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

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Hydrogen sulfide (H\textsubscript{2}S) 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\textsubscript{2}S. The metabolic pathway leading to H\textsubscript{2}S formation in periodontal pockets has not been determined. We used a variety of thiol compounds as substrates for *T. denticola* to produce H\textsubscript{2}S. Our results indicate that glutathione, a readily available thiol source in periodontal pockets, is a suitable substrate for H\textsubscript{2}S production by this microorganism. In addition to H\textsubscript{2}S, 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\textsubscript{2}S, 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 Nα-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\textsubscript{2}S), could contribute to pathogenic changes in the host tissues (16). High levels of H\textsubscript{2}S (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\textsubscript{2}S 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\textsubscript{2}S 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\textsubscript{2}S 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\textsubscript{2}S, 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\textsubscript{2}S 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\textsubscript{2}S 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.

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\textsubscript{2}, 10% H\textsubscript{2}, 85% N\textsubscript{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\textsubscript{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 35409 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\textsuperscript{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\textsubscript{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$_2$S was quantitated 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$_2$O) and reagent II (0.5% [wt/vol] NaOH, 16% [wt/vol] Na$_2$HPO$_4$, and 0.49% [wt/vol] sodium hydrochloride in distilled H$_2$O) were prepared fresh prior to use; 0.2 ml of an ultratitration 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 x 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 O.D$_{600}$ 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 ultracentrifugation with a Centricon filter (Millipore 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 2600 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-Fluorborate 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 2600 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$_2$S, NH$_3$, pyruvate, glutamic acid, and glycine, were added separately at a concentration of 4 mM to 2 x 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.

Hemolysis and hemoglobin assay. Hemolysis activity was determined with sheep red blood cells by using the method of Leech and Smith (27). Hemolysis activity was determined as described previously (7).

Characterization of enzymatic activities in _T. denticola_ that produce H$_2$S from glutathione, Cys-Gly, and cysteine. _T. denticola_ cells and cellular extracts were examined for activity of glutathione (reduced form), and Cys-Gly resulted in production of significantly more H$_2$S than incubation in S-deficient medium (basic GM-1 medium without cysteine). In the presence of appropriate thiol substrates, H$_2$S 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$_2$S 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$_2$S, the oxidized form of glutathione had no effect. Identical results were obtained with _T. denticola_ ATCC 35404, ATCC 35405, and ATCC 33520 and clinical isolates GM-1 and MS25 (data not shown). By contrast, _T. pectinovorum_, _T. vincentii_, and _T. socranskii_ were not able to produce H$_2$S 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$_2$S per ml compared to the >0.4 nmol of H$_2$S 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$_2$S from the substrate cysteine. This protein, cystalysin, appears to play a pivotal role in H$_2$S production in _T. denticola_. Since _T. denticola_ can utilize glutathione to generate H$_2$S (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$_2$S, ammonia, and pyruvate. As Table 1 shows, _T. denticola_ generated H$_2$S in the presence of cysteine, Cys-Gly, or glutathione (reduced form). Ammonia
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$_2$S. Cystathionine consists of cysteine and homocysteine, and the enzyme products obtained with cystathionin should be homocysteine, pyruvate, and ammonia (10). Consistent with the results reported for cystathionin (10, 24), *T. denticola* was not able to metabolize methionine, 2-ME, DTT, Cys-Leu, Glu-Cys, homocysteine, and other compounds. However, purified cystathionin displayed a specific substrate preference for L-cysteine and cysteine (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$_2$S, ammonia, and pyruvate were produced. The same products were detected after L-cysteine degradation catalyzed by cystathionin. 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$_2$S. As a control, oxidized glutathione, which is not a suitable metabolic substrate for *T. denticola*, had no hemoxidative and hemolytic activities. Significant, although cystathionine was metabolized by the bacterium, generating ammonia and pyruvate, it did not release hemoxidative and hemolytic activities. These results support the hypothesis

![H$_2$S production by *T. denticola* in the presence of thiol compounds.](image)

**FIG. 1.** H$_2$S production by *T. denticola* in the presence of thiol compounds. Solid bars, H$_2$S production by *T. denticola* incubated for 30 min with thiol compounds; grey bars, H$_2$S contents of supernatants of 2-day *T. denticola* cultures grown in the presence of various thiol compounds. H$_2$S 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.

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

<table>
<thead>
<tr>
<th>Organism or enzyme</th>
<th>Substrate</th>
<th>End products of enzymatic reaction</th>
<th>Hemoxidative activity (U/mg of protein)</th>
<th>Hemolysis activity (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H$_2$S</td>
<td>NH$_3$</td>
<td>Pyruvate</td>
</tr>
<tr>
<td><em>T. denticola</em></td>
<td>Cysteine</td>
<td>+/+/a</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Cystine</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Glutathione (reduced form)</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Glutathione (oxidized form)</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Cys-Gly (reduced form)</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Cys-Gly (oxidized form)</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Coenzyme A glutathione disulfide</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Cystathionine</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>2-ME</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Dithiothreitol</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Homocysteine</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>γ-Glu-Cys</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td></td>
<td>Glu-Cys-Leu</td>
<td>+/+</td>
<td>+/+</td>
<td>±/+</td>
</tr>
<tr>
<td>Purified cystathionin</td>
<td>Cysteine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cystine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Glutathione (reduced form)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Glutathione (oxidized form)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cys-Gly (reduced form)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cystathionine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$ product detectable; $-$, product undetectable; $\pm$, 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.
that H$_2$S 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, glutathione (reduced form), and 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$_2$S (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$_2$S 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$_2$S, 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$_2$S, 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$_2$S 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$_2$S, ammonia, and pyruvate were targeted as products of glutathione and Cys-Gly metabolism by using chemical reaction methods. However, metabo-
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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{T. denticola}.

**Enzymatic activities responsible for stepwise metabolism of glutathione.** Our results suggested that there is stepwise metabolism of glutathione in \textit{T. denticola}, leading to production of glutamate, glycine, and cysteine. Cysteine is further degraded into H$_2$S, ammonia, and pyruvate in a reaction catalyzed by \textit{cystalysin}. Since glutathione is not a substrate of purified \textit{cystalysin} (Table 1), we evaluated the characteristics of the enzymatic activities responsible for metabolism of this tripeptide. To do this, we determined the H$_2$S-producing capacity of \textit{T. denticola}.

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

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control</th>
<th>With glutathione</th>
<th>With Cys-Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Asn</td>
<td>0.011</td>
<td>0.949</td>
<td>0.012</td>
</tr>
<tr>
<td>Ser</td>
<td>0.726</td>
<td>0.969</td>
<td>0.916</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>0.042</td>
<td>4.712$^b$</td>
<td>0.029</td>
</tr>
<tr>
<td>Gly</td>
<td>0.077</td>
<td>3.321$^b$</td>
<td>3.081$^b$</td>
</tr>
<tr>
<td>His</td>
<td>0.949</td>
<td>0.632</td>
<td>0.868</td>
</tr>
<tr>
<td>Arg</td>
<td>0.006</td>
<td>0.188</td>
<td>0.004</td>
</tr>
<tr>
<td>Thr</td>
<td>0.033</td>
<td>0.079</td>
<td>0.063</td>
</tr>
<tr>
<td>Ala</td>
<td>0.081</td>
<td>0.189</td>
<td>0.129</td>
</tr>
<tr>
<td>Pro</td>
<td>0.109</td>
<td>0.094</td>
<td>0.086</td>
</tr>
<tr>
<td>Cys</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.785</td>
<td>0.074</td>
<td>0.288</td>
</tr>
<tr>
<td>Val</td>
<td>0.124</td>
<td>0.066</td>
<td>0.077</td>
</tr>
<tr>
<td>Met</td>
<td>0.011</td>
<td>0.004</td>
<td>0.062</td>
</tr>
<tr>
<td>Lys</td>
<td>0.104</td>
<td>0.212</td>
<td>0.149</td>
</tr>
<tr>
<td>Ile</td>
<td>0.062</td>
<td>0.044</td>
<td>0.035</td>
</tr>
<tr>
<td>Leu</td>
<td>0.152</td>
<td>0.090</td>
<td>0.091</td>
</tr>
<tr>
<td>Nle</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Phe</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ \textit{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

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Glutathione</th>
<th>Cys-Gly</th>
<th>Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 3.9</td>
<td>100.0 ± 2.5</td>
<td>100.0 ± 6.8</td>
</tr>
<tr>
<td>With proteinase inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLCK</td>
<td>2.5 ± 0.2</td>
<td>5.5 ± 0.6</td>
<td>98.4 ± 4.6</td>
</tr>
<tr>
<td>PMSF</td>
<td>80.5 ± 2.3</td>
<td>85.9 ± 7.6</td>
<td>94.5 ± 6.6</td>
</tr>
<tr>
<td>Benazidine</td>
<td>75.4 ± 4.8</td>
<td>90.4 ± 6.5</td>
<td>97.6 ± 3.9</td>
</tr>
<tr>
<td>With cystalysin inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanoborohydride</td>
<td>20.4 ± 3.4</td>
<td>15.3 ± 2.8</td>
<td>10.5 ± 1.1</td>
</tr>
<tr>
<td>β-Chloroalanine</td>
<td>40.5 ± 5.1</td>
<td>42.5 ± 5.6</td>
<td>45.6 ± 6.5</td>
</tr>
<tr>
<td>With proteinases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinase K</td>
<td>5.5 ± 0.8</td>
<td>6.9 ± 0.7</td>
<td>96.4 ± 6.6</td>
</tr>
<tr>
<td>Trypsin</td>
<td>15.9 ± 2.1</td>
<td>20.5 ± 1.9</td>
<td>98.5 ± 5.8</td>
</tr>
<tr>
<td>With thiol compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-ME</td>
<td>166.5 ± 18.6</td>
<td>178.2 ± 16.8</td>
<td>150.5 ± 12.4</td>
</tr>
<tr>
<td>DTT</td>
<td>151.6 ± 13.8</td>
<td>158.7 ± 20.5</td>
<td>144.6 ± 13.8</td>
</tr>
<tr>
<td>Treatments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heating (56°C)</td>
<td>7.5 ± 1.1</td>
<td>5.8 ± 0.6</td>
<td>90.5 ± 3.4</td>
</tr>
<tr>
<td>Freezing (−20°C)</td>
<td>18.9 ± 2.1</td>
<td>20.5 ± 1.8</td>
<td>98.2 ± 4.3</td>
</tr>
<tr>
<td>Sonication</td>
<td>30.5 ± 1.8</td>
<td>25.4 ± 1.2</td>
<td>121.5 ± 5.8</td>
</tr>
<tr>
<td>Immunodepletion</td>
<td>13.5 ± 2.6</td>
<td>12.48 ± 1.6</td>
<td>95.1 ± 1.8</td>
</tr>
</tbody>
</table>

*a* The results are expressed as percentages of the control value. The control contained T. denticola lysate or 2 × 10⁶ cells per ml, and preparations were incubated at 37°C for 30 min. H₂S 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.

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 H₂S production. Suppression of cystalysin by inhibitors (cyanoborohydride and β-chloroalanine) or immunodepletion not only significantly blocked H₂S production from cysteine but also suppressed H₂S 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 H₂S 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 H₂S, 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 H₂S is critical for hemoxicative, 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 H₂S 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, H₂S, is toxic to host cells, another product, pyruvate, promotes bacterial growth. Such a mechanism not only could damage the periodontium 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. socranski was able to use glutathione as a substrate to generate H₂S 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 H₂S. 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 H₂S, 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 H₂S from glutathione and Cys-Gly, these peptides are good substrates for H₂S 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-glutamytransferase; CGase, cysteinyld glycine.>
GLUTATHIONE METABOLISM IN T. DENTICOLA

accumulation of five end products, glutamate, glycine, H$_2$S, 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 dipetide 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 can be catalyzed by a cysteinyldi- lase (1, 45), which is a highly conserved enzyme specifically involved in Cys-Gly hydrolysis. The participation of other enzymes, including cystein aminopeptidase (1), appears to be less likely, since Cys-Leu was not a good substrate for H$_2$S production in *T. denticola* (Table 1). The last step of glutathione catabolism is the degradation of cysteine, which is catalyzed by cysteasyn and releases H$_2$S, 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$_2$S may be critical for the virulence of the bacterium. Results obtained in this study suggested that H$_2$S plays an essential role in hemoxidation and hemolysis (Table 1). Thiol compounds, including glutathione, Cys-Gly, and cysteine, facilitate H$_2$S production by *T. denticola* and exhibit high hemoxidation and hemolysis activities. These observations, along with previous work which showed that H$_2$S is cytotoxic (4, 10, 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$_2$S 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$_2$S 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$_2$S, ammonia, and pyruvate. While H$_2$S 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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