinases and their inhibitors

Proteinase K/pronase (100 μg/ml) and 2 mM of each proteinase inhibitor, sodium-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF) and benzamidine (BN) were added to 2 μg/ml (final concentration) *A. actinomycetemcomitans* GTT in reaction buffer. After incubation at 37°C for 30 min, 1 mM GNA was added for another 60 min. GTT enzyme activity was determined as described above.

Heating

A. actinomycetemcomitans GTT at a concentration of 2 μg/ml was heated at 37, 40, 42, 45, 50, 60 and 80°C for 30 min in reaction buffer (pH 7.2), and then cooled on ice to 4°C. The enzyme was incubated with 1 mM GNA for 30 min at 37°C and its activity was measured.

Contribution of recombinant *A. actinomycetemcomitans* GGT to H_2S production from glutathione

Recombinant *A. actinomycetemcomitans* GGT and cystalysin were purified from *E. coli* cells previously described (12). The reaction buffer contained the final concentration of each enzyme at 2 μg/ml, 0.2 mM $MnCl_2$ and 2 mM 2-ME in PBS at pH 7.2. The whole cells of *A. actinomycetemcomitans* were added to the reaction buffer to a final concentration of 0.5 mg protein/bacterium/ml. Glutathione was added (1 mM final concentration) and the reaction was allowed to proceed for 60 min at 37°C.

Distribution of GGT activity and presence of the *ggt* gene among various strains of *A. actinomycetemcomitans*

Based on the amino acid sequence of GGTs, different strains of other bacterial species such as *T. denticola*, *Salmonella* and *Shigella* spp. and *A. actinomycetemcomitans* HK1651 and JP-2 are identical at the N and C terminals (L. Chu, X. Xu, Z. Dong, D. Cappelli, JL. Ebersole, unpublished

data). Primers were designed dependent on the *A. actinomycetemcomitans ggt* gene sequence from HK1651 (forward: 5′-ATGAAGAAATTCACAACAAAATG-3′; reverse: 5′-ATACCCGACCGTTTTCCC-3′) and used to identify the presence of *ggt* genes in other strains of *A. actinomycetemcomitans*. The *A. actinomycetemcomitans* genomic DNAs were used as templates to identify the *ggt* genes using PCR. After an initial denaturing step (95°C for 5 min), the DNA was amplified through 40 cycles of amplification (95°C for 1 min; 58°C for 2 min; 72°C for 2 min). PCR products were resolved by electrophoresis on 0.7% agarose gels and stained with ethidium bromide. Genomic DNA was isolated and purified by the detergent-proteinase K lysis method (9).

Results

Analysis of GGT and CGase activities of *A. actinomycetemcomitans*

Previous reports indicate that only a few oral bacterial species have the capacity to degrade glutathione and so produce H_2S (5). *A. actinomycetemcomitans*, including strains HK1651 and JP-2, demonstrated significant GGT activity. To understand glutathione catabolism in *A. actinomycetemcomitans* HK1651 cells were examined for GGT, CGase and cystalysin activities. As seen in Fig. 1, HK1651 cells were able to catabolize or degrade GNA and Cys-Gly. In contrast, they could not use L-cysteine to generate H_2S. The results suggest that *A. actinomycetemcomitans* has significant GGT and CGase activities but does not have significant activities of cystalysin. The ability of the *A. actinomycetemcomitans* whole cells to hydrolyse GNA for GGT activity and hydrolyse Cys-Gly for CGase activity was compared. The native GGT had a K_m = 0.27 mM GNA and CGase had a K_m = 0.55 mM Cys-Gly.

Cloning and sequencing of the *A. actinomycetemcomitans ggt* gene

To understand glutathione catabolism in *A. actinomycetemcomitans*, we amplified the GGT DNA sequence of *A. actinomycetemcomitans* HK1651 using a PCR-based method. *A. actinomycetemcomitans* HK1651 and JP-2 genomic DNAs were used separately as templates for amplification. An approximate 1800-bp PCR product was obtained from each amplification, ligated into a plasmid pRSETA and the construct was transformed into *E. coli* BL21 (DE3) cells.

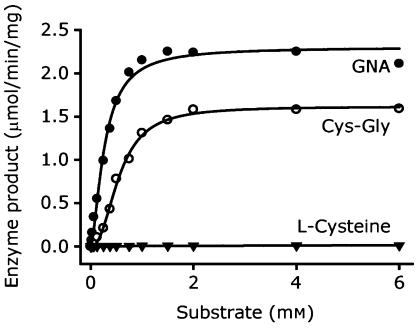

Fig. 1. γ-Glutamyltransferase, cysteinylglycinase (Cys-Gly) and cystalysin activities from *Aggregatibacter actinomycetemcomitans*. *N*-γ-L-glutamyl-4-nitroaniline (GNA), Cys-Gly, or L-cysteine was added to *A. actinomycetemcomitans*. GNA hydrolysis, L-cysteine production from Cys-Gly, or H_2S production from L-cysteine was determined to indicate the activity of γ-glutamyltransferase, cysteinylglycinase (CGase) or cystalysin activities. The results demonstrated significant GGT and CGase activities in *A. actinomycetemcomitans* whole cells, but no detectable cystalysin activity was found. Standard deviations were less 10% of means.

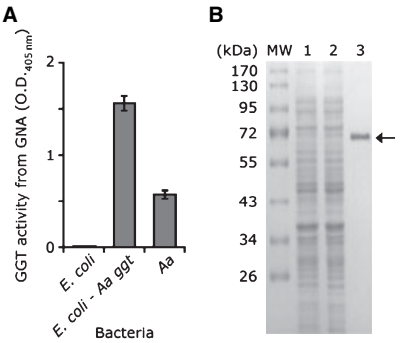

Fig. 2. Expression of *Aggregatibacter actinomycetemcomitans* γ-glutamyltransferase (GGT) activity from *Escherichia coli* (A) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the purified recombinant *A. actinomycetemcomitans* GGT (B). The transformants carrying pRSETA plasmid harboring the *ggt* gene from *A. actinomycetemcomitans* (*E. coli–A. actinomycetemcomitans ggt*) and native *A. actinomycetemcomitans* showed significant GGT activity; however, the native *E. coli* strain did not express detectable GGT activity (A). SDS–PAGE analysis demonstrated the purified recombinant *A. actinomycetemcomitans* GGT protein (B). Error bars indicate standard deviations (A).

A positive colony for each strain was selected and the plasmid harboring the *ggt* gene of *A. actinomycetemcomitans* was then isolated and sequenced. The open reading frame of the *A. actinomycetemcomitans* JP-2 *ggt* matched AA01845 of HK1651 containing 1767 bp encoding 589 amino acids (data not shown), which was identical to the GGT that was deduced from the *A. actinomycetemcomitans* genome of strain HK1651. The molecular mass of GGT from *A. actinomycetemcomitans* JP-2 is 63,161 Da and its isoelectric point is 6.87. The enzyme contains only one L-cysteine, 74 basic amino acids (46 Lys, 16 Arg, 12 His), and 54 acidic amino acids (34 Glu, 20 Gln). The *A. actinomycetemcomitans* HK1651 *ggt* gene was also cloned and sequenced. The amino acid sequence was identical to that in the database. Indeed, only two of the 589 amino acids of the whole GGT molecule were different from HK1651 (35-A and 545-T) and HK1651 (35-T and 545-A).

Expression and purification of recombinant *A. actinomycetemcomitans* GGT from *E. coli*

To understand the properties of GGT from *A. actinomycetemcomitans*, the positive recombinant colony containing the *A. actinomycetemcomitans ggt* gene from the HK1561 strain was selected for recombinant enzyme purification. As seen in Fig. 2(A), the native *E. coli* BL-21 (DE3) did not show detectable GGT activity after inoculation at 37°C, even when cultured with IPTG; however, the *E. coli* BL-21(DE3) containing the plasmid with the *ggt* gene from *A. actinomycetemcomitans* demonstrated significant GGT activity (Fig. 2A). Approximately 70% of GGT activity (measured using GNA as the substrate) existed in the soluble fraction of the cell after sonication and the protein was purified by affinity chromatography eluted with 140–210 mM imidazole. An 80-mM imidazole elution removed all the loosely bound proteins with no detectable enzyme activity and the last step of purification resulted in an approximately 120-fold purification of protein with a yield of 68% of the total enzyme activity in the soluble fraction. The purified recombinant GGT of *A. actinomycetemcomitans* was obtained with a molecular mass of approximately 66 kDa. The recombinant GGT was purified to homogeneity using SDS–PAGE (Fig. 2B).

Identification of recombinant *A. actinomycetemcomitans* GGT

To identify the purified recombinant protein, the protein was extracted from the gel, reduced, alkylated, digested with trypsin and the resulting peptides were subjected to mass spectrometry. The peptide map obtained from the recombinant protein band was identical to the predicted

63 kDa protein deduced from the genome at AA01845 of *A. actinomycetemcomitans* strain HK1651 (http://www.genome.ou.edu/act.html). Based on these results and the fact that the amino acid sequence of this protein was highly homologous to the other reported GGTs (18, 26, 35, 38, 45), we conclude that the 63 kDa protein is the GGT of *A. actinomycetemcomitans* encoded by the genome AA01845.

Determination of the enzymatic properties of *A. actinomycetemcomitans* GGT

The capacity of recombinant *A. actinomycetemcomitans* GGT to hydrolyse glutathione was examined. HPLC analysis detected two end-enzymatic products, glutamic acid and Cys-Gly (Fig. 3A). Similar molarities of glutamic acid and Cys-Gly were recovered from the reaction, which reflected the complete hydrolysis of glutathione because the absorbance of 1 mM glutathione disappeared after 2 h of incubation (data not shown). The enzymatic properties were then determined using the purified recombinant GGT. The optimal pH of the GGT enzyme reaction was between 6.9 and 7.1 and maximum enzymatic activity was observed at 42°C. The enzyme activity was increased between 1.5- and 2.1-fold by the addition of 2-ME and dithiothreitol but was reduced 95% using TLCK. However, PMSF and benzamidine did not significantly alter the activity of the enzyme (data not shown).

Kinetic analysis indicated that the K_m and K_{cat} of the GGT were 0.135 mM and 4.2×10^3/min, respectively by using GNA as the substrate (Fig. 3B). The K_{cat}/K_m of the recombinant GGT from GNA as substrate was 31/μM/min. Further enzymatic analysis of the purified *A. actinomycetemcomitans* GGT demonstrated that oxidized glutathione, Cys-Gly either in the oxidized or reduced form, and L-cysteine were not the substrates of the enzyme (data not shown), indicating that glutathione was the specific substrate of the *A. actinomycetemcomitans* GGT.

The addition of *T. denticola* cystalysin to whole *A. actinomycetemcomitans* resulted in significant H_2S production from glutathione. This suggests that *A. actinomycetemcomitans* CGase activity is important in glutathione catabolism by providing L-cysteine to bacteria, which can take up this amino acid and degrade it by cystalysin (Fig. 4).

Recently, we identified the *T. denticola* CGase gene from a database, which belongs to the same family as leucyl aminopeptidase in the M17 family (13). After searching the *A. actinomycetemcomitans* genome (ftp://ftp.genome.ou.edu/pub/act/act_annotated_genome.txt), there were two gene products at 1,198,597–1,200,009 and 497,580–496,690, which were identified as potential amino-peptidases from the *A. actinomycetemcomitans* genome. These gene products share 31–33% homology with the *T. denticola* CGase gene, suggesting that the two gene products from the *A. actinomycetemcomitans* genome may contribute to the CGase function detected in this study.

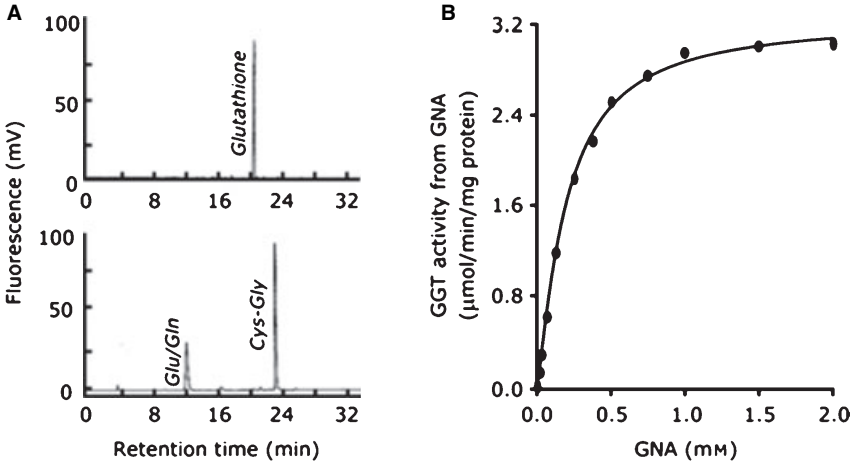

Fig. 3. Catalytic analysis of *Aggregatibacter actinomycetemcomitans* γ-glutamyl transferase (GGT) with glutathione as substrate by high-performance liquid chromatography (A) and enzymatic kinetics of recombinant *A. actinomycetemcomitans* GGT from *N*-γ-L-glutamyl-4-nitroaniline (GNA) as substrate (B). (A) shows a pre-reaction of glutathione (top) and two enzyme products, cysteinylglycine (Cys-Gly) and glutamic acid/glutamine (Glu/Gln), after reaction of glutathione with *A. actinomycetemcomitans* GGT. Kinetic analysis of recombinant *A. actinomycetemcomitans* GGT using GNA as a substrate was demonstrated in (B).

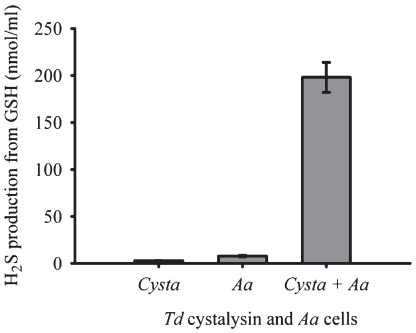

Fig. 4. Effect of *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* cystalysin on H_2S production with glutathione as substrate. The addition of *T. denticola* cystalysin (Cysta) enhanced the capability of *A. actinomycetemcomitans* (*Aa*) to produce H_2S, while *Aa* alone and cystalysin (Cysta) alone did not show significant activity. Error bars indicate standard deviations.

Distribution of GGT activity and gene (*ggt*) from various *A. actinomycetemcomitans* strains

All *A. actinomycetemcomitans* strains used in this study, including HK1651, 33384 and 29523 strains, JP-2 and clinical isolate C031, exhibited similarly strong GGT and CGase activities (data not shown). To determine whether similar GGT capacity is caused at the genetic level, we examined the distribution of the *ggt* gene in the various *A. actinomycetemcomitans* strains. To this end, PCR with primers designed according to the sequence of the *ggt* gene from HK1651 were amplified using an identical fragment (1.8 kb) from the genomic DNAs isolated from all of the *A. actinomycetemcomitans* strains including HK1651, ATCC 33384 and 29523 strains, as well as clinical isolate C031 (Fig. 5). As expected, no amplification was detected on the DNAs from the other bacteria, including *P. gingivalis* and *T. forsythia*. Therefore, the results suggested that all *A. actinomycetemcomitans* strains contain the *ggt* gene with a high identity to the genes from *A. actinomycetemcomitans* HK1651.

Discussion

A. actinomycetemcomitans, a gram-negative, facultative anaerobic bacterium, has been identified as an important periodontopathic bacterium (24, 25, 41). It is not only commonly regarded as a pathogen of local aggressive periodontal diseases (31, 36, 42), but it is also frequently recovered from sites that exhibit chronic periodontal disease (16, 34, 43). While several oral

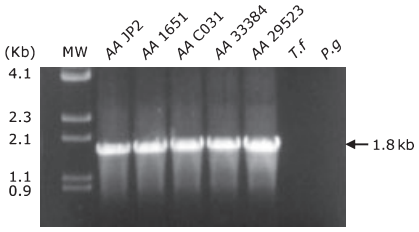

Fig. 5. Polymerase chain reaction (PCR) analysis of GGT in genomic DNA from oral bacteria. PCR primers were designed according to the sequence of the *Aggregatibacter actinomycetemcomitans* HK1651 gene. Lanes in the figure represent results for: *A. actinomycetemcomitans* (*AA*) ATCC and clinic isolate strains and *Porphyromonas gingivalis* (*P.g*) W83 and *Tannerella forsythia* (*T.f*) 43037 as controls. Only one band with an approximately 1.8-kilobase (kb) PCR fragment from each *A. actinomycetemcomitans* strain as template, but no band was observed from controls.

bacteria may be involved in H_2S production using different substrates (4, 5, 7, 29), *A. actinomycetemcomitans* was unable to produce H_2S from glutathione or L-cysteine. Therefore, unlike *T. denticola*, *A. actinomycetemcomitans* lacks some of the key enzymes required to catalyse glutathione to produce H_2S. This study examined glutathione catabolism and H_2S production in *A. actinomycetemcomitans*.

Carlsson et al. reported the capacities of oral bacteria to use three substrates, glutathione, Cys-Gly and L-cysteine for H_2S production (4, 5, 7, 29, 36). In our previous studies, three enzymes were identified in a three-step pathway in *T. denticola* (9, 12, 13). In this study, we characterized the GGT activity and also demonstrated CGase activity from *A. actinomycetemcomitans*, which can hydrolyse glutathione and Cys-Gly to produce glutamic acid, glycine and L-cysteine, but cannot convert glutathione into H_2S, ammonia and pyruvate. Interestingly, the combination of *A. actinomycetemcomitans* and *T. denticola* cystalysin could convert glutathione into H_2S, pyruvate and ammonia. Therefore, this study demonstrated the roles of *A. actinomycetemcomitans* on glutathione catabolism, suggesting that *A. actinomycetemcomitans* together with other oral bacteria (5, 7, 29) may produce H_2S from glutathione.

To confirm these findings, the *ggt* gene from *A. actinomycetemcomitans* HK1651 was cloned and sequenced; the recombinant GGT from *E. coli* was identified; and the GGT enzyme properties were characterized. In addition to GGT activity we also showed CGase activity in *A. actinomycetemcomitans*. The *A. actinomycetemcomitans* degraded glutathione and released Cys-Gly and L-cysteine. These products may be degraded by other bacteria, releasing H_2S and ammonia.

Compared with *T. denticola* GGT (molecular weight 26 kDa) (9, 23), the *A. actinomycetemcomitans* GGT has a larger molecular mass. *Helicobacter pylori*, *E. coli* and other pathogens were reported to contain *ggt* genes (18, 26, 35, 45), but *E. coli* exhibited lower levels of GGT activity than other organisms (17). While these bacteria express GGT activity under normal culture conditions (37°C), *E. coli* did not express significant GGT activity. The weak GGT activity in *E. coli* reflects the conditional expression at lower temperatures (approximately 25°C) (17, 35). *A. actinomycetemcomitans* GGT shares a much greater homology (54–59%) to the GGTs from gastrointestinal pathogens, *Salmonella typhimurium* LT2, *Shigella flexneri* and *H. pylori*, but a lesser homology (29–33%) to the GGTs from both human and pig (19, 27, 38).

The present study indicates that glutathione catabolism may be achieved in the periodontal pockets by a combination of different species. For example, one bacterium, like *A. actinomycetemcomitans* as hypothesized in Fig. 6, provides GGT and/or CGase while another bacterium, such as *Peptostreptococcus micros*, *Fusobacterum* whole cells (4, 5, 7), *P. gingivalis* extract (29), *T. forsythia* whole cells (29), *T. denticola* (12, 13) whole cells and enzymes and some others (5, 29), make CGase and/or cystalysin or CGase (4, 5, 12, 29). As a result, all three enzymes required for glutathione catabolism are produced by different bacteria and present in the periodontal pocket to produce H_2S. Our current study only provides limited evidence that different species of bacteria may collaborate to use glutathione to produce H_2S. Of note, the transporter systems for glutathione, Cys-Gly, and L-cysteine in oral bacteria are complex and big differences in affinity exist for these substrates among the various bacterial species (4, 5, 36). Therefore, the observed differences in substrate catabolism/degradation in various bacteria may also be caused by differences in substrate transport. Further research should determine whether the catabolism defect(s) in a specific bacterial species are the result of the absence of a critical enzyme(s) or of failed substrate transport. In addition, further studies should examine the interdependence among the various bacterial species in terms of glutathione utilization.

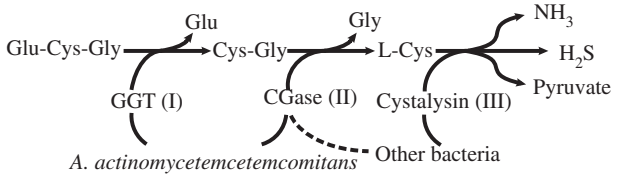

Fig. 6. Hypothetical model for role of *Aggregatibacter actinomycetemcomitans* on glutathione catabolism and H_2S production from glutathione. GGT, γ-glutamyltransferase; CGase, cysteinylglycinase; Cys-Gly, cysteinylglycine; L-Cys, L-cysteine; Cystalysin, an L-cysteine desulfhydrase. (I), (II) and (III) represent each step of the three-step pathway. *A. actinomycetemcomitans* GGT and CGase activities are hypothesized to play a potential role in glutathione catabolism and H_2S production in oral bacteria was outlined in this model. Other bacteria in the figure may provide cystalysin (activities), which include *Peptostreptococcus micros*, *Fusobacterum* whole cells (4, 5), *Porphyromonas gingivalis* extract (29), *Tannerella forsythia* whole cells (29), *Treponema denticola* (12, 13) whole cells and enzymes and some others (4, 29).

Acknowledgments

This study was supported by National Institutes of Health grant DE-13819. We are grateful to Steven L. Mouton for his scientific input in HPLC analyses and the Mass Spectrometry Laboratory for their technical assistance. We thank Drs. David Kolotrubetz and Howard Kuramitsu for their collaboration and criticism of this work. We also thank Dr. Kuramitsu and Dr. John Rugh for reviewing this manuscript.

References

1. Bauer JP, Ackermann PG, Toro G. Clinic laboratory methods. St Louis, MO: C. V. Company, 1974: 399–401.
2. Beauchamp RO Jr, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA. A critical review of the literature on hydrogen sulfide toxicity. CRC Crit Rev Toxicol 1984: **13**: 25–97.
3. Beilzer M, Lauterburg BH. Glutathione metabolism in activated human neutrophils: stimulation of glutathione synthesis and consumption of glutathione by reactive oxygen species. Eur J Clin Invest 1991: **21**: 316–322.
4. Carlsson J, Larsen JT, Edlund MB. *Peptostreptococcus micros* has a uniquely high capacity to form hydrogen sulfide from glutathione. Oral Microbiol Immunol 1993: **8**: 42–45.

5. Carlsson J, Larsen JT, Edlund MB. Utilization of glutathione (L-gamma-glutamyl-L-cysteinylglycine) by *Fusobacterium nucleatum* subspecies. Oral Microbiol Immunol 1994: **9**: 297–300.
6. Cellini B, Bertoldi M, Borri Voltattorni C. *Treponema denticola* cystalysin catalyzes beta-desulfination of L-cysteine sulfinic acid and beta-decarboxylation of L-aspartate and oxalacetat. FEBS Lett 2003: **554**: 306–310.
7. Claesson R, Edlund MB, Persson S, Carlsson J. The production of volatile sulfur compounds by various *Fusobacterium* species. Oral Microbiol Immunol 1990: **5**: 137–142.
8. Chu L, Dong Z, Xu X, Cochran DL, Ebersole JL. Role of glutathione metabolism of *Treponema denticola* in bacterial growth and virulence expression. Infect Immun 2002: **70**: 1113–1120.
9. Chu L, Xu X, Dong Z, Cappelli D, Ebersole JL. Role for recombinant γ-glutamyltransferase from *Treponema denticola* in glutathione metabolism. Infect Immun 2003: **71**: 335–342.
10. Chu L, Ebersole JL, Holt SC. Hemoxidation and binding of the 46-kDa cystalysin of *Treponema denticola* leads to an L-cysteine-dependent hemolysis of human erythrocytes. Oral Microbiol Immunol 1999: **14**: 293–303.
11. Chu L, Ebersole JL, Kurzben GP, Holt SC. Cystalysin, a 46-kDa L-cysteine desulfhydrase from *Treponema denticola*: biochemical and biophysical characterization. Clin Infect Dis 1999: **28**: 442–450.
12. Chu L, Ebersole JL, Kurzben GP, Holt SC. Cystalysin, a 46-kilodalton cysteine desulfhydrase from *Treponema denticola*, with hemolytic and hemoxidative activities. Infect Immun 1997: **65**: 3231–3238.
13. Chu L, Xu X, Lai Y et al. A 52-kDa leucyl aminopeptidase from *Treponema denticola* is a cysteinylglycinase that mediates the second step of glutathione metabolism. J Biol Chem 2008: **283**: 19351–19358.
14. Fahey CR, Brown WC, Adams WB, Worsham MB. Occurrence of glutathione in bacteria. J Bacteriol 1978: **133**: 1126–1129.
15. Gaitonde MK. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem J 1967: **104**: 627–633.
16. Haase EM, Bonstein T, Palmer RJ, Scannapieco FA. Environmental influences on *Actinobacillus actinomycetemcomitans* biofilm formation. Arch Oral Biol 2006: **51**: 299–314.
17. Hashimoto W, Suzuki H, Yamamoto K, Kumagai H. Analysis of low temperature inducible mechanism of gamma-glutamyltranspeptidase of *Escherichia coli* K-12. Biosci Biotechnol Biochem 1997: **61**: 34–39.
18. Ishiye M, Yamashita M, Niwa M. Molecular cloning of the gamma-glutamyltranspeptidase gene from a *Pseudomonas* strain. Biotechnol Prog 1993: **9**: 323–331.
19. Jin Q, Yuan Z, Xu J et al. Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. Nucleic Acids Res 2002: **30**: 4432–4441.
20. Kennell W, Holt SC. Comparative studies of the outer membranes of *Bacteroides gingivalis*, strains ATCC 33277, W50, W83, 381. Oral Microbiol Immunol 1990: **5**: 121–130.
21. Kurzben GP, Chu L, Ebersole JL, Holt SC. Sulfhemoglobin formation in human erythrocytes by cystalysin, an L-cysteine desulfhydrase from *Treponema denticola*. Oral Microbiol Immunol 1999: **14**: 153–164.
22. Lai Y, Chu L. A novel mechanism for aerobic growth of anaerobic *Treponema denticola*. Appl Environ Microbiol 2008: **74**: 73–79.
23. Mäkinen PL, Mäkinen KK. Gamma-glutamyltransferase from the outer cell envelope of *Treponema denticola* ATCC 35405. Infect Immun 1997: **65**: 685–691.
24. Meyyer DH, Fiver-Taylor PM. The role of *Actinobacillus actinomycetemcomitans* in the pathogenesis of periodontal disease. Trends Microbiol 1997: **5**: 224–228.
25. Mombelli A, Gmur R, Gobbi C, Lang NP. *Actinobacillus actinomycetemcomitans* in adult periodontitis. II. Characterization of isolated strains and effect of mechanical periodontal treatment. J Periodontol 1994: **65**: 820–826; 827–834.
26. Nakayama R, Kumagai H, Tochikura T. Purification and proterties of γ-glutamyltransferase from *Proteus mirabilis*. J Bacteriol 1984: **160**: 341–346.
27. McClelland M, Sanderson KE, Spieth J et al. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 2001: **413**: 852–856.
28. Persson S. Hydrogen sulfide and methyl mercaptan in periodontal pockets. Oral Microbiol Immunol 1992: **7**: 378–379.
29. Persson S, Edlund MB, Claesson R, Carlsson J. The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. Oral Microl Immunol 1990: **5**: 195–201.
30. Rizzo AA. The possible role of hydrogen sulfide in human periodontal disease. I. Hydrogen sulfide production in periodontal pockets. Periodontics 1967: **5**: 233–236.
31. Schacher B, Baron F, Rossberg M, Wohlfeil M, Arndt R, Eickholz P. *Aggregatibacter actinomycetemcomitans* as indicator for aggressive periodontitis by two analysing strategies. J Clin Periodontol 2007: **34**: 566–573.
32. Siegel LM. A direct microdetermination for sulfide. Anal Biochem 1965: **11**: 126–132.
33. Spitznagel J Jr, Kraig E, Kolodrubetz D. Regulation of leukotoxin in leukotoxic and nonleukotoxic strains of *Actinobacillus actinomycetemcomitans*. Infect Immun 1991: **59**: 1394–1401.
34. Slots J. Update on *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease. J Int Acad Periodontol 1999: **1**: 121–126.
35. Suzuki H, Kumagai H, Echigo T, Tochikura T. DNA sequence of the *Escherichia coli* K-12 gamma-glutamyltranspeptidase gene, *ggt*. J Bacteriol 1989: **171**: 5169–5172.
36. Tang-Larsen J, Claesson R, Edlund MB, Carlsson J. Competition for peptides and amino acids among periodontal bacteria. J Periodontal Res 1995: **30**: 390–395.
37. Thomas JA, Hardies SC, Rolando M et al. Complete genomic sequence and mass spectrometric analysis of highly diverse, atypical *Bacillus thuringiensis* phage 0305phi8-36. Virology 2007: **368**: 405–421.
38. Tomb JF, White O, Kerlavage AR et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature 1997: **388**: 539–547.
39. US National Research Council. Hydrogen sulfide. Baltimore, MD: University Park Press, 1979: 10–50.
40. Williams GD, Holt SC. Characteristics of the outer membrane of selected oral *Bacteroides* species. Can J Microbiol 1985: **31**: 238–250.
41. Wilson M, Henderson B. Virulence factors of *Actinobacillus actinomycetemcomitans* relevant to the pathogenesis of inflammatory periodontal diseases. FEMS Microbiol Rev 1995: **17**: 365–379.
42. Yang HW, Asikainen S, Doğan B, Suda R, Lai CH. Relationship of *Actinobacillus actinomycetemcomitans* serotype b to aggressive periodontitis: frequency in pure cultured isolates. J Periodontol 2004: **75**: 592–599.
43. Yang HW, Huang YF, Chan Y, Chou MY. Relationship of *Actinobacillus actinomycetemcomitans* serotypes to periodontal condition: prevalence and proportions in subgingival plaque. Eur J Oral Sci 2005: **113**: 28–33.
44. Zheng L, White RH, Cash VL, Jack RF, Dean DR. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci U S A 1993: **90**: 2754–2758.
45. Xu K, Strauch MA. Identification, sequence, and expression of the gene encoding γ-glutamyltranspeptidase in *Bacillus subtilis*. J Bacteriol 1996: **178**: 4319–4322.