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Role of Glutathione Metabolism of *Treponema denticola* in Bacterial Growth and Virulence Expression

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Hydrogen sulfide (H_2S) is a major metabolic end product detected in deep periodontal pockets that is produced by resident periodontopathic microbiota associated with the progression of periodontitis. *Treponema denticola*, a member of the subgingival biofilm at disease sites, produces cystalysin, an enzyme that catabolizes cysteine, releasing H_2S. The metabolic pathway leading to H_2S formation in periodontal pockets has not been determined. We used a variety of thiol compounds as substrates for *T. denticola* to produce H_2S. Our results indicate that glutathione, a readily available thiol source in periodontal pockets, is a suitable substrate for H_2S production by this microorganism. In addition to H_2S, glutamate, glycine, ammonia, and pyruvate were metabolic end products of metabolism of glutathione. Cysteinyl glycine (Cys-Gly) was also catabolized by the bacteria, yielding glycine, H_2S, ammonia, and pyruvate. However, purified cystalysin could not catalyze glutathione and Cys-Gly degradation in vitro. Moreover, the enzymatic activity(ies) in *T. denticola* responsible for glutathione breakdown was inactivated by trypsin or proteinase K, by heating (56°C) and freezing (−20°C), by sonication, and by exposure to *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). These treatments had no effect on degradation of cysteine by the purified enzyme. In this study we delineated an enzymatic pathway for glutathione metabolism in the oral spirochete *T. denticola*; our results suggest that glutathione metabolism plays a role in bacterial nutrition and potential virulence expression.

Treponema denticola is a predominant cultivable spirochete found in the gingival crevice and has been implicated in the development of the subgingival ecology of periodontal pockets (18, 33, 41, 43). A number of studies have shown that there is a relationship between the emergence of oral treponemes and the transition from health to periodontitis (21, 30, 39, 47). It has also been proposed that *T. denticola* belongs to Socransky's Red complex, which may be related to biofilm virulence (21). While it has been shown in vitro that *T. denticola* produces multiple potential virulence factors (16, 17, 20, 25, 26, 35, 44), the exact role or activity of these factors in the in vivo environment remains to be determined. One of these factors, the production of volatile sulfur compounds, including hydrogen sulfide (H_2S), could contribute to pathogenic changes in the host tissues (16). High levels of H_2S (up to 2 mM) have been detected in infected periodontal pockets, while low levels have been detected in clinically healthy sites (22, 34, 36, 42). In vitro, H_2S has been shown to be cytotoxic for a variety of host cells, including gingival fibroblasts and epithelial cells (4, 10, 40, 48).

Several reports have described H_2S formation from metabolism of human serum proteins, cysteine, and glutathione by oral bacteria (6, 37, 38). Despite these observations, the metabolic pathways leading to H_2S production from glutathione by oral bacteria have not been delineated. Previously, we identified a 46-kDa protein, cystalysin, in *T. denticola* (7, 8, 9). This 46-kDa protein participates in the degradation of L-cysteine and the production of H_2S, pyruvate, and ammonia. Cystalysin also participates in the destruction of red blood cells, exhibiting hemoxidative and hemolytic activities (8, 10, 11, 23, 24). In the present study, we screened substrates for H_2S production by *T. denticola*, as well as by purified cystalysin. Our results show that whereas the bacterium is able to metabolize the peptides glutathione and cysteinyl glycine (Cys-Gly), purified recombinant cystalysin can use only cysteine as a substrate for H_2S production. Analysis of the end products of glutathione metabolism in *T. denticola* suggested that there is a metabolic pathway consisting of multiple steps and that the last step of cysteine degradation is catalyzed by cystalysin.

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MATERIALS AND METHODS

Materials. Recombinant cystalysin was produced and purified as described previously (11). Unless otherwise indicated, all chemicals and reagents were obtained from Sigma Chemical Company, St. Louis, Mo.

Bacterial strains and culture conditions. *T. denticola* ATCC 35404 (= TD-4), ATCC 35405 (= TD-5), ATCC 33520, GM-1, and MS25 (50), *Treponema pectinovorum* ATCC 33768, *Treponema vincentii* ATCC 35580, and *Treponema socranskii* ATCC 35536 were cultured anaerobically in a Coy anaerobic chamber (5% CO_2, 10% H_2, 85% N_2) in GM-1 medium (50). The bacteria were inoculated at a ratio of 1/10 into the medium. After 2 days of growth, the optical density at 660 nm (OD_{660}) was approximately 0.26, and the culture was used as the inoculum for the next culture. Since the initial experiments showed that the responses of different strains of *T. denticola* were similar, ATCC 35405 was used for the majority of the analyses.

Measurement of bacterial growth. To determine the effects of various chemicals on bacterial growth, 2-day cultures were added to 5 ml of basic GM-1 medium (GM-1 broth without cysteine) at a starting concentration of approximately 5×10^8 cells/ml. Each of the chemicals was added at a final concentration of 6 mM to the broth. The cultures were incubated anaerobically at 37°C, and the OD_{660} was determined at different times to monitor the growth of bacteria.

Enzymatic reaction catalyzed by cystalysin. Purified recombinant cystalysin was added at a concentration of 1 or 4 μg/ml to reaction buffer consisting of cold phosphate-buffered saline (PBS) and substrates at a concentration of 0.5 or 2 mM. The reaction mixtures were incubated for 1 h at 37°C. End products of the

enzymatic reactions were analyzed by chemical methods or high-performance liquid chromatography (HPLC).

Chemical analysis. All analyses were carried out in triplicate unless otherwise indicated. H_2S was quantified by a method modified from the method of Siegel (46); the method has been described previously (11). Pyruvate was analyzed by the method described by Zheng et al. (51), with minor modifications described previously (11). Ammonia contents were determined by a method modified from the method of Bauer et al. (3). Reagent I (1% [vol/vol] phenol and 0.05% [wt/vol] sodium nitroprussic acid in distilled H_2O) and reagent II (0.5% [wt/vol] NaOH, 16% [wt/vol] $Na_2HPO_4 \cdot H_2O$, and 0.49% [wt/vol] sodium hypochloride in distilled H_2O) were prepared fresh prior to use; 0.2 ml of an ultrafiltration reaction mixture (10) was added to 1 ml of reagent I and 1 ml of reagent II in a 10-ml glass test tube, and the preparation was mixed thoroughly by vortexing, sealed with Parafilm, and incubated in a 37°C water bath for 30 min. The absorbance at 620 nm was determined, and the concentration of ammonia in the filtrate was calculated by using a standard curve constructed with ammonium sulfate.

Catabolism of substrates by *T. denticola* and HPLC analysis of amino acids. To determine the amino acids released from catabolism of glutathione and Cys-Gly by the treponeme, *T. denticola* ATCC 35405 was harvested from 2-day cultures by centrifugation for 10 min at 6,000 × *g* and 4°C. The cell pellet was washed twice with ice-cold 10 mM PBS (pH 7.4) and resuspended in distilled water to an OD_{660} of 3.0. Substrates to be tested were dissolved in water and added to *T. denticola* suspensions at a concentration of 6 mM. Each mixture was gently vortexed and incubated for 1 h at 37°C. The supernatants were separated by ultrafiltration with a Centricon filter (Millpore Corp., Bedford, Mass.) with a 10-kDa cutoff, and the resultant <10-kDa fractions were subsequently concentrated with a SpeedVac sc100 (Savant Co., New York, N.Y.) and used for HPLC analysis.

HPLC analysis of amino acids was performed with a 2690 Separations Module (Waters Co.). Samples to be analyzed were not hydrolyzed prior to derivatization. The samples (5 μl) were placed in clean, 1.5-ml microcentrifuge tubes and dried in a SpeedVac concentrator. Identical microcentrifuge tubes containing 5 μl of a Sigma amino acid standard or 5 μl of water were also dried. AccQ-Fluor borate buffer (Waters Co.) was added to each tube, the contents were mixed by vortexing for 30 s, the AccQ-Fluor reagent was added, the preparation was vortexed for an additional 30 s, and the sample was placed into a 55°C heating block for 15 min. The samples, 100 pM standard, and blank were transferred to the Waters 2690 Separations Module for analysis.

Kinetic analysis of metabolite absorption by *T. denticola*. Bacteria from 2-day cultures were used to perform a kinetic analysis of metabolite absorption by *T. denticola*. Metabolic end products, including H_2S, NH_3, pyruvate, glutamic acid, and glycine, were added separately at a concentration of 4 mM to 2×10^{10} *T. denticola* cells, and the preparations were incubated at 37°C. Samples were collected after 15, 30, 60, or 90 min of incubation, and the levels of each end product remaining in the incubation media were determined. Loss of specific products from the media was considered an indication that the products were absorbed by the bacteria.

Hemoxidation and hemolysis assays. Hemoxidation activity was determined with sheep red blood cells by using the method of Leahy and Smith (27). Hemolysis activity was determined as described previously (7).

Characterization of enzymatic activities in *T. denticola* that produce H_2S from glutathione, Cys-Gly, and cysteine. *T. denticola* cells and cellular extracts were examined for metabolism of glutathione (reduced form), Cys-Gly, and L-cysteine. The *T. denticola* cells were harvested from 1-day cultures by centrifugation at 6,000 × *g* for 10 min and then washed once with PBS. To prepare cellular extracts, the bacterial cells were lysed in 1% Triton X-100 in PBS at room temperature for 20 min, and the supernatants (soluble lysates) were collected after 5 min of centrifugation at 14,000 × *g*. Production of H_2S in reactions was determined by chemical methods as described above.

Effects of proteinase inhibitors and proteinases. The proteinase inhibitors *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (2 mM), phenylmethylsulfonyl fluoride (2 mM), and benzamidine (2 mM), proteinase K (100 μg/ml), or trypsin (100 μg/ml) was added to Triton X-100 lysates of *T. denticola*. After incubation at 37°C for 30 min, a substrate (glutathione, Cys-Gly, or cysteine) was added to each mixture at a concentration of 0.5 mM, and the preparation was incubated for another 60 min. H_2S production was determined. Cyanoborohydride is an inactivator of cystalysin (24), and β-chloroalanine is a substrate for this enzyme and was used as a competitive inhibitor (24). These chemicals were each added at a concentration of 10 mM to a *T. denticola* lysate (1 mg of protein per ml) with a substrate (glutathione, Cys-Gly, or cysteine), and the preparation was incubated for 30 min at 37°C. H_2S production was determined as described above.

Effects of selected treatments. For heating, *T. denticola* (2×10^9 cells/ml) was heated at 56°C for 30 min in PBS and then cooled on ice. The cells were separately incubated with substrates (1 mM glutathione, 1 mM Cys-Gly, or 1 mM cysteine) for 60 min at 37°C, and H_2S production in each suspension was determined. For freezing, *T. denticola* (2×10^9 cells/ml) was incubated overnight at −20°C. After the bacteria were thawed at room temperature, they were incubated with substrates, and H_2S production was determined. For sonication, *T. denticola* (2×10^9 cells/ml) was sonicated for 5 min on ice, and the resultant mixture was used to determine H_2S formation in the presence of different substrates.

Immunodepletion of cystalysin from *T. denticola* lysate. Rabbit antiserum to cystalysin was prepared as previously described (8). Sepharose 4B (3 g [wet weight]; Pharmacia) was activated with 1 g of cyanogen bromide (13). Rabbit anti-cystalysin immunoglobulin G (100 mg) was coupled with the activated Sepharose 4B and resuspended in 6 ml of distilled H_2O. For immunodepletion, 1×10^{10} *T. denticola* cells were lysed with 1% Triton X-100 in PBS containing 10 mM 2-mercaptoethanol (2-ME). The lysate was collected by centrifugation at 12,000 × *g* for 5 min. One milliliter of the lysate was combined with 0.3 ml of immunoglobulin G-Sepharose 4B, and the preparation was mixed gently by shaking at 20°C for 30 min. After incubation, the supernatant was collected and used for analysis of H_2S production in the presence of various substrates. Bacterial lysates that were not exposed to anti-cystalysin serum were used as controls. The residual cystalysin after immunodepletion was barely detectable by immunoblot analysis (data not shown).

Statistical analysis. One-way analysis of variance and the Student-Newman-Keuls test were used to examine differences between groups of data. The level of statistical significance was $P < 0.05$.

RESULTS

Screening of substrates for H_2S production in *T. denticola*. The effects of various thiol compounds on H_2S production in *T. denticola* ATCC 35405 are shown in Fig. 1. Nine chemicals were tested, and incubation of whole cells with cysteine, glutathione (reduced form), and Cys-Gly resulted in production of significantly more H_2S than incubation in S-deficient medium (basic GM-1 medium without cysteine). In the presence of appropriate thiol substrates, H_2S production was detected in *T. denticola* cells, as well as culture supernatants (Fig. 1). When methionine, cystathionine, cysteamine, 2-ME, dithiothreitol (DTT), and sodium sulfate were used, essentially no H_2S was obtained with whole cells or culture supernatants. Interestingly, while glutathione (reduced form) and Cys-Gly (reduced or oxidized form) were used as substrates by *T. denticola* to generate H_2S, the oxidized form of glutathione had no effect. Identical results were obtained with *T. denticola* ATCC 35404, ATCC 35405, and ATCC 33520 and clinic isolates GM-1 and MS25 (data not shown). By contrast, *T. pectinovorum*, *T. vincentii*, and *T. socranskii* were not able to produce H_2S from glutathione (reduced form) and Cys-Gly under the conditions tested in this study. In the presence of 1 mM glutathione or 1 mM cysteine, *T. pectinovorum* ATCC 33768, *T. vincentii* ATCC 35580, and *T. socranskii* generated less than 0.02 nmol of H_2S per ml, compared to the >0.4 nmol of H_2S per ml produced by the *T. denticola* strains (data not shown).

Substrate specificity of *T. denticola* and cystalysin. In previous studies (11, 24), we described a 46-kDa protein which catalyzes the production of H_2S from the substrate cysteine. This protein, cystalysin, appears to play a pivotal role in H_2S production in *T. denticola*. Since *T. denticola* can utilize glutathione to generate H_2S (Fig. 1), we determined the substrate specificity of *T. denticola* and cystalysin with a variety of thiol compounds. To do this, we first analyzed the end products of enzymatic pathways, including H_2S, ammonia, and pyruvate. As Table 1 shows, *T. denticola* generated H_2S in the presence of cysteine, Cys-Gly, or glutathione (reduced form). Ammonia

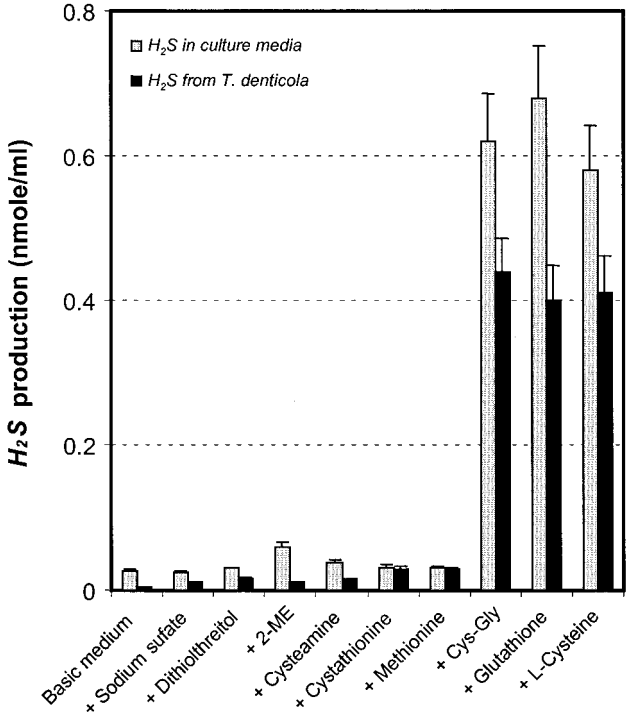

FIG. 1. H_2S production by *T. denticola* in the presence of thiol compounds. Solid bars, H_2S production by *T. denticola* incubated for 30 min with thiol compounds; grey bars, H_2S contents of supernatants of 2-day *T. denticola* cultures grown in the presence of various thiol compounds. H_2S formation was determined by chemical analysis as described in Materials and Methods. As determined by statistical analysis, the differences between the control (Basic medium) and cultures grown with Cys-Gly, glutathione, or L-cysteine were significant ($P < 0.05$); the differences between the control and the other groups were not significant.

and pyruvate were also produced. While the oxidized form of Cys-Gly was a suitable substrate for production of the three end products in *T. denticola*, the oxidized form of glutathione was not. Interestingly, in the presence of cystathionine, *T. denticola* produced ammonia and pyruvate but did not produce H_2S. Cystathionine consists of cysteine and homocysteine, and the enzyme products obtained with cystalysin should be homocysteine, pyruvate, and ammonia (10). Consistent with the results reported for cystalysin (10, 24), *T. denticola* was not able to metabolize methionine, 2-ME, DTT, Cys-Leu, Glu-Cys, homocysteine, and other compounds. However, purified cystalysin displayed a specific substrate preference for L-cysteine and cystine (Table 1); neither glutathione nor Cys-Gly was a suitable substrate for this enzyme.

Hemoxidative and hemolytic activities of *T. denticola* in the presence of glutathione and Cys-Gly. When *T. denticola* was cultured with glutathione and Cys-Gly, H_2S, ammonia, and pyruvate were produced. The same products were detected after L-cysteine degradation catalyzed by cystalysin. Since hemoxidative and hemolytic activities have been observed for *T. denticola* cultured with L-cysteine, similar biological effects of glutathione and Cys-Gly were predicted. As shown in Table 1, *T. denticola* cultured with glutathione (reduced form) or Cys-Gly exhibited approximately 20- and 10-fold greater hemoxidative and hemolytic activities, respectively, than *T. denticola* cultured with other compounds which are not substrates of *T. denticola* that yield H_2S. As a control, oxidized glutathione, which is not a suitable metabolic substrate for *T. denticola*, had no hemoxidative and hemolytic activities. Significantly, although cystathionine was metabolized by the bacterium, generating ammonia and pyruvate, it did not release hemoxidative and hemolytic activities. These results support the hypothesis

TABLE 1. Thiol substrate specificity of *T. denticola* and purified cystalysin

Organism or enzyme	Substrate	End products of enzymatic reaction			Hemoxidation activity (U/mg of protein)	Hemolysis activity (U/mg of protein)
		H_2S	NH_3	Pyruvate		
T. denticola	Cysteine	+/+[a]	+/+	±/+	26.5 ± 1.8[b]	6.8 ± 0.6
	Cystine	+/+	+/+	±/+	15.6 ± 2.1	4.6 ± 0.3
	Glutathione (reduced form)	+/+	+/+	±/+	28.4 ± 3.3	7.6 ± 0.5
	Glutathione (oxidized form)	−	−	−	1.2 ± 0.3	0.4 ± 0.1
	Cys-Gly (reduced form)	+/+	+/+	±/+	24.6 ± 2.8	6.5 ± 0.3
	Cys-Gly (oxidized form)	+/+	+/+	±/+	26.4 ± 2.1	6.8 ± 0.5
	Coenzyme A glutathione disulfide	−	−	−	1.1 ± 0.2	0.3 ± 0.0
	Methionine	−	−	−	1.6 ± 0.2	0.5 ± 0.1
	Cystathionine	−	+/+	±/+	1.3 ± 0.1	0.4 ± 0.0
	2-ME	−	−	−	1.6 ± 0.4	0.3 ± 0.1
	Dithiothreitol	−	−	−	1.8 ± 0.3	0.5 ± 0.1
	Homocysteine	−	−	−	1.6 ± 0.2	0.4 ± 0.0
	γ-Glu-Cys	−	−	−	1.2 ± 0.1	0.3 ± 0.0
	Glu-Cys-Leu	−	−	−	1.3 ± 0.2	0.4 ± 0.1
Purified cystalysin	Cysteine	+	+	+	586.5 ± 66.8	185.6 ± 25.3
	Cystine	+	+	+	444.2 ± 38.9	143.3 ± 11.8
	Glutahtione (reduced form)	−	−	−	1.9 ± 0.3	0.9 ± 0.1
	Cys-Gly (reduced form)	−	−	−	1.5 ± 0.4	0.6 ± 0.1
	Cys-Gly (oxidized form)	−	−	−	1.6 ± 0.2	0.8 ± 0.1
	Cystathionine	−	+	+	1.3 ± 0.1	1.0 ± 0.1

[a] +, product detectable; −, product undetectable; ±, product weakly detectable; +/+, product detectable in both whole cells and lysate; ±/+, product weakly detectable in whole cells but detectable in lysate.
[b] Mean ± standard error.

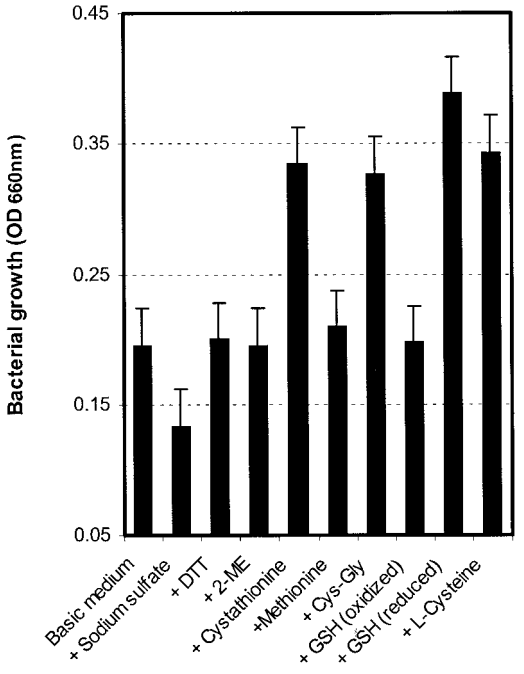

FIG. 2. Effects of thiol compounds on the growth of *T. denticola*. *T. denticola* was cultured in basic GM-1 medium or basic medium containing various chemicals at a concentration of 6 mM. After 2 days, the OD_{660} was determined to monitor bacterial growth. GSH, glutathione. The bars and error bars indicate means and standard deviations, respectively ($n = 3$). The values for cultures grown in the presence of glutathione, Cys-Gly, cysteine, and cystathionine are significantly ($P < 0.05$) higher than the values for other cultures.

that H_2S production is related to hemoxidation and hemolysis by *T. denticola.*

Growth-promoting effects of glutathione in *T. denticola*. Compared to the growth of *T. denticola* in GM-1 medium alone (basic medium), growth of *T. denticola* with L-cysteine, glutathione (reduced form), Cys-Gly (reduced or oxidized form), and cystathionine was significantly greater. *T. denticola* growth in the presence of L-cysteine, *T. denticola* growth in the presence of glutathione (reduced form), and *T. denticola* growth in the presence of cystathionine were approximately 33, 50, and 33% greater, respectively. In contrast, other thiol compounds tested, including methionine, 2-ME, DTT, and sodium sulfate, had no effect on growth of the bacteria (Fig. 2). Our results indicate that pyruvate, the other major metabolic product obtained from glutathione, stimulates bacterial growth. Figure 3 shows the kinetics of growth of *T. denticola* in the presence of substrates or various enzyme products. Pyruvate was comparable to glutathione in terms of growth stimulation. Ammonia had only a small effect on growth, while other glutathione metabolites, including H_2S (Fig. 3), glutamate, and glycine (data not shown), had no effect on growth. These results indicate that *T. denticola* may also utilize glutathione to generate pyruvate for nutrition.

Kinetic analysis of pyruvate metabolism by *T. denticola*. The results of three experiments suggested that a more detailed examination of pyruvate utilization by *T. denticola* is needed. First, exogenous pyruvate was a suitable substrate to stimulate bacterial growth (Fig. 3). Second, when *T. denticola* was incubated with the reduced form of glutathione, H_2S and ammonia were readily detected in the media, whereas only a small amount of pyruvate was detected (Fig. 4A). Similar results were obtained when L-cysteine and Cys-Gly were used as substrates for *T. denticola* (data not shown). Third, similar concentrations of pyruvate, H_2S, and ammonia were produced following incubation of *T. denticola* lysate with substrates, including glutathione (Fig. 4B), Cys-Gly, and cysteine (Table 1). Therefore, we monitored the removal of glutathione metabolites, including glutamate, glycine, ammonia, H_2S, and pyruvate, by *T. denticola* (Fig. 4C). After pyruvate was mixed with a bacterial culture, more than 85% of this compound disappeared from the medium within 90 min (Fig. 4C). In contrast, only minimal loss of either ammonia or H_2S was observed. Approximately 15% of the glutamate and approximately 15% of the glycine were lost during incubation, suggesting that additional degradation or absorption occurred. The disappearance of the pyruvate in the medium suggests that this compound is rapidly absorbed by *T. denticola* cells, which is consistent with the bacterial growth effects of this metabolite.

HPLC analysis of glutathione metabolism in *T. denticola*. In the experiments described above, H_2S, ammonia, and pyruvate were targeted as products of glutathione and Cys-Gly metabolism by using chemical reaction methods. However, metabo-

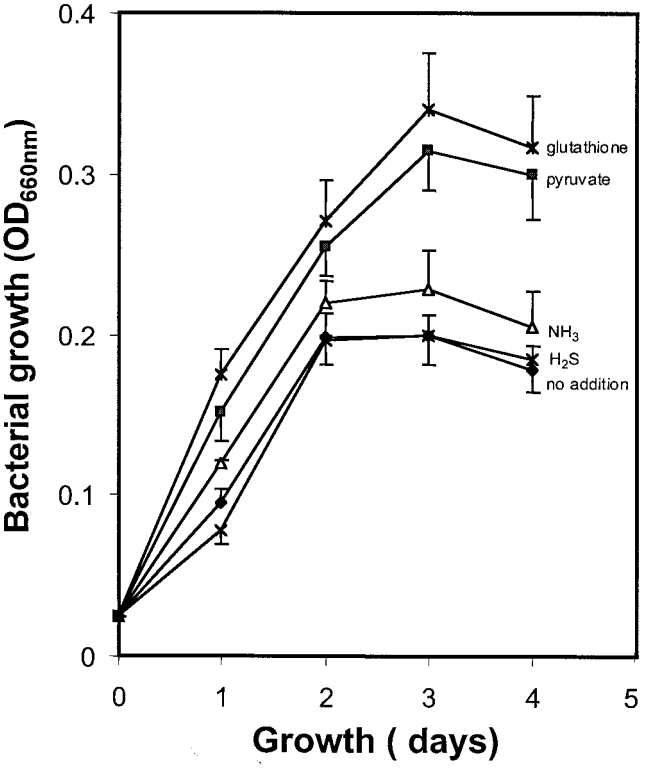

FIG. 3. Effects of glutathione metabolites on the growth of *T. denticola*. *T. denticola* was cultured in basic GM-1 medium (no addition) or in basic GM-1 medium containing 12 mM glutathione, 12 mM pyruvate, 12 mM H_2S, 12 mM NH_3, 12 mM glutamate (Glu), or 12 mM glycine (Gly). OD_{660} of the cultures were determined for 1 to 4 days to monitor bacterial growth. The data points and error bars indicate means and standard deviations ($n = 3$). Other results (data not shown) indicated that glutamate and glycine, added either separately or in combination, had no effect on bacterial growth.

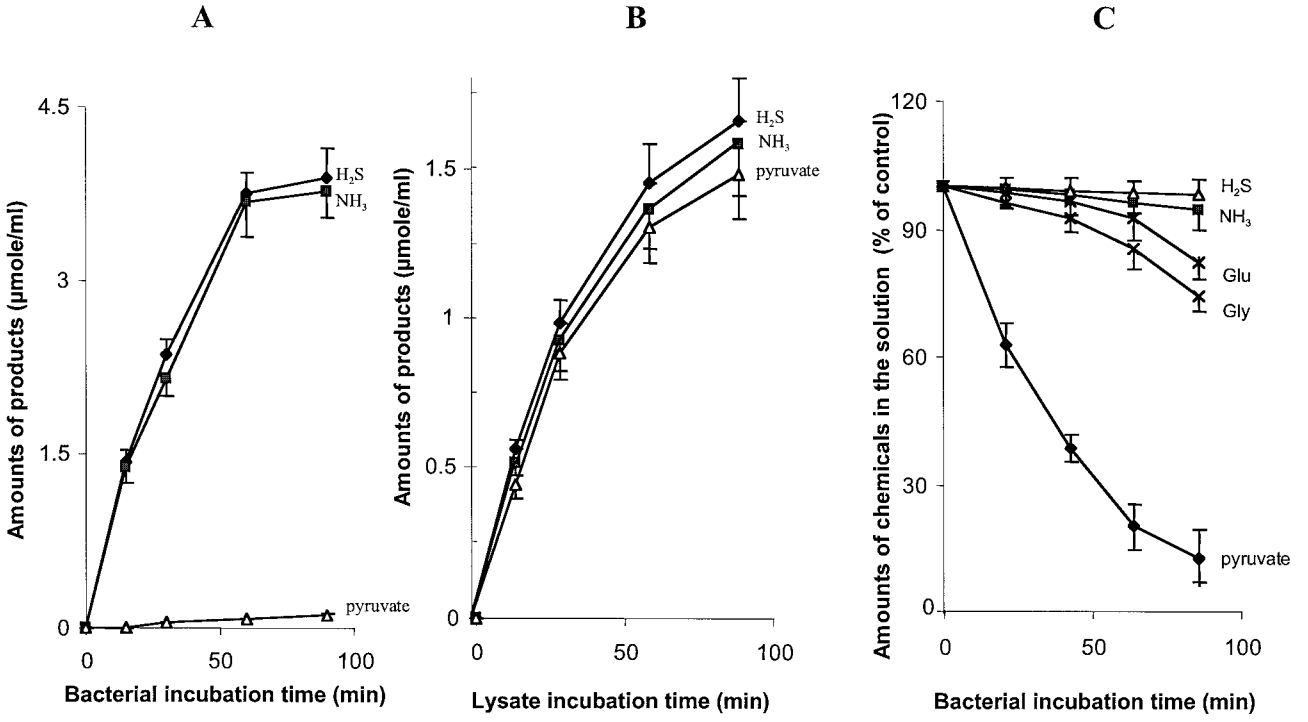

FIG. 4. Kinetic analysis of pyruvate metabolism. (A) Glutathione (4 mM) incubated with *T. denticola* cells. The amounts of H_2S, NH_3, and pyruvate in the incubation medium were measured. (B) Glutathione (4 mM) added to *T. denticola* cell lysate . (C) Glutamate (Glu) (4 mM), glycine (Gly) (4 mM), pyruvate (4 mM), H_2S (4 mM), and NH_3 (4 mM) mixed with *T. denticola* cells. After 15, 30, 60, or 90 min of incubation, the amounts of chemicals remaining in the incubation media were measured. The data points and error bars indicate means and standard deviations, respectively ($n = 3$). Virtually no pyruvate was lost in the control solution without *T. denticola* (data not shown).

lism of glutathione, the tripeptide glutamatyl-cysteinyl glycine, should lead to accumulation of other products, such as glutamate and glycine. Therefore, we analyzed these metabolites by HPLC. The results of a representative experiment are shown in Table 2. After 60 min of incubation of glutathione with *T. denticola*, significant amounts of glutamate and glycine were detected. The difference between the amount of glutamate (4.712 nmol/ml) and the amount of glycine (3.321 nmol/ml) in the medium could have been due to quicker absorption of the latter compound by *T. denticola* (Fig. 4C). As predicted, only glycine was detected when the dipeptide Cys-Gly was used as the substrate. The control suspension, without exogenously added glutathione, contained limited amounts of glutamate and glycine, indicating that leakage of these amino acids from *T. denticola* was insignificant. Cysteine was undetectable in control mixtures and following addition of glutathione or Cys-Gly, presumably reflecting the potent cysteine-degrading activity of cystalysin. Small amounts of serine, histidine, and tyrosine were found in control reaction mixtures and reaction mixtures supplemented with glutathione and Cys-Gly. The HPLC analysis showed that Cys-Gly was catabolized into glycine and that glutamate and glycine were produced from glutathione, suggesting that a stepwise degradation pathway is involved in glutathione metabolism in *T. denticola*.

Enzymatic activities responsible for stepwise metabolism of glutathione. Our results suggested that there is stepwise metabolism of glutathione in *T. denticola*, leading to production of glutamate, glycine, and cysteine. Cysteine is further degraded into H_2S, ammonia, and pyruvate in a reaction catalyzed by cystalysin. Since glutathione is not a substrate of purified cystalysin (Table 1), we evaluated the characteristics of the enzymatic activities responsible for metabolism of this tripeptide. To do this, we determined the H_2S-producing capacity of *T. den-*

TABLE 2. Amino acids derived from *T. denticola* metabolism of glutathione and Cys-Gly

Amino acid	Concn (nmol/ml)[a]		
	Control	With glutathione	With Cys-Gly
Asp/Asn	0.011	0.949	0.012
Ser	0.726	0.969	0.916
Glu/Gln	0.042	4.712[b]	0.029
Gly	0.077	3.321[b]	3.081[b]
His	0.949	0.632	0.868
Arg	0.006	0.188	0.004
Thr	0.033	0.079	0.063
Ala	0.081	0.189	0.129
Pro	0.109	0.094	0.086
Cys	—	—	—
Tyr	0.785	0.074	0.288
Val	0.124	0.066	0.077
Met	0.011	0.004	0.062
Lys	0.104	0.212	0.149
Ile	0.082	0.044	0.035
Leu	0.152	0.090	0.091
Nle	0.001	0.001	0.001
Phe	—	—	—

[a] *T. denticola* was incubated with buffer alone or with buffer containing 6 mM reduced glutathione or 6 mM Cys-Gly. The amino acids in the incubation solutions were analyzed by HPLC. —, product undetectable.
[b] Value significantly different.

TABLE 3. Characterization of enzymatic activities responsible for glutathione degradation in *T. denticola*

Prepn	H_2S-producing activity (%) with the following substrates[a]:		
	Glutathione	Cys-Gly	Cysteine
Control	100.0 ± 3.9	100.0 ± 2.5	100.0 ± 6.8
With proteinase inhibitors			
TLCK	2.5 ± 0.2	5.5 ± 0.6	98.4 ± 4.6
PMSF[b]	80.5 ± 2.3	85.9 ± 7.6	94.5 ± 6.6
Benzamidine	75.4 ± 4.8	90.4 ± 6.5	97.6 ± 3.9
With cystalysin inhibitors			
Cyanoborohydride	20.4 ± 3.4	15.3 ± 2.8	10.5 ± 1.1
β-Chloroalanine	40.5 ± 5.1	42.5 ± 5.6	45.6 ± 6.5
With proteinases			
Proteinase K	5.5 ± 0.8	6.9 ± 0.7	96.4 ± 6.6
Trypsin	15.9 ± 2.1	20.5 ± 1.9	98.5 ± 5.8
With thiol compounds			
2-ME	166.5 ± 18.6	178.2 ± 16.8	150.5 ± 12.4
DTT	151.6 ± 13.8	158.7 ± 20.5	144.6 ± 13.8
Treatments			
Heating (56°C)	7.5 ± 1.1	5.8 ± 0.6	90.5 ± 3.4
Freezing (−20°C)	18.9 ± 2.1	20.5 ± 1.8	98.2 ± 4.3
Sonication	30.5 ± 1.8	25.4 ± 1.2	121.5 ± 5.8
Immunodepletion	13.5 ± 2.6	12.48 ± 1.6	9.5 ± 1.8

[a] The results are expressed as percentages of the control value. The control contained *T. denticola* lysate or 2×10^9 cells per ml, and preparations were incubated at 37°C for 30 min. H_2S concentrations were determined in order to determine the activities responsible for degradation of glutathione, Cys-Gly, and cysteine. The methods used are described in Materials and Methods. Reagents, including proteinase K and trypsin, were purchased from Sigma Chemical Co.

[b] PMSF, phenylmethylsulfonyl fluoride.

ticola in the presence of glutathione, Cys-Gly, or cysteine subjected to various treatments (Table 3). The enzymatic activities responsible for processing glutathione and Cys-Gly into cysteine can clearly be dissociated from the activities responsible for degrading cysteine. Cysteine-degrading activity was significantly resistant to TLCK inhibition, trypsin or proteinase K digestion, temperatures that included heating and freezing, or sonication. Cystalysin was processed into fragments after proteinase K digestion and yet retained cysteine degradation activity (unpublished data). However, each of these treatments substantially decreased the enzymatic activities responsible for processing glutathione and Cys-Gly into cysteine for subsequent H_2S production. Suppression of cystalysin by inhibitors (cyanoborohydride and β-chloroalanine) or immunodepletion not only significantly blocked H_2S production from cysteine but also suppressed H_2S production from glutathione and Cys-Gly. These results, which are consistent with our previous observations, indicate that cystalysin is an important *T. denticola* enzyme that is responsible for H_2S production following glutathione processing by other enzymes.

DISCUSSION

In this study we examined glutathione metabolism in *T. denticola*, an important pathogen of periodontal diseases. A stepwise pathway involving the production of glutamate, glycine, and cysteine is proposed. Ultimately, cysteine generated as a result of glutathione catabolism is degraded into H_2S, pyruvate, and ammonia. While cystalysin is a key enzyme for cysteine degradation, it is dissociated from the enzymatic activities responsible for glutathione processing into glutamate and glycine. The potential biological significance of glutathione metabolism by *T. denticola* appears to be at least twofold. The production of H_2S is critical for hemoxidative, hemolytic, and other toxic activities that could occur in vivo. Pyruvate, a product of glutathione metabolism, can be utilized as a nutrient to support bacterial growth. These observations suggest that glutathione metabolism plays a significant role in pathogenic processes accompanying *T. denticola* colonization. It is possible that cysteine degradation in the presence of cystalysin is only the last step of glutathione metabolism in *T. denticola*; more than likely, there are multiple degradative pathways for H_2S production in whole cells of *T. denticola* for other compounds (i.e., glutathione and Cys-Gly). Significantly, glutathione metabolism could be important for virulence expression in *T. denticola*, since reasonably high levels of this tripeptide substrate are present in periodontal pockets (28, 31, 32). While one of the metabolites of glutathione, H_2S, is toxic to host cells, another product, pyruvate, promotes bacterial growth. Such a mechanism not only could damage the periodotium but also could facilitate bacterial expansion, resulting in further development of tissue pathology.

Glutathione metabolism appears to be a universal property of *T. denticola* and has been found in strains ATCC 35404, ATCC 35405, and ATCC 33520 and clinical isolates GM-1 and MS25. On the other hand, neither *T. pectinovorum*, *T. vincentii*, nor *T. socranskii* was able to use glutathione as a substrate to generate H_2S or pyruvate. The clear difference in glutathione metabolism between *T. denticola* and other oral treponemes is at least in part determined by the production of cystalysin. Previously, we have shown that cystalysin is the key enzyme used by *T. denticola* for cysteine degradation to release H_2S. The lack of cystalysin in the other treponemes tested was confirmed by Southern hybridization and PCR amplification with suitable primers (data not shown). The results provide convincing evidence that while cystalysin is expressed in all *T. denticola* strains, it is not present in other oral treponemes. Since the final step of glutathione catabolism involves cysteine degradation, the lack of cystalysin prevents these treponemes from using glutathione to generate H_2S, ammonia, and pyruvate.

A stepwise pathway of glutathione degradation in *T. denticola* was suggested by our findings (Fig. 5). Our results showed that while purified cystalysin cannot release H_2S from glutathione and Cys-Gly, these peptides are good substrates for H_2S production in *T. denticola*. Chemical and HPLC analyses indicated that glutathione metabolism in this bacterium leads to

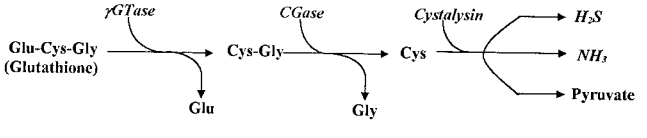

FIG. 5. Hypothetical model for stepwise degradation of glutathione in *T. denticola*. γGTase, γ-glutamyltransferase; CGase, cysteinyl glycinase.

accumulation of five end products, glutamate, glycine, H_2S, ammonia, and pyruvate. In recent studies workers have isolated a γ-glutamyltransferase from the outer cell envelope of *T. denticola* (29). This enzyme appears to be a good candidate for the enzyme responsible for the first step of glutathione breakdown, which results in the production of glutamate and Cys-Gly (1, 29). If the reaction stopped here, we would expect accumulation of the dipeptide Cys-Gly. However, HPLC analysis revealed that a significant amount of glycine is produced. Thus, these results suggest that there is a second step of glutathione catabolism, which breaks Cys-Gly into cysteine and glycine. This reaction could be catalyzed by a cysteinyl glycinase (1, 45), which is a highly conserved enzyme specifically involved in Cys-Gly hydrolysis. The participation of other enzymes, including cystinyl aminopeptidase (1), appears to be less likely, since Cys-Leu was not a good substrate for H_2S production in *T. denticola* (Table 1). The last step of glutathione catabolism is the degradation of cysteine, which is catalyzed by cystalysin and releases H_2S, ammonia, and pyruvate (10, 11, 12, 24). The stepwise pathway enables *T. denticola* to digest and utilize glutathione in an efficient manner.

Based on end product analysis, glutathione metabolism in *T. denticola* may have multiple biologic consequences. First, production of H_2S may be critical for the virulence of the bacterium. Results obtained in this study suggested that H_2S plays an essential role in hemoxidation and hemolysis (Table 1). Thiol compounds, including glutathione, Cys-Gly, and cysteine, facilitated H_2S production by *T. denticola* and exhibited high hemoxidation and hemolysis activities. These observations, along with previous work which showed that H_2S is cytotoxic (4, 10, 40, 48), indicate that production of this volatile sulfur toxin following glutathione degradation can contribute significantly to the virulence of *T. denticola*. Another end product of glutathione catabolism, pyruvate, was shown to enhance bacterial growth (Fig. 3). This implies that while H_2S is more directly involved in bacterial virulence (14), other metabolites of glutathione might play important roles in bacterial expansion. In this context, such mechanisms could be predicted to maintain and exacerbate the pathogenic actions of *T. denticola*.

In eukaryotes, glutathione is important for the maintenance of cellular homeostasis. This compound has been implicated in macromolecule synthesis, transport, and enzymatic regulation, as well as in cellular defense against oxidative stress (31, 32). As a result, high (millimolar) levels of glutathione are usually present in the cells. However, much less glutathione is found in bacteria. In fact, little or no glutathione has been detected in anaerobic bacteria (2, 15, 28, 31). Previous work suggested that short peptides were not good nutrients for these organisms (19, 49). Recent studies of Carlsson et al. (6) provided compelling evidence that glutathione is actively transported and utilized by *Peptostreptococcus*. Our experiments delineated a glutathione metabolism pathway in *T. denticola*, an anaerobic pathogen that causes periodontal diseases. Therefore, the abilities of different species of anaerobic bacteria to metabolize glutathione may vary greatly. A potentially enormous resource of glutathione for bacteria is the eukaryotic cells in specific microenvironments. For example, *T. denticola* lives in a glutathione-rich medium, the periodontal pocket. In infected periodontal pockets, a predominant cell type is polymorphonuclear leukocytes, which contain up to 4 mM glutathione (5). Interactions of *T. denticola* with these leukocytes may provide a large reservoir of glutathione that can be used for H_2S and pyruvate production, enhancing the virulence of this bacterium.

In conclusion, this study provided substantial evidence that there is a stepwise pathway of glutathione metabolism in *T. denticola*, which results in the production of glutamate, glycine, H_2S, ammonia, and pyruvate. While H_2S may play a profound role in the virulence of *T. denticola*, another glutathione metabolite, pyruvate, enhances bacterial growth. Since glutathione is a predominant thiol resource in periodontal pockets, our results suggest that glutathione metabolism plays important roles in pathogenic processes mediated by *T. denticola*.

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