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Glutathione Catabolism by *Treponema denticola* Impacts Its Pathogenic Potential

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Abstract

Treponema denticola is a spirochete that is etiologic for periodontal diseases. This bacterium is one of two periodontal pathogens that have been shown to have a complete three step enzymatic pathway (GTSP) that catabolizes glutathione to H_2S. This pathway may contribute to the tissue pathology seen in periodontitis since diseased periodontal pockets have lower glutathione levels than healthy sites with a concomitant increase in H_2S concentration. In order to be able to demonstrate that glutathione catabolism by the GTSP is critical for the pathogenic potential of *T. denticola*, allelic replacement mutagenesis was used to make a deletion mutant (Δ*ggt*) in the gene encoding the first enzyme in the GTSP. The mutant cannot produce H_2S from glutathione since it lacks gamma-glutamyltransferase (GGT) activity. The hemolytic and hemoxidation activities of wild type *T. denticola* plus glutathione are reduced to background levels with the Δ*ggt* mutant and the mutant has lost the ability to grow aerobically when incubated with glutathione. The Δ*ggt* bacteria with glutathione cause less cell death in human gingival fibroblasts (hGFs) *in vitro* than do wild type *T. denticola* and the levels of hGF death correlate with the amounts of H_2S produced.

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Conflict of interest

On behalf of all authors, the corresponding author, Dr. Kolodrubetz, states that there is no conflict of interest.

Importantly, the mutant spirochetes plus glutathione make significantly smaller lesions than wild type bacteria plus glutathione in a mouse back lesion model that assesses soft tissue destruction, a major symptom of periodontal diseases. Our results are the first to prove that *T. denticola* thiol-compound catabolism by its gamma-glutamyltransferase can play a significant role in the in the types of host tissue damage seen in periodontitis.

Keywords

Treponema denticola; *ggt* mutant; Periodontal disease; Gingival fibroblast death; Glutathione catabolism

Introduction

Periodontal diseases are among the most prevalent human bacterial infections; roughly half of the adult population in the United States suffers from mild to severe periodontitis [1,2]. Although the symptoms are often mild, periodontitis can cause severe pain and tooth loss from connective tissue damage [3]. This disease is due to a complex mixture of inflammation and other host responses to polymicrobial infections [4–8]. *Treponema denticola* is an anaerobic spirochete that is considered to be a periodontal pathogen since its levels increase in the subgingival microbiome of periodontally-diseased patients [5,9,10], relative to healthy individuals, and it can cause disease symptoms (soft tissue destruction and alveolar bone loss) in animal models [11–13]. Dozens of virulence determinants have been identified in this bacterium [14,15] including potential roles for metabolism and metabolic end-products in virulence [15]. One such metabolic pathway, and end product, that may play a role in *T. denticola* pathogenesis is the glutathione three-step pathway which metabolizes glutathione into H_2S. These two thiol compounds are of particular interest because their levels differ in the gingival crevicular fluid (GCF) of healthy versus periodontally-diseased individuals. Specifically, the levels of glutathione, the predominant thiol compound in human GCF, are lower in the GCF of periodontitis patients [16–18]. This decrease in glutathione levels may contribute to the disease process since glutathione is an antioxidant that can moderate host cell damage [19–21] and since glutathione can also reduce the inflammatory response [22,23]. On the other hand, H_2S is present at high levels in pockets associated with periodontitis [24–27] and thus could play a role in periodontal pathology. For example, H_2S can induce apoptosis in human periodontal epithelial, ligament and fibroblast cells [28–30] consistent with the finding that the proportion of apoptotic cells in gingival tissue is higher in periodontitis patients [31–33]. H_2S has also been shown to cause hemoxidation and hemolysis of human red blood cells, and its activity is enhanced if H_2S is generated from cysteine by the *T. denticola* cystalysin enzyme [34]. Finally, the high levels of the reducing agent H_2S could play a role in creating the low levels of oxygen found in diseased periodontal pockets [35,36]. Low oxygen conditions are necessary for the growth of many periodontal pathogens, which are often anaerobes [5,7]. Taken together, these results suggest that alterations in the levels of glutathione and H_2S in the periodontal pocket will play a critical role in the extent of tissue/bone damage seen in diseased patients.

Thus, one distinct possibility is that the catabolism of glutathione into hydrogen sulfide by periodontal bacteria plays a significant role in periodontal disease symptomology. Although numerous oral bacteria can generate H_2S from cysteine or methionine [37–39], *T. denticola* and *Fusobacterium nucleatum* are the only well-established periodontal pathogens that have been shown to be able to make H_2S from glutathione [40–42]. We, and others, have identified, purified and characterized the enzymes in a three-step-pathway (GTSP) for glutathione metabolism in *T. denticola*. Initially, glutathione is cleaved into Cys-Gly and glutamate by gamma-glutamyltransferase (GGT) [40,43]. Then, Cys-Gly is degraded into glycine and cysteine by cysteinylglycinase [44]. Finally, cysteine is hydrolyzed by cystalysin [45–51], releasing ammonia, pyruvate and H_2S. Since *T. denticola* can, by itself, convert glutathione into H_2S [40] and since H_2S has been shown, as described above, to have toxic effects on host cells *in vitro*, it is reasonable to hypothesize that the GTSP pathway will play a key role in periodontal pathology. However, this has not been proven. In this report, experiments are described which definitively test the role of the *T. denticola* GTSP in functional assays related to periodontitis symptoms. We have constructed a *ggt*-deletion strain and demonstrated that this mutant *T. denticola* no longer makes H_2S from glutathione. This mutant was then used to show that the GTSP is required for maximal induction of host cell death when human gingival fibroblasts are incubated with *T. denticola*, for hemoxidation and hemolysis of erythrocytes by this spirochete and for this bacterium to grow aerobically. Most importantly, the mutant plus glutathione formed significantly smaller lesions than wild type *T. denticola* plus glutathione in a mouse back lesion model of soft tissue destruction, an *in vivo* manifestation of periodontal disease.

Materials and Methods

Bacterial growth conditions.

Unless otherwise noted, *Treponema denticola* strain ATCC 33520 was grown in GM-1 medium [52] which contains 1.5% heat-inactivated rabbit serum and 6 mM L-cysteine. The bacteria were incubated in a Coy anaerobic chamber (5% CO_2, 10% H_2, 85% N_2). Before use, the culture purity was checked by dark-field microscopy at a magnification of ×500.

Construction of *ggt* mutant in *T. denticola*.

We followed the allelic replacement protocol of Xu et al [53] to construct a *ggt* mutant in *T. denticola*. First, PCR and standard recombinant DNA procedures were used to construct plasmid pGGT:*erm*, in which an erythromycin resistance cassette [54] replaced part of the *ggt* coding region. The *erm*^{res} gene was flanked by an ~1.1 kb fragment of genomic DNA that is normally upstream of the *ggt* gene and by an ~1.0 kb fragment of genomic DNA that contained the last four-fifths of the *ggt* gene and some downstream sequences. The plasmid was linearized by *Xho*1 and electroporated into *T. denticola* [53]. After electroporation, the cells were incubated overnight in 2 ml GM-1 broth without erythromycin and then plated on TYGVS containing 0.85% sea plaque agarose with 40 μg/ml erythromycin. Antibiotic resistant colonies, which appeared after 7–12 days, were tested by PCR for the presence of the *erm*^{res} gene in the *ggt* locus. PCR fragments containing the junctions between the *erm*^{res} gene and the *T. denticola* genome were sequenced from the isolate used in these studies.

Measuring enzyme activities in *T. denticola*.

Gamma-glutamyl transferase activity (GGT) in *T. denticola* cells was assayed as described [55] with minor modifications. Briefly, two day bacterial cultures were harvested, suspended in PBS buffer (to one-fourth of original volume) and sonicated. After removing large particles by centrifugation at 14,000 × g for 10min, the soluble material was used for assessing enzyme activity. One ml of soluble material (equal to 4 ml bacterial culture) was added to 1 ml reaction mixture containing 2 mM β-mercaptoethanol, 20 mM Tris buffer at pH 8.0 and 2 mM Na-γ-glutamyl–4-nitroaniline (GNA). After incubation for 2 hr at 37°C, the absorption of each sample at $OD_{405\ nm}$ was determined.

Cysteinylglycinase activity was measured using L-Leu-*p*-NA as substrate [56]. One ml of concentrated whole bacterial isolate, prepared as described for the GGT assay, was added to 1 ml reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.2 mM $MnCl_2$ and 2 mM L-Leu-*p*-NA (final concentrations). The reaction was incubated at 37°C for 60 min before being stopped by cooling on ice. The concentration of the reaction product was measured by the absorbance at OD_{405nm}.

Cystalysin activity was measured as the production of ammonia from L-cysteine, as described by Chu et al [48]. Briefly, the reaction mixture consisted of soluble cell fraction material, prepared as described above, which was incubated with L-cysteine (2 mM) for 60 min at 37°C. The samples were then filtered through an Amicon Centricon filter (10-kDa cutoff) and the material in the flow-through was measured for ammonia using the method of Bauer et al [57].

Determination of H_2S production by *T. denticola*.

Hydrogen sulfide produced by *T. denticola*, after growth in various conditions, was assayed as described by Siegel [58] with some modifications. Briefly, after removal of bacterial cells by centrifugation, a portion (180 μl) of the supernatant was removed to a fresh tube for H_2S determination. Ten μl of 0.02 M *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 10 μl of 0.3 M $FeCl_3$ in 1.2 N HCl were added sequentially to the tube. After color development for 30 min at room temperature, the absorbance at 620 nm was measured. The sulfide concentration was determined from an Na_2S standard curve.

Hemoxidation and hemolytic analyses.

Hemoxidation (HeO) activity was assessed as described by Leahy and Smith [59] with some modifications [45]. Briefly, 2 ml of 2% (vol/vol) sheep red blood cells (RBCs), after being washed twice to remove soluble hemoglobin, was added to 2 ml of *T. denticola* cell sonicates containing 6 mM glutathione. Samples were incubated at 37°C with slow rotation (90 rpm) and aliquots were removed at 0, 1, 2, 4, 6, and 8 hrs. The formation of methemoglobin (or sulfhemoglobin) in the RBCs was then determined [60], where 1 HeO unit is defined as 20% methemoglobin (or sulfhemoglobin) formation from the RBCs.

Hemolytic activity (HeA) was determined using sheep red blood cells as described by Chu and Holt [45] with some modifications. Briefly, 2 ml of 2% (vol/vol) sheep RBCs, after being washed twice to remove soluble hemoglobin, was added to 2 ml of *T. denticola* cell

sonicates containing 6 mM glutathione. Samples were incubated at 37°C with slow rotation (90 rpm) and aliquots of the supernatants were removed at various times The amount of hemoglobin (or its oxidized form) released from the RBCs was measured as the optical density of each sample at OD_{560nm}. The percentage (%) hemolysis was calculated relative to the amount of hemoglobin released from the same number of RBCs lysed by hypotonic distilled water (100% lysis).

Testing aerobic growth of *T. denticola*.

The ability of *T. denticola* strains to grow in aerobic conditions was assessed as detailed previously, with some modifications [36]. Bacterial cultures that had been grown anaerobically to an optical density of approximately 0.26 (about 4.9×10^8 cells/ml) were diluted to a starting OD_{660nm} of 0.09 by inoculation into a set of 10 ml tubes each containing six ml (final volume) of basic GM-1 medium with all supplements except L-cysteine. Glutathione, Cys-Gly, or L-cysteine were then added separately to a final concentration of 6 mM. After incubation at 37°C for 48 hrs in aerobic conditions without shaking, 2 ml from each sample was diluted into 4 ml of fresh media of the same composition as in each sample's original tube. After incubation at 37°C for an additional 48 hrs in aerobic conditions without shaking, bacterial growth in each tube was determined spectrophotometrically at OD_{660nm}.

Evaluating induction of host cell death by *T. denticola*.

Primary cultures of human gingival fibroblast cells (hGFs) were established from biopsies of gingiva from healthy individuals, with the fully informed consent of the donors and using de-identified samples, following published methods [61,62]. Briefly, gingival tissues were sliced into small pieces and put into tissue culture plates with DF10 media that contained 100 U/ml penicillin and 100mg/ml streptomycin. The samples were grown at 37 °C in 5% CO_2. Once cells grew, the cultures were expanded and stocks were frozen in liquid nitrogen. HGF cells between passages 10–16 were used for all experiments. To prepare cells for cell death experiments, the hGFs were grown in T25 flasks containing 50 ml DMEM/10% fetal bovine serum at 37°C in a 5% CO_2 incubator [30]. When the hGFs reached ~80% confluency, wild type *T. denticola* or *Δggt* mutant bacteria were added to different flasks to a final bacterial concentration of 5×10^8 cells in 50 ml for each flask. Glutathione (final concentration of 2 mM) was added to some flasks. Control flasks contained hGF cells plus glutathione without bacteria. After 24 hours, hGF cell death was quantified by annexinV/propidium iodide staining followed by flow cytometry, as described in Zhang et al [30]. This hGF cell death experiment was done four times (biological replicates). H_2S levels were also measured, as described in a previous section, in the supernatant of the cultures from the sample set of one experiment.

Assessing lesion formation by wild type and Δ*ggt T. denticola* in the presence of glutathione.

Four groups (8 mice/group) of BALB/c mice were infected subcutaneously, as described by Kesavalu et al [63], on the back with 5×10^{10} of (i) wild type *T. denticola*, (ii) wild type bacteria and glutathione (16 mM), (iii) Δ*ggt* mutant cells, (iv) Δ*ggt* mutant cells and glutathione (16 mM). The bacteria were grown with glutathione only if they were to be used

for the “and glutathione” inoculations. A fifth group of mice (control) was challenged with media plus glutathione (16 mM) alone. After challenge, the animals were monitored for symptoms of infection. Subcutaneous abscess and/or necrotic skin lesion sizes (length and width) were measured daily for 15 days and the area was determined and expressed in square millimeters. The maximum lesion size for each mouse was used to generate the mean lesion size shown for each group of mice. All mouse studies were done following protocols approved by the UTHSCSA Institutional Animal Care and Use Committee (IACUC).

Statistical analyses.

The data for enzyme activities, aerobic growth, hGF cell death and mouse lesion formation are expressed as means ± standard deviations. For the hGF cell death and murine lesion formation experiments, the statistical significance was evaluated for independent experiments using the Tukey’s multiple comparison test. Differences between samples were considered statistically significant if the P value was <0.05.

Results

A Δ*ggt* mutant specifically lacks gamma-glutamyl transferase activity and does not produce H_2S from glutathione.

In order to evaluate the biological role of the three-steppathway for glutathione metabolism (GTSP), a *T. denticola* mutant in the *ggt* gene was created by allelic replacement mutagenesis [53]. One erythromycin-resistant strain was characterized by sequencing PCR products generated from the region around the *ggt* gene. The sequencing showed that the ermF-ermAM erm^{res} cassette had replaced the genomic DNA segment encoding amino acids 17–36 of *T. denticola* GGT (Gen Bank EMB43424.1). As expected, the rest of the DNA around the erm^{res} cassette matched the genomic sequence of *T.* d*enticola* strain ATCC 33520 [http://www.homd.org/index.php].

The sequence analysis, indicating that the disrupted *ggt* gene should not produce GGT, was corroborated by testing the mutant strain for GGT activity. As predicted, the Δ*ggt* cells had no GGT activity above background whereas wild type bacteria had significant levels of GGT (Fig. 1A). These data also indicate that *T. denticola* does not have a second, unidentified *ggt* gene. As expected, the activities or expression of the two other enzymes in the GTSP, CGase and cystalysin, were not affected by the Δ*ggt* mutation (Figs. 1B and 1C).

Since the Δ*ggt* strain does not have GGT activity, this mutant should not be able to produce H_2S from glutathione via the GTSP, unless there is an alternate catabolic pathway that can carry out the same overall reaction. To rule out this possibility, the production of H_2S from three substrates was examined in wild type and mutant cells. As seen previously [40], wild type *T. denticola* was able to make H_2S from glutathione, Cys-Gly or L-cysteine (Fig. 2). However, the *Δggt* mutant could not catabolize glutathione into H_2S (Fig. 2), although it made normal levels of H_2S from Cys-Gly and L-cysteine. This proves that this disruption of the *ggt* gene inactivates the first step in the glutathione three enzyme glutathione catabolism pathway and that the GTSP is the only way for *T. denticola* to produce H_2S from glutathione.

The *T. denticola* Δ*ggt* mutant has decreased hemolytic and hemoxidative activity.

T. denticola has been shown to be able to lyse red blood cells and oxidize hemoglobin to methemoglobin in the presence of the GTSP substrates glutathione, Cys-Gly or L-cysteine [40]. This suggests, but does not prove, that the GTSP plays a key role in these hemolytic (HeA) and hemoxidative (HeO) functions. To assess the relative contribution of the GTSP to the HeA and HeO activities of *T. denticola*, their levels were measured in the *Δggt* mutant. Wild type *T. denticola* had high levels of HeA and HeO activity when glutathione was added, but the amount of activity in the Δ*ggt* cells was the same as in the media alone controls (Fig. 3). When Cys-Gly or L-cysteine were used as substrates in the assays instead of glutathione, the *Δggt* mutant and wild type strain had similar HeA and HeO activities (data not shown) indicating that the GGT protein does not play a direct role in hemolysis and hemoxidation. These results demonstrate that the GTSP has a major role in the ability of *T. denticola* to hemoxidize and lyse erythrocytes and support the hypothesis that H_2S production is needed for these functions.

T. denticola does not grow aerobically without GGT.

The growth rates of the *Δggt* mutant and wild type cells were the same when *T. denticola*, an obligate anaerobe, was grown anaerobically in GM-1 media with L-cysteine. This was not surprising since the mutant still has cystalysin activity and thus can produce pyruvate, which is used by *T. denticola* to increase its growth [40]. If the bacteria were grown anaerobically in GM-1 media with glutathione instead of L-cysteine, the *Δggt* cells grew more slowly and reached a lower final cell density than wild type cells since the mutant cannot produce pyruvate from glutathione (data not shown). Interestingly, even though *T. denticola* is an obligate anaerobe, it can grow in aerobic conditions when glutathione or L-cysteine are included in the media [36]. The ability to grow aerobically correlates with the production of H_2S by *T. denticola*, leading to the hypothesis that the organism's GTSP is required for aerobic growth. To test this inference, the aerobic growth of wild type and *Δggt* cells were compared. As expected, wild type *T. denticola* grew aerobically when incubated with glutathione, Cys-Gly or L-cysteine (Fig. 4). The *Δggt* cells also grew aerobically when the media was supplemented with Cys-Gly or L-cysteine but not when glutathione was the only supplement added (Fig. 4). To determine why the mutant cells could not grow aerobically with glutathione, the levels of oxygen and H_2S were measured in the samples that had been grown with glutathione. The media with wild type *T. denticola* had high levels of H_2S (2.1 ± 0.090 mM) whereas the media with the mutant spirochetes had significantly ($p<0.001$) less H_2S (0.07 ± 0.02 mM). Conversely, wild type *T. denticola* samples had low levels of dissolved oxygen (0.05 ± 0.006 mM) and the samples from the mutant bacteria had significantly ($p<0.001$) higher levels of dissolved oxygen (2.1 ± 0.115 mM). These results prove that the GTSP is essential for *T. denticola* to grow aerobically and reinforce the concept that it is the production of H_2S by the GTSP that leads to the reduction of oxygen levels allowing this anaerobe to grow "aerobically".

Catabolism of glutathione to H_2S by the *T. denticola* GTSP induces gingival fibroblast cell death.

H_2S has been shown to cause apoptosis in human gingival fibroblast (hGF) cells and periodontal ligament cells [30]. Thus, it is reasonable to postulate that catabolism of glutathione by the *T. denticola* GTSP, which produces H_2S, will induce host cell death. To assess this possibility, wild type and Δ*ggt* cells were incubated with hGF cells, in the presence or absence of glutathione. After 24 hours, Annexin V and propidium iodide staining was used to measure cell death. Dead hGFs include early apoptotic cells (Annexin V positive/propidium iodide low), late apoptotic cells (V^+/propidium iodide high) and necrotic hGFs (propidium iodide high) (Fig. 5A). Gingival fibroblasts incubated with wild type *T. denticola* and glutathione had significantly more cell death than hGFs grown with wild type bacteria without glutathione (Fig. 5B). This induction required the GTSP since Δ*ggt* cells with glutathione induced much less host cell death (12%) than did wild type bacteria with glutathione (52%). These results correlated well (Pearson correlation coefficient = 0.97) with the levels of H_2S in the same samples (Fig. 5C). For example, the media from hGFs incubated with mutant *T. denticola* and glutathione had 0.13 mM H_2S, whereas wild type bacteria produced 1.56 mM H_2S from glutathione. The results demonstrate that the GTSP plays a major role in the ability of *T. denticola* to induce cell death in human gingival fibroblasts and strongly argue that it is the production of H_2S from glutathione that leads to gingival fibroblast death.

The *T. denticola* Δ*ggt* mutant makes smaller lesions in a mouse back abscess model.

Since physiologically relevant concentrations of glutathione (2mM) were used in the experiments showing that GGT had a major role in the ability of *T. denticola* to cause host cell death *in vitro*, we hypothesized that the GTSP would play a similarly important role in causing host tissue damage *in vivo*. Thus, a murine back abscess model [63] was used to assess the role of the GTSP in soft tissue destruction, one of the hallmarks of periodontal disease. Animals were challenged with wild type *T. denticola* or the *Δggt* mutant, with or without glutathione being added, and the necrotic skin lesion sizes were measured over time. Importantly, mice challenged with Δ*ggt* bacteria and glutathione had significantly ($p = 0.0007$) smaller lesions (113.1 mm^2) than the animals inoculated with wild-type *T. denticola* plus glutathione (148.6 mm^2) (Fig. 6). This result proves our hypothesis that GTSP catabolism of glutathione by *T. denticola* plays a role in soft tissue destruction *in vivo*, most likely because of its production of H_2S.

Discussion

The construction of a defined *ggt* deletion mutant (Δ*ggt*) in *T. denticola* has allowed us to show definitively that the catabolism of glutathione is necessary for several virulence activities of the bacterium *in vitro*. The hemolytic and hemoxidation activities of wild type *T. denticola* plus glutathione are absent in the Δ*ggt* mutant and the mutant cannot grow aerobically when incubated with glutathione whereas wild type *T. denticola* can. Most relevant to a role for the *T. denticola* GTSP in tissue damage in periodontal disease is the fact that the Δ*ggt* bacteria with glutathione cause less cell death in human gingival fibroblasts (hGFs) *in vitro* than do wild type spirochetes. Since the levels of hGF death

correlate with the amounts of H_2S produced in these studies, the results are consistent with the hypothesis that it is the production of H_2S from glutathione by the GTSP of *T. denticola* that leads to gingival fibroblast death.

Surprisingly, there were higher levels of H_2S (0.32 mM) when wild type *T. denticola* was incubated with hGFs without exogenous glutathione than in the sample incubated with the Δ*ggt* mutant without glutathione (0.05 mM H_2S) (Fig. 5C). This correlated with the significant increase in hGF cell death seen with wild type bacteria without glutathione (26%) versus the mutant samples without glutathione (7%) (Fig. 5B). This outcome implies that there is endogenous glutathione that the wild type spirochetes can use to make H_2S which then causes cell death. In fact there are two potential sources for endogenous glutathione in all of the samples: the fetal bovine serum (FBS) added to the media and the hGF cells. Although the amount of glutathione in FBS is not routinely measured, the literature clearly shows that FBS can contain glutathione [64,65] although the amount can vary as much as thirty-fold depending upon the lot of FBS [66]. In addition, host cells can generate glutathione from the basic amino acids that are present in the DMEM component of the growth media. Although most of the host-cell-generated glutathione is retained inside the cell, where it serves essential functions in antioxidant defense and the regulation of metabolic functions [67], some is secreted. The amount secreted is typically in the μM range [68,69]. The amount of glutathione made by hGF cells has not been measured, but it could be higher than seen in other host cells since hGFs could be a source for the higher levels of glutathione (0.5 – 2.5 mM) found in gingival crevicular fluid [16,69]. Thus, clearly there will be some endogenous glutathione that can be used by the GTSP of wild type *T. denticola* to generate H_2S in the absence of exogenously added glutathione.

Another unexpected result, since we have shown that the Δ*ggt* mutant does not make H_2S from glutathione, is the observation that the addition of glutathione to hGF cells and Δ*ggt* spirochetes led to an increase in H_2S levels (from 0.052 to 0.13 mM, Fig 5C) and more cell death (from 7% to 12%, Fig. 5B). Although this increase in cell death was not statistically significant, all four paired samples had the same pattern. The rise in cell death seen when glutathione was added to hGF cells and Δ*ggt* spirochetes would appear to be an additive effect from two other ways that H_2S, which leads to increased cell death, was being generated in this experiment. First, the hGF cells by themselves (no bacteria) can make H_2S (0.045 mM) in the presence of glutathione (Fig. 5C). Second, Δ*ggt* mutant bacteria can make H_2S (0.052 mM) in the absence of glutathione (Fig. 5C); this H_2S is presumably being made from L-cystine (a dimer of cysteine), which is present in the media, by the still-present cystalysin in Δ*ggt T. denticola*. Since these two means of making H_2S are independent of each other, they should produce an additive amount of H_2S (0.045 mM + 0.052 = 0.097 mM H_2S). Indeed, 0.097 mM H_2S is close to the 0.13 mM H_2S seen in the sample with hGF cells plus the Δ*ggt* mutant and glutathione. This increase in H_2S concentration presumably leads to the increased level (12%) of host cell death seen when hGF cells, the Δ*ggt* mutant and glutathione are added together (Fig. 5B), as opposed to separately. A possible alternate explanation for the 7% cell death seen when mutant bacteria are added to hGF cells in the absence of glutathione is that other *T. denticola* molecules can cause host cell death. This is certainly a possibility since the *T. denticola* Sip protein has been shown to induce apoptosis in human T cells [70] and outer membrane extracts from *T. denticola* can increase apoptosis

in porcine endothelial cells [71]. However, we do not think these other *T. denticola* virulence factors contribute much to hGF cell death in our experiments since the correlation between H_2S levels and the percentage of cell death is so tight (Pearson correlation coefficient = 0.97) in our data (Fig. 5).

Importantly, the *in vitro* results demonstrating that glutathione catabolism by *T. denticola* is involved in the pathogenic potential of the organism were confirmed *in vivo*. Specifically, the mutant spirochetes plus glutathione made significantly smaller lesions than wild type bacteria plus glutathione in a mouse back lesion model that assesses soft tissue destruction, a major symptom of periodontal diseases. These results are the first to prove that *T. denticola* thiol-compound catabolism can play a critical role *in vivo* in the types of host tissue damage seen in periodontitis. Somewhat unexpectedly, wild type *T. denticola* appeared (p=0.26) to induce larger lesions (132.5 mm^2) than *ggt* mutant spirochetes (118 mm^2) even in the absence of exogenous glutathione. The most likely explanation is that wild type bacteria can make H_2S from the low levels of glutathione found in blood serum (1 – 4 μM as opposed to 0.5 – 2.5 mM in gingival crevicular fluid) [16,69] and cause tissue damage even without exogenous glutathione.

Even though our results demonstrate that the GTSP is important for *T. denticola*'s ability to cause soft tissue destruction, lesions still formed in mice challenged with the *Δggt* mutant, which cannot make H_2S from glutathione (Fig. 6). This data indicates that either (1) there is enough endogenous cysteine in mice to generate H_2S using the still-functional *T. denticola* cystalysin, and/or, (2) there are other proteins or metabolites produced by *T. denticola* that could also contribute to host tissue damage. In fact, the latter is likely to be true; dozens of potential *T. denticola* virulence factors have been identified in various *in vitro* experiments (14,15,72). However, only a handful of these non-GTSP proteins have been tested for their pathogenic potential in animal models. Deletion mutants in the genes encoding two *T. denticola* proteases have been tested for their virulence in the mouse back abscess model. A mutant in dentilisin, which alters the structural organization of *T. denticola*'s outer sheath, formed lesions that were 25–40% smaller than the lesions made by wild type spirochetes (73). The cysteine protease dentipain is also important for soft tissue destruction since a *T. denticola* dentipain mutant made 33% smaller lesions than wild type bacteria, although the difference is only seen at later time points (days 4–6) postinoculation (74). Bian et al (75) have characterized a mutant in the cyclic di-GMP binding protein (TDE0214) *in vitro* and *in vivo*. The Δ*tde0214* strain was less motile than wild type and did not form biofilms very well. When tested in the murine back abscess model, the lesions made by Δ*tde0214* bacteria were 25% the size of those made by wild type *T. denticola*. The *T. denticol*a neuraminidase TDE0471 removes sialic acid from host serum proteins and plays a role in bacterial virulence *in vivo*; a TDE0471 deletion mutant formed lesions in the back abscess model that were half the size of lesions made by wild type spirochetes (76). Finally, Miller et al (77) assessed the effect of a *T. denticola* mutant in FhbB, a surface-exposed lipoprotein that binds to the complement system regulatory protein factor H and blocks dentilisin-mediated cleavage of factor H. In the mouse back abscess model, the Δ*fhbB* mutant had lesions that were 50% the size of those made by wild type *T. denticola*. Thus, multiple molecules/ pathways, including the generation of H_2S by the catabolism of glutathione, which we have demonstrated here, contribute to the soft tissue destruction caused by *T. denticola*. The

relative contributions of the various *T. denticola* virulence determinants to lesion formation can be evaluated only by constructing combinations of virulence protein mutants and comparing them directly to each other in the back abscess model.

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Highlights

- *T. denticola* lacking γ-glutamyl transferase (*Δggt*) cannot make H_2S from glutathione
- The *T. denticola Δggt* mutant has decreased hemolytic and hemoxidative activity
- *T. denticola* does not grow aerobically without γ-glutamyl transferase activity
- Without GGT, *T. denticola* does not fully induce cell death in gingival fibroblasts
- The *T. denticola Δggt* mutant makes smaller lesions in a mouse back abscess model

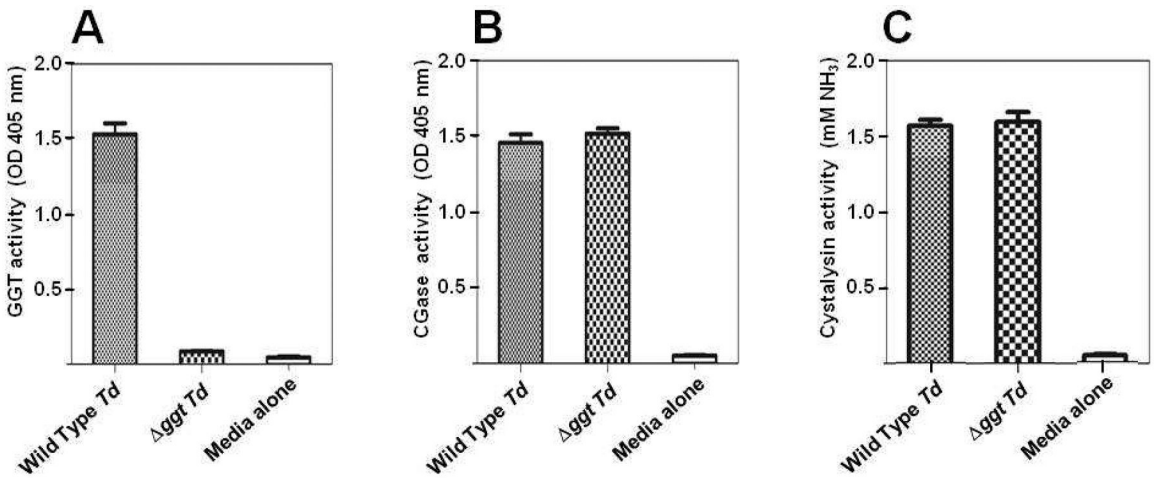

Fig. 1. GTSP enzyme activity levels in wild type *T. denticola* and its isogenic Δ*ggt* mutant.

The levels of enzyme activity for each of the three GTSP proteins were measured in equal amounts of clarified extract from wild type and mutant (Δ*ggt*) *T. denticola* (*Td*) cells. The Media alone control contained buffer with no bacteria. (A) The gamma-glutamyl transferase activity was assayed using GNA as the substrate. The levels of the resulting product were determined by the absorption of each sample at 405 nm. (B) Cysteinylglycinase activity was measured using L-Leu-*p*-NA as the substrate. The levels of the resulting product, 4-nitroaniline, were determined by the absorption of each sample at 405 nm. (C) Cystalysin activity was assessed using L-cysteine as the substrate. The resulting levels of NH_3 were determined as detailed in Materials and Methods. Each experiment was done three times. The bars indicate standard deviations of the mean.

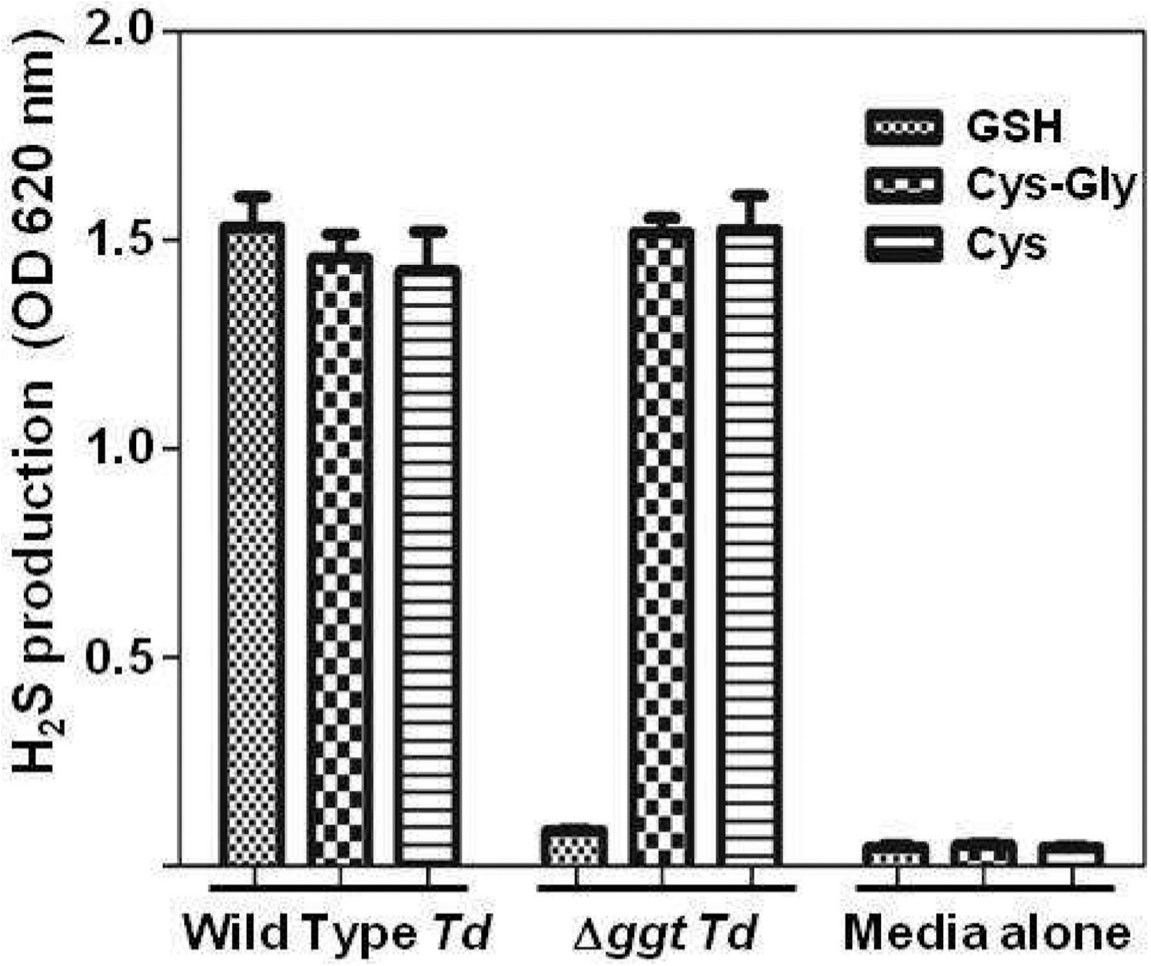

Fig. 2. Production of H_2S from wild type *T. denticola* and its isogenic Δ*ggt* mutant.

Parental *T. denticola* (wild type *Td*) and its isogenic Δ*ggt* mutant were grown anaerobically to an $OD_{620\ nm}$ of 0.320. The indicated substrates, GSH (glutathione), Cys-Gly (L-cysteine-L-glycine), or Cys (L-cysteine) were then added individually to each sample. H_2S production (as OD at 620 nm) was measured 1 hr later. Control = media alone with no bacteria. Each experiment was done three times. Error bars indicate standard deviations of the mean.

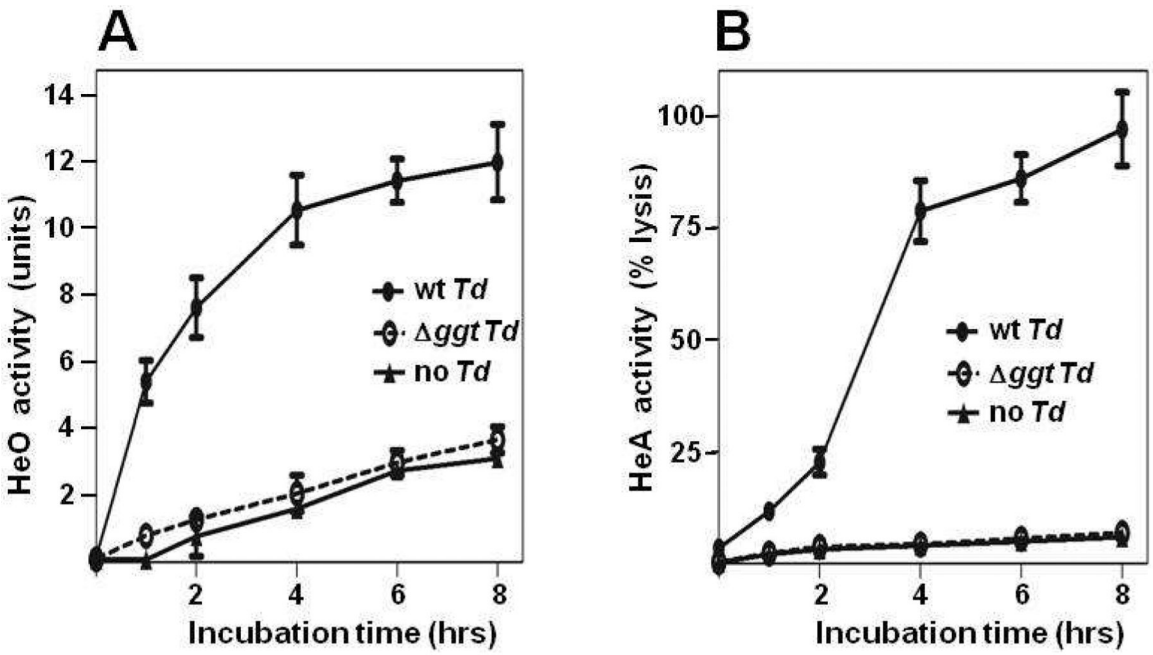

Fig. 3. Hemoxidation and hemolytic activity in wild type *T. denticola* and the Δ*ggt* mutant.

Material from sonicates of wild type *T. denticola* (wt *Td*) cells or Δ*ggt* mutant cells were added to sheep red blood cells (RBCs) in media with glutathione. Control (no *Td*) = red blood cells in media with glutathione but no bacteria. (A) Aliquots of RBCs were removed at the indicated times and hemoxidation activity (HeO), the formation of methemoglobin (or sulfhemoglobin) in the RBCs, was measured. One HeO unit is defined as 20% methemoglobin (or sulfhemoglobin) formation from the RBCs. (B) Aliquots of sample supernatants were removed at the indicated times. Hemolytic activity (HeA) was assayed by measuring the amount of hemoglobin (or its oxidized form) released from the RBCs which was determined by the optical density of the RBC supernatant at 560 nm. The percentage hemolysis was calculated relative to the amount of hemoglobin released from RBCs by hypotonic lysis, which was set as 100%.

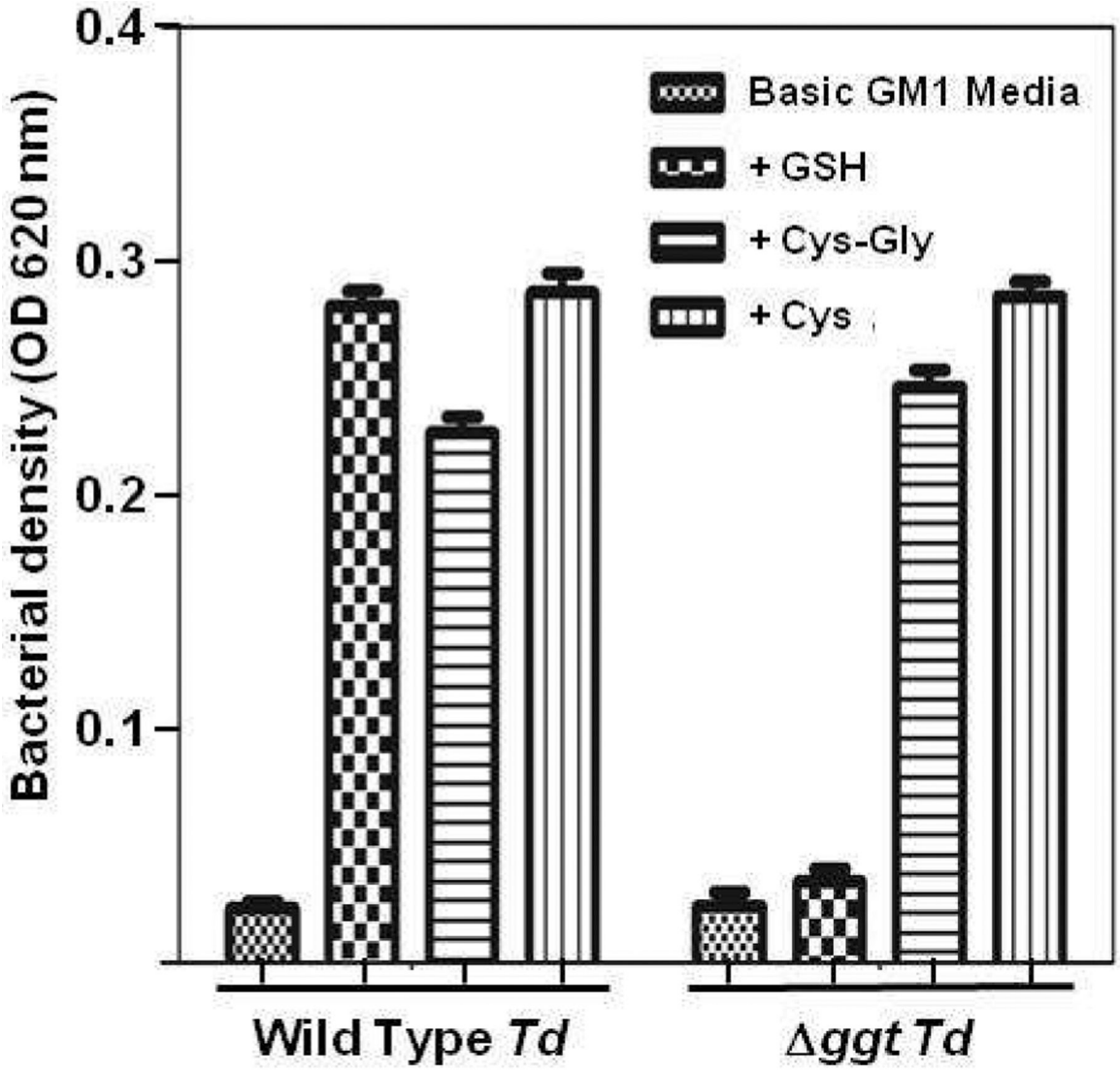

Fig. 4. Testing aerobic growth of wild type and mutant *T. denticola*.

Wild type *T. denticola* and the isogenic Δ*ggt* mutant were inoculated, to an initial OD_{620nm} of 0.09, into basic GM-1 media without L-cysteine. Glutathione (GSH), L-cysteine-L-glycine (Cys-Gly) and L-cysteine (Cys) were added individually to different tubes as indicated and the samples were incubated aerobically. After 48 hr each sample was diluted three-fold into fresh media with the same additions and incubated aerobically for another 48 hr. Growth was then measured as the absorbance of each sample at $O.D_{620nm}$. Basic GM-1 media alone (without Cys, GSH or CysGly) was used as a negative control. Each experiment was done three times. Error bars indicate standard deviations of the mean.

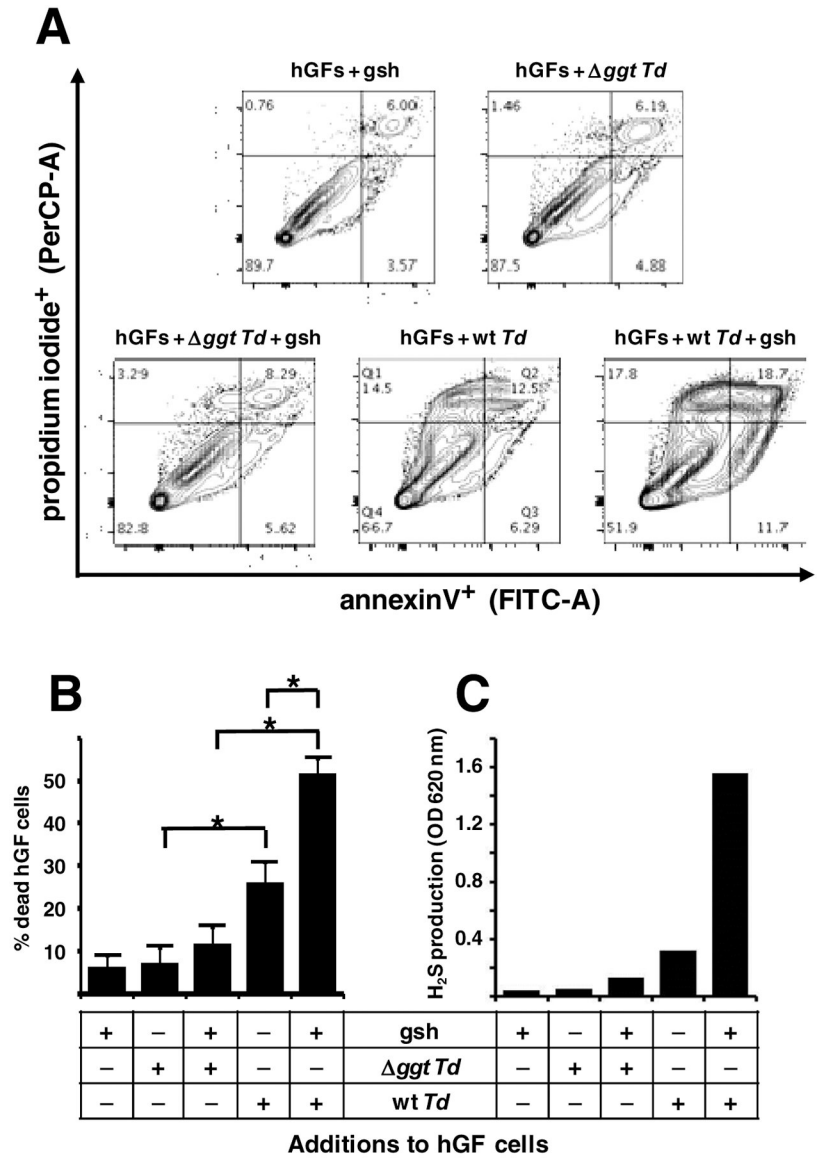

Figure 5. Effect of *T. denticola ggt* gene deletion on death of hGF cells.

Wild type *T. denticola* (wt *Td*) or *Δggt* mutant bacteria and glutathione (gsh) were added in various combinations to different tissue culture flasks of human gingival fibroblast cells (hGFs) at near80% confluency. After 24 hours incubation, cell death (apoptosis plus necrosis) of hGFs was measured by flow cytometry. The experiment was done four times (biological replicates). The levels of H_2S in the media were also measured in one experiment. (A) The hGF cells from the samples indicated were stained with annexin V and propidium iodide followed by flow cytometry analysis (30). Annexin V levels were measured with a FITC-A filter set and propidium iodide staining was measured with a PerCP-A filter set. The results of one representative experiment (of four) are shown. (B) Quantification of the four flow cytometry plots, where % death of hGF cells in the indicated samples equals the percentage of cells that stained with Annexin V, propidium iodide, or

both. (C) H_2S levels in the culture supernatants of the indicated samples. *, p = 0.001 per Tukey's test analysis.

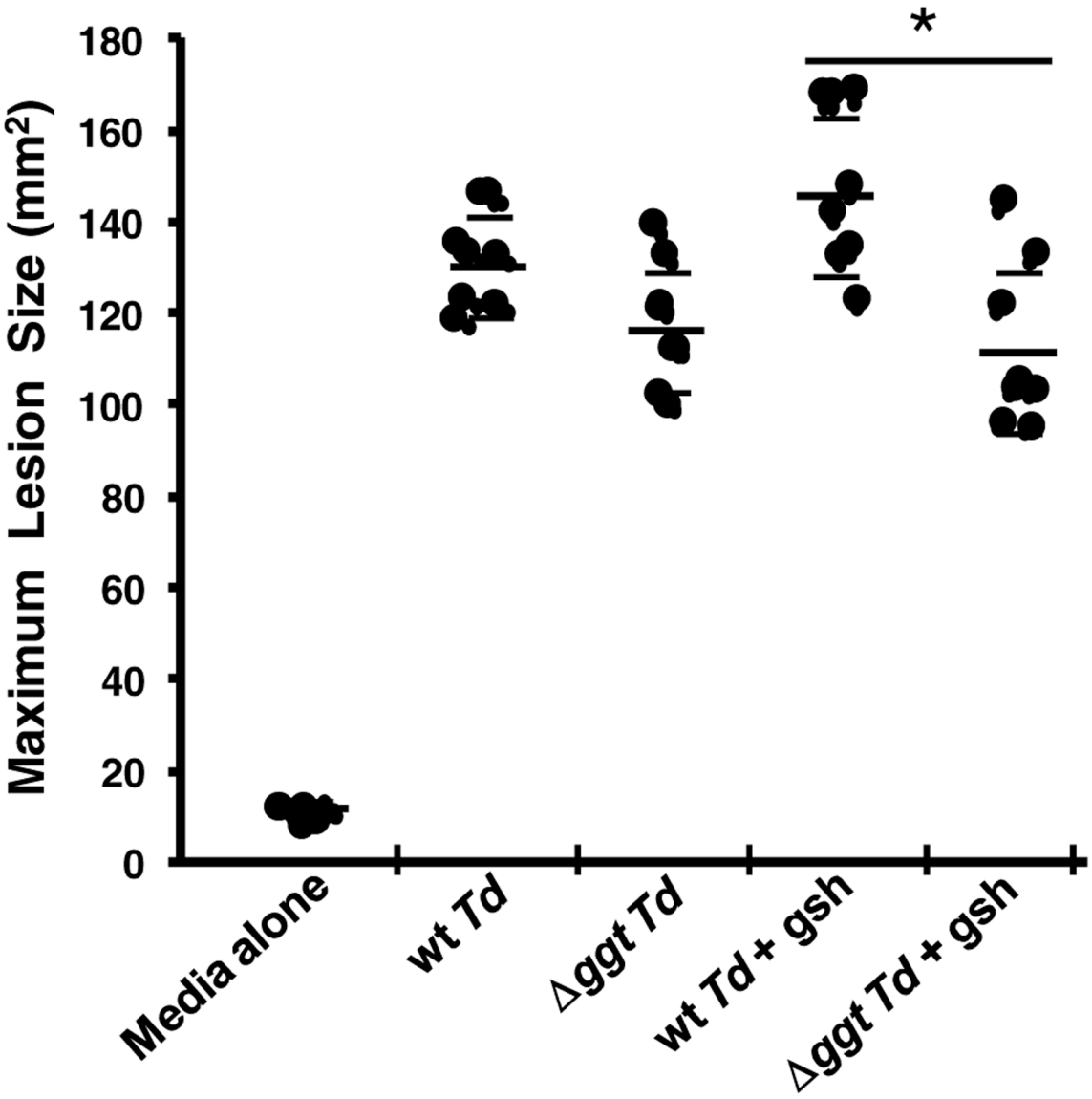

Figure 6. Effect of *T. denticola ggt* gene deletion on murine back lesion size.

Wild type *T. denticola* (wt *Td*) or *Δggt* mutant bacteria with or without glutathione (gsh) were injected subcutaneously into the backs of mice. Control mice were injected with media alone (no bacteria, no glutathione) The size of each lesion was measured as its area (mm^2) daily for 15 days. The maximum lesion size for each mouse was used to generate the mean lesion size shown as a longer, thicker bar for each group of mice (eight mice per group). The thinner, shorter bars delimit the standard deviations of the mean for each group of mice. *, p = 0.0007 per Tukey's test analysis.