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Novel Mechanism for Conditional Aerobic Growth of the Anaerobic Bacterium *Treponema denticola*▽

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***Treponema denticola*, a periodontal pathogen, has recently been shown to exhibit properties of a facultative anaerobic spirochete, in contrast to its previous recognition as an obligate anaerobic bacterium. In this study, the capacity and possible mechanism of *T. denticola* survival and growth under aerobic conditions were investigated. Factors detrimental to the growth of *T. denticola* ATCC 33405, such as oxygen concentration and hydrogen sulfide (H_2S) levels as well as the enzyme activities of gamma-glutamyltransferase, cysteinylglycinase, and cystalysin associated with the cells were monitored. The results demonstrated that *T. denticola* grew only at deeper levels of broth (≥3 ml in a 10-ml tube), high inoculation ratios (≥20% of culture in medium), and short cultivation times (≤4 days for one passage) and in media containing L-cysteine or glutathione as the substrate for H_2S production during aerobic growth. The determination of the factors showed that oxygen levels were always lower (0 to 0.6%) with significantly higher concentrations of H_2S and higher activities of the three enzymes in all cultures grown aerobically. Further data revealed that H_2S production from the *T. denticola* enzymes plus their substrates resulted in removal of dissolved O_2 in the growth cultures in a dose-dependent manner. These results demonstrated that *T. denticola* was able to generate microanaerobic environments in growth media for its survival and growth under aerobic conditions. Furthermore, the organism can be defined as a true obligate anaerobic spirochete. These findings suggest that spirochetes may play a significant role in maintaining the anaerobic environment at diseased sites in periodontitis.**

Oral spirochetes have been strongly implicated as part of the periodontopathic microbiota. They are present as the predominant organisms associated with the progression of periodontal diseases. *Treponema denticola*, a small cultivable oral spirochete, is frequently isolated from periodontal pockets (27, 43). Using in vitro studies, this spirochete was shown to express a variety of potential virulence factors, i.e., adhesins, proteinases, cytotoxic factors, chemotaxis, and peptidases as well as other virulence factors (15, 20, 21, 23, 24).

In long-term studies of periodontal diseases, two types of gaseous properties were detected. First, low oxygen levels were observed in periodontal pockets. Since periodontal pockets are generally considered to be highly anaerobic, anaerobes compose the major flora, especially in deep periodontal pockets. *T. denticola* was classified as an obligate anaerobe which cannot grow in the presence of atmospheric oxygen because it initiated growth only when there was less than 0.5% oxygen in the cultures (16, 25). The predominance of anaerobic bacteria in subgingival plaque samples suggests that the pocket environment is anaerobic. Mettraux et al. (26, 30) reported that the oxygen tension (pO_2) at the bottom of pockets ranged from 5 to 27 mm Hg, with a mean value of 13.3 mm Hg, and the oxygen sensitivities of resident organisms coincided with their proportional distribution in pockets at various pO_2s and depths. Deep pockets have a lower pO_2 than moderate pockets (7 to 10 mm/12.0 mm Hg versus 5 to 6 mm/15.7 mm Hg) and contain higher proportions of spirochetes.

In addition to oxygen differences, volatile thiol compounds were found in all periodontal pockets deeper than 3 mm and most often in the deepest regions of these pockets. Hydrogen sulfide (H_2S) was predominant among these thiol compounds at elevated concentrations of up to 2 mM (22, 34, 37, 41) in the pockets associated with periodontitis. Several reports have also indicated that some oral bacteria, including spirochetes, can produce H_2S from the metabolism of human serum proteins, L-cysteine, glutathione, and other sulfur-containing compounds (7, 8, 13, 38, 39), all of which are available in periodontal pockets (1, 52). Only low levels of L-cysteine (around 0.08 mM) are present in infected periodontal pockets. However, since polymorphonuclear leukocytes contain up to 4 mM of glutathione and significant amounts of glutathione may be released when polymorphonuclear leukocytes are damaged in the pockets, the tripeptide may be the major substrate for hydrogen sulfide formation in the pockets (3, 7, 32, 36). Despite these observations, the metabolic pathway leading to H_2S production from glutathione in periodontal pockets has not been determined. *T. denticola* resides at the bottom of deep pockets which contain high levels of H_2S and low pO_2. Nevertheless, the relationship between the two gases in the pockets is unknown. H_2S has been shown to be cytotoxic for a variety of host cells, such as gingival fibroblasts, epithelial cells, and HeLa cells (2, 9, 19, 29, 34, 35, 40, 49). Whether this microbial metabolite possesses other etiological and pathogenic properties in periodontal pockets still remains to be determined.

The strict anaerobe *T. denticola* was found to be capable of growing in aerobic gaseous environment (31, 47). Therefore, the spirochete has recently been described as a facultative anaerobic spirochete, which can grow with and without oxygen (4), in contrast to its earlier classification as an anaerobic

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bacterium (44, 45). Since *T. denticola* did not grow with the use of normal cultivation procedures aerobically, the more recent observations that the spirochete can grow under aerobic conditions may need to be reevaluated in relation to the contributions of this organism to pathogenicity in periodontal pockets. While evidence for *T. denticola* being able to grow under aerobic conditions was reported, neither the oxygen nor the H_2S concentrations in the cultures were documented in those studies.

Recently, we have described a metabolic pathway for H_2S production from glutathione as a substrate in *T. denticola* (8). We reasoned that investigations regarding the two gases, oxygen and H_2S, in in vitro cultures and in in vivo periodontal pockets are needed to further define pathogenicity in periodontal diseases. In the present study, we have investigated the capacity of *T. denticola* to survive and grow under conditional aerobic conditions as well as a likely mechanism to explain this property.

MATERIALS AND METHODS

Materials. Unless otherwise indicated, all chemicals and reagents were obtained from Sigma Chemical Company, St. Louis, MO.

Bacterial strains and culture conditions. *T. denticola* ATCC 35405, ATCC 35404, and ATCC 33520 and *Treponema vincentii* ATCC 35580 were obtained from the American Type Culture Collection (Manassas, VA). *T. denticola* GM-1 was isolated from human periodontal pockets (33, 51). All strains were stored at −78 °C in 15% glycerol medium. For normal cultivation, the spirochetes were grown in GM-1 medium (51), which contains 2% heat-inactivated rabbit serum and 6 mM L-cysteine, in screw-cap tubes (120 by 9 to 10 mm). The bacteria were incubated either anaerobically in a Coy anaerobic chamber (5% CO_2, 10% H_2, and 85% N_2) or aerobically in a 37°C warm room for the times appropriate for the experimental design. The culture purity was checked by dark-field microscopy at a magnification of ×400. *T. denticola* reference strain 35405 was used for the majority of the analyses.

Determination of bacterial growth. To determine the effects of various factors on bacterial growth, 1-day cultures, whose optical density at a wavelength of 660 nm (OD_{660}) was approximately 0.16 (about 3×10^8 cells/ml), were inoculated into complete or basic GM-1 medium (GM-1 broth without L-cysteine) at the indicated volumes (mostly 6 ml) and starting concentrations (mostly an OD_{660} of 0.032). Each of the supplements was added at a final concentration of 6 mM to the broth. The spirochetes were grown routinely in static culture at 37°C, and the growth rate was determined spectrophotometrically by OD_{660} with a Beckman DU-65 spectrophotometer at different times.

Determination of dissolved oxygen. Oxygen levels in the cultures were assessed by using a SM600 dissolved oxygen meter (Milwaukee Instruments, Inc.). Based on the manufacturer's instructions, the electrode was calibrated to zero before every measurement and the concentration of oxygen in each sample was measured at room temperature.

Hydrogen sulfide detection. Spent growth supernatant harvested from bacterial cultures by centrifugation was used for samples. Hydrogen sulfide production in the cultures was quantified as described by Siegel (42) with some modifications. Briefly, 3 μl of 0.02 M *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 3 μl of 0.3 M $FeCl_3$ in 1.2 N HCl were added sequentially to 54 μl of spent growth supernatant in a 1.5-ml microcentrifuge tube. The reaction mixture was mixed thoroughly by vortexing and sealed with Parafilm. The absorbance at 620 nm was determined after color development for 30 min at room temperature. The sulfide concentration was determined from an Na_2S standard curve. All analyses were carried out in triplicate unless otherwise indicated.

Standard curve for H_2S. The method of Carlsson et al. (14) was used to determine H_2S production. A 200 mM concentration of disodium sulfide (Na_2S) was stored at −20°C as a stock solution. H_2S was produced by neutralizing disodium sulfide in phosphate-buffered saline (PBS) or GM-1 medium to pH 7.4 with the same molarity of HCl. Production of H_2S in the reactions was determined by chemical methods as described above.

Determination of enzymatic activities. The synthetic substrates for the measurement of gamma-glutamyltransferase (GGT), cysteinylglycinase (CGase), and cystalysin activities were Na-γ-glutamyl-4-nitroaniline, Cys-Gly, and L-cysteine, respectively. These substrates represent the means to determine the three peptidase activities involved in a stepwise enzymatic pathway previously characterized in our laboratory for *T. denticola* (6). Spirochete cells were harvested from 1-day cultures by centrifugation at 6,000 × *g* for 10 min, washed twice with 0.1 M PBS (pH 7.0), and resuspended in the same buffer to an OD_{660} of 6.0. In the controls the spirochete suspension was replaced by PBS. All related measurements were carried out in triplicate.

GGT was assayed by the method of Makinen and Makinen (28) with minor modifications. The reaction mixture contained 44 μl of 0.1 M PBS at pH 7.0 and 6 μl of 20 mM Na-γ-glutamyl-4-nitroaniline in a 1.5-ml microcentrifuge tube. The reaction was initiated by the addition of substrate, and the mixture was incubated at 37 °C for 1 h. Absorption at 405 nm was monitored in a Multiskan MCC (Fisher Scientific).

CGase activity was measured by a modification of the procedures of Cappiello et al. (6) based on the specific reaction between cysteine and ninhydrin (46). The reaction mixture (final volume, 80 μl) contained 0.4 mM $MnCl_2$, 10 mM dithiothreitol, and 2 mM Cys-Gly in 50 mM Tris-HCl buffer (pH 7.3). The reaction was started by the addition of a 12-μl aliquot of washed spirochetes. After incubation for 1 hour at 37°C, the reaction was stopped by adding 5% trichloroacetic acid and the incubation mixture was centrifuged at 12,000 × *g* for 1 min. Supernatant (50 μl) was added to 50 μl of glacial acetic acid and 50 μl of a reagent prepared by dissolving 250 mg of ninhydrin in 10 ml of acetic acid–0.6 M phosphoric acid (3:2). The mixture was placed in a boiling bath for 10 min and then cooled on ice. After cooling, 150 μl of the mixture was diluted with 0.3 ml of 95% ethanol and used for spectrophotometric measurements at 540 nm.

Cystalysin (cysteine desulfhydrase) activity was determined by the colorimetric assay described by Chu et al. (10) with a reaction mixture consisting of 47.6 μl of 0.1 M PBS at pH 7.0, 10-μl aliquots of washed spirochetes, and 2.4 μl of 50 mM L-cysteine. The mixture was incubated at 37 °C for 1 h. The end product H_2S was determined by the chemical assay described above.

Specific experimental design and methods. (i) Effect of inoculum concentrations on cell growth. As starting cultures, *T. denticola* cultures at an OD_{660} of 0.16 were inoculated into GM-1 medium at the indicated concentrations in 6-ml volumes for 72 h of incubation at 37°C. The growth yield of the cells was determined for comparison between anaerobic and aerobic gaseous conditions.

(ii) Effect of culture depth on the proliferation of the spirochete. The 1-day cultures of *T. denticola* 35405 were diluted to an OD_{660} of 0.032 with GM-1 medium and then placed in tubes measuring 9 to 10 mm by 120 mm. Volumes of 1, 2, 3, 6, and 10 ml yielded liquid column heights of about 0.6, 1.5, 2.3, 5.3, and 7.1 cm, respectively. The growth yield was determined every 24 h or 72 h of incubation at 37°C in an anaerobic chamber or under aerobic conditions (warm room), and these cultures were passaged at 30% inoculation densities as indicated.

(iii) Correlation between growth yields, H_2S production, and O_2 reduction in *T. denticola* cultures. As starting cultures, *T. denticola* cultures at an OD_{660} of 0.16 were inoculated into GM-1 medium at the indicated final concentrations in 6-ml volumes for 24 h of incubation at 37 °C.

(iv) Kinetics of H_2S formation and oxygen reduction in *T. denticola* cultures. This experiment was to test the kinetic changes of H_2S production and oxygen reduction after the addition of *T. denticola* cells into the medium. Before the experiment, the GM-1 medium was stored under normal room conditions, i.e., air-saturated medium with an initial O_2 concentration of 4.6 mg/liter (5.8% O_2). One-day 20% (culture volume/medium volume) cultures of *T. denticola* were added to the medium to an OD_{660} of 0.04 (final concentration) and cultivated under aerobic conditions. Samples were taken at the indicated incubation times, and the concentrations of H_2S and O_2 were determined.

(v) Hydrogen sulfide addition and oxygen removal in the medium. In this experiment, sodium sulfide (Na_2S) was used to generate H_2S in the culture medium, since Na_2S will be converted to H_2S at neutral pH. The same molarity of Na_2S and HCl at the indicated concentrations (0, 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mM) was added to 6-ml volumes of GM-1 medium. After 30 min of incubation, O_2 levels were determined versus H_2S concentrations; kinetic analysis of O_2 reduction with the addition of 2 mM Na_2S in the media at various times was performed by determining the concentrations of oxygen in the medium.

(vi) Effects of S-containing compounds and substrates of cystalysin on the growth of *T. denticola* and H_2S production under aerobic conditions. Basic GM-1 medium without L-cysteine (including all other medium compounds and supplements) was used in the experiment. Each of the selected S- or thiol-containing compounds (cystathionine, sodium sulfate, glutathione, L-cysteine, and D-cysteine) and other compounds as substrates of cystalysin, including alanine, was used to replace L-cysteine as a supplement in the basic GM-1 medium. *T. denticola* was inoculated into the basic GM-1 medium separately containing each of the compounds at a concentration of 6 mM under aerobic and anaerobic

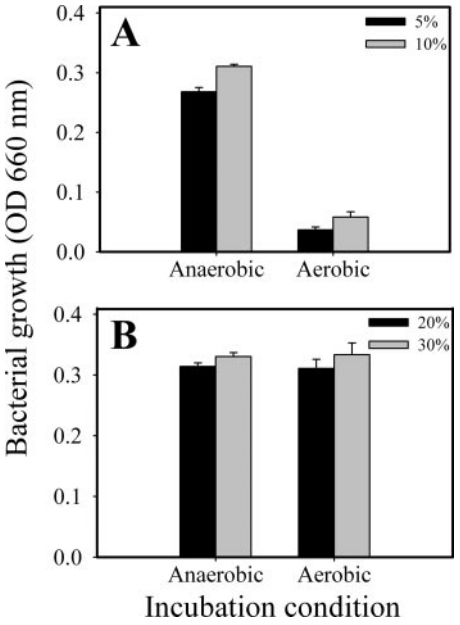

FIG. 1. Effect of inoculum concentration on cell growth under both anaerobic and aerobic gasses with regular (routine) (A) and conditional (B) inoculation. *T. denticola* cultures with an initial OD_{660} of 0.16 were inoculated into GM-1 medium under the indicated conditions in 6-ml volumes for 72 h of incubation. The regular (routine) inoculum (A) represents ≤10% inoculation (culture volume/broth volume), and the conditional inoculum (B) represents ≥20% inoculation (culture volume/broth volume). Error bars indicate standard deviations.

conditions. After 2 days of aerobic incubation, the cell growth and H_2S formation were determined for each sample.

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

RESULTS

Effect of inoculum concentration on cell growth. The growth yields of the cells determined with various inoculum concentrations under both anaerobic and aerobic gaseous conditions are shown in Fig. 1. As predicted, while the cultivation results demonstrated that the organisms were able to grow at all tested inoculation rates in the anaerobic chamber with an OD_{660} of at least 0.25 (Fig. 1A and B), the spirochete failed to grow (OD_{660} of <0.08) with the regular (routine) inocula (≤10% inoculation [culture volume/broth volume]) under aerobic conditions (Fig. 1A). These results showed that *T. denticola* was not like other facultatively anaerobic organisms and could not grow under aerobic conditions. However, the organism was able to grow well under aerobic conditions when the inoculum density was increased to ≥20% (Fig. 1B). These results indicated that *T. denticola* was able to conditionally grow in an aerobic environment and that the inoculum densities were important for this property. If the inoculum density was sufficient to support its initial survival and proliferation, the gaseous environments had less effect on the growth yields. The ODs of the cultures grown under aerobic conditions were similar to those of cultures grown under anaerobic conditions.

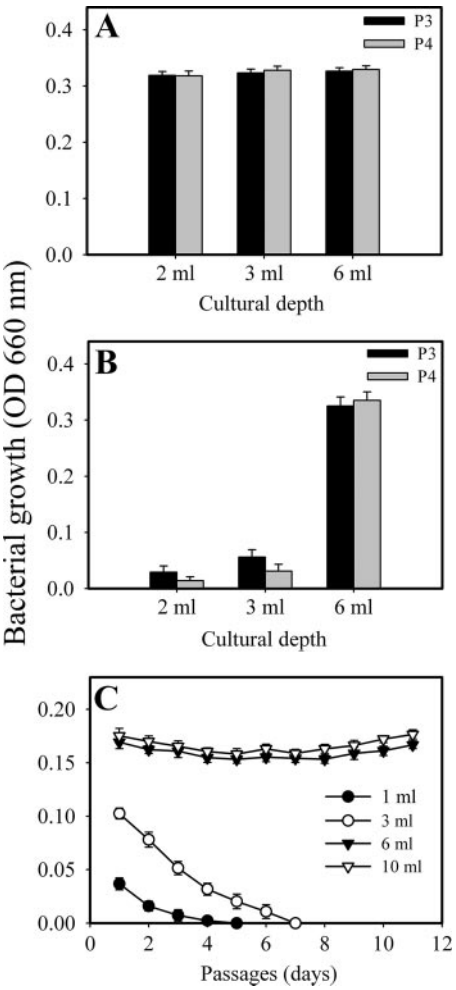

FIG. 2. Effect of broth depth on cell proliferation under both anaerobic (A) and aerobic (B and C) conditions. *T. denticola* cultures at a final OD_{660} of 0.032 were placed in tubes at the indicated volumes. The growth yield was determined every 72 h (A and B) or every 24 h (C), and the bacteria were passaged as indicated (A and B) or 30% (C). Panel C represents the growth following continuous inoculation. Error bars indicate standard deviations.

The microscopic morphologies and motilities of the organisms were comparable (data not shown).

Effect of culture depth on proliferation of the spirochete. The results showed that the organism grew and could be passaged at all culture depths in the anaerobic chamber (Fig. 2A). Under aerobic conditions, the organism can be continuously transferred over 11 passages at ≥6 ml of broth depth. However, the spirochete survived and grew for only a few passages at ≤3 ml of broth depth and dramatically failed to grow in 1 ml of broth depth (Fig. 2B and C). These results clearly show that the depth of the cultures significantly influenced the ability of the spirochete to grow under aerobic conditions and indicated that *T. denticola* only conditionally grew aerobically at appropriate broth depths.

Correlation between growth yield, H_2S production, and O_2 reduction in *T. denticola* cultures under aerobic conditions. To address the question of why *T. denticola* was able to grow only at higher inoculum densities and in deeper cultures, potential

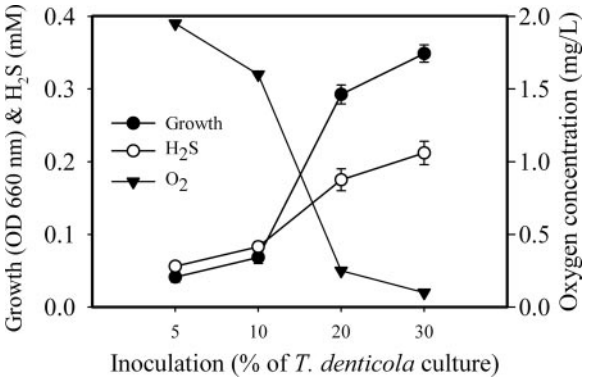

FIG. 3. Correlation between growth rate, H_2S production, and O_2 reduction in cultures of *T. denticola* under aerobic condition. *T. denticola* cultures at a starting OD_{660} of 0.16 were added to GM-1 medium at the indicated concentrations in 6-ml volumes for 72 h of incubation. Data are shown for bacterial growth and H_2S and O_2 levels in the cultures. Error bars indicate standard deviations.

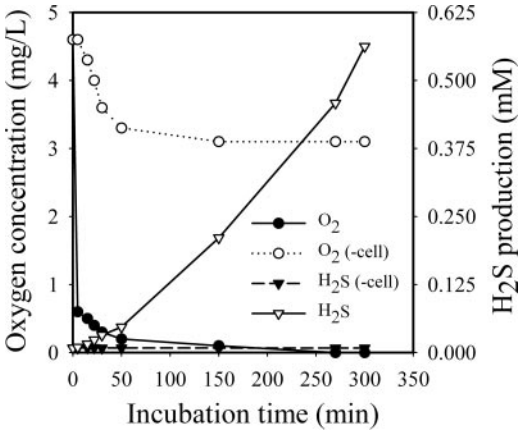

FIG. 4. Kinetic analysis of H_2S production and O_2 reduction in *T. denticola* aerobic cultivations. *T. denticola* cultures (OD_{660} = 0.048, final concentration) were cultivated under aerobic conditions. After 0, 5, 15, 22,30, 50, 150, 270, and 300 min of incubation, the changes in O_2 levels were determined for cell-free medium (○) and medium plus bacteria (●). Changes in H_2S levels are shown for medium without cells (▼) and for medium with cells (▽).

correlations between growth yield, H_2S production, and O_2 reduction in *T. denticola* cultures under aerobic conditions were examined. As seen in Fig. 3, hydrogen sulfide generation was significantly increased with significant growth of *T. denticola*. No significant levels of H_2S were determined in poorly growing cultures with regular inocula equal to or less than 10% (OD_{660} = 0.016) under aerobic conditions. However, the dissolved H_2S concentrations were determined to be over 0.2 mM when the spirochete grew well (with an OD_{660} of more than 0.3). Interestingly, in contrast to the changes in H_2S, the dissolved oxygen levels were significantly decreased with increased cellular growth. When the organism grew very poorly, oxygen concentrations of more than 1.6 mg/liter (2% O_2) were detected, which is about 85% of that in control media without bacteria (data not shown) and simulates microaerophilic conditions (31). In actively growing cultures, the oxygen concentrations dramatically dropped to less than 0.25 mg/liter (0.3% O_2), which is less than 10% of that of air-saturated water (data not shown) or the oxygen levels in an anaerobic jar (25). These results indicated that cell growth yields were positively correlated with H_2S production and negatively correlated with O_2 concentration during aerobic cultivation. It is further suggested that microanaerobic environments can be generated in *T. denticola* cultures under aerobic conditions.

Kinetics of H_2S formation and oxygen reduction in *T. denticola* cultures. To understand whether H_2S production from *T. denticola* cells will result in a reduction of oxygen in the growth cultures, H_2S and oxygen levels in the culture medium were determined after the addition of *T. denticola* compared to broth alone. The results presented in Fig. 4 show the changes of O_2 and H_2S levels in cell-free medium versus medium containing bacteria. As indicated (Fig. 4), the O_2 levels fell sharply in the initial a few minutes and were lowest over 150 min. The O_2 levels decreased to less than 0.2 mg/liter (0.25% O_2) versus about 3.1 mg/liter (3.9% O_2) for medium without cells. On the other hand, a significant rise in H_2S levels in the fresh cultures was observed at 15 min after the addition of *T. denticola* cells, and a maximum concentration of about 0.5 mmol/liter was determined at 5.5 h. No measurable H_2S was observed either in the medium without *T. denticola* or in the medium without L-cysteine and with *T. denticola* (data not shown). These results strongly suggest that the spirochete itself or H_2S produced by the organism from its substrate L-cysteine, or both, had the ability to remove almost all of the O_2 from the cultures.

Hydrogen sulfide addition and oxygen reduction in the medium. In order to directly determine whether or not H_2S was capable of removing O_2 from aerobic cultures, oxygen reduction was determined following the addition of equal molar concentrations of Na_2S and HCl. The addition of 0.5 mM Na_2S/H_2S was able to remove 27.0 ng oxygen from GM-1 medium; i.e., this caused complete removal of oxygen (98%) (Fig. 5A). When 2 mM Na_2S plus HCl was added to the culture medium, H_2S dramatically reduced oxygen levels to undetectable within 6 min (Fig. 5B). These results strongly indicated that the concentrations of H_2S alone were sufficient to rapidly remove O_2 almost completely from the culture medium and resulted in the formation of an anaerobic environment in the medium.

Effects of S-containing compounds and substrates of cystalysin on the growth of *T. denticola* and H_2S production under aerobic conditions. We have described an enzyme pathway for H_2S production from glutathione, Cys-Gly, and L-cysteine as substrates (8, 12). To further investigate the possible mechanism for the ability of *T. denticola* to grow under aerobic conditions, some sulfur-containing compounds and substrates of cystalysin were selected for the replacement of L-cysteine as a supplement in GM-1 medium for supporting *T. denticola* growth under aerobic conditions. The organism grew well in the presence of L-cysteine or glutathione, and such growth was accompanied by significant levels of H_2S ($P < 0.01$) (Fig. 6A). In contrast, when L-cysteine was replaced by alanine or cystathionine, the substrates for cystalysin and other thiol-containing compounds in the basic GM-1 medium, essentially no H_2S was detected in the cultures and the spirochete failed to grow (Fig. 6A). A dose-dependent positive correlation with L-cysteine, H_2S production, and treponeme growth was observed

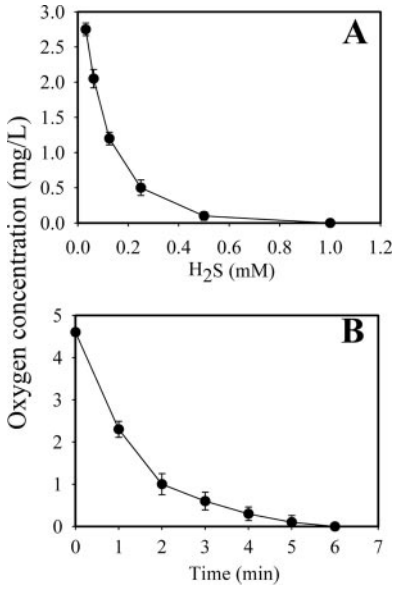

FIG. 5. Demonstration of oxygen removal by the addition of H_2S in GM-1 medium (A) and kinetic analysis of O_2 reduction with the addition of H_2S (B). Various concentrations of H_2S (Na_2S and HCl at the same molarity) were separately added to 6-ml volumes of GM-1 medium in tubes. After 30 min of incubation, O_2 levels were determined versus H_2S concentrations (A). Kinetics of O_2 reduction were assayed following the addition of 2 mM H_2S (B). Error bars indicate standard deviations.

(Fig. 6B). Similar results were obtained in the presence of glutathione (data not shown). H_2S as one of the enzyme products is not required for *T. denticola* growth under anaerobic conditions (data not shown). These results support the hypothesis that only those thiol-containing compounds effective as substrates for cystalysin or gamma-glutamyltransferase producing H_2S can support *T. denticola* growth under aerobic conditions. Identical results were obtained with *T. denticola* 35404 and 33520 and the clinical isolate GM-1 (data not shown), each of which contained the intact three-enzyme pathway. By contrast, *T. vincentii* failed to grow under the conditions tested in this study, since it was not able to produce H_2S from glutathione, Cys-Gly, or L-cysteine (data not shown).

DISCUSSION

In the present study, we investigated the effect of H_2S production, O_2 availability, and changes in the levels of L-cysteine on the in vitro growth conditions for *T. denticola*. The results suggested a model for *T. denticola* growth under aerobic conditions, based upon several observations. First, it was demonstrated that *T. denticola* did not survive and grew under routine aerobic conditions (<20% inoculation) (Fig. 1A). However, the spirochete was conditionally capable of growth under these atmospheric conditions (Fig. 1B, 2, and 3). This observation is not in agreement with previous reports (16, 25, 44, 45) which suggested that this spirochete is an obligate anaerobe. Nevertheless, others have previously proposed that *T. denticola* was a facultative anaerobic spirochete (31, 47). In contrast, our findings revealed that the organism is only conditionally capable of surviving and growing under strict aerobic conditions (Fig. 1B and 3). The second relevant observation is the demonstration that the significant levels of dissolved O_2 in the cultures of *T. denticola* grown under normal atmospheric conditions were reduced to <0.5%, facilitating the growth of the spirochete under microanaerobic conditions (Fig. 4). Furthermore, our data suggest that the addition of *T. denticola* cells resulted in the coupling of O_2 removal from the culture medium to the simultaneous production of H_2S (Fig. 4). Third, it was observed that H_2S production from *T. denticola* cultures was always accompanied by the removal of dissolved O_2 in the culture medium under aerobic conditions (Fig. 4). Furthermore, the coupling of H_2S production with O_2 removal was found to be dose dependent (Fig. 5). Therefore, H_2S production might be the real trigger for removal of dissolved O_2 in the growth cultures and the creation of anaerobic conditions for promoting the growth of *T. denticola*. Finally, following a pro-

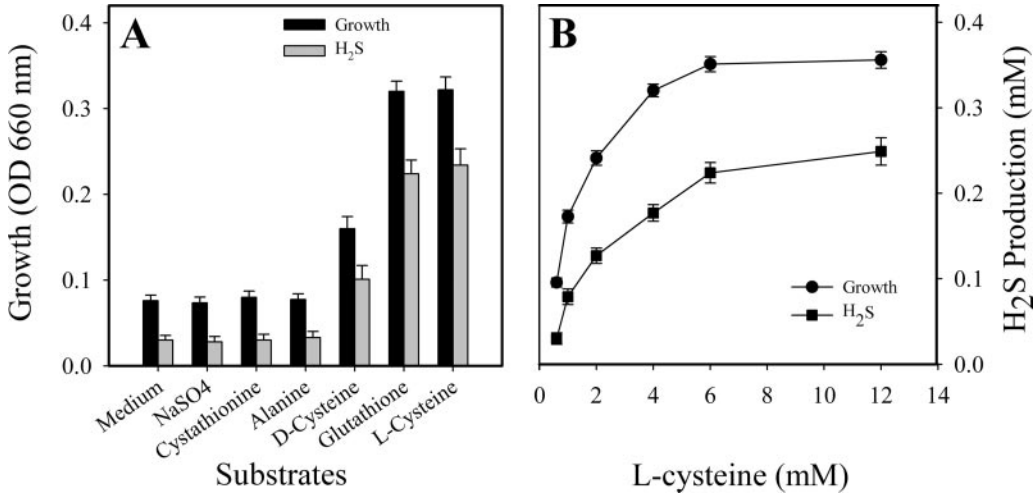

FIG. 6. Effects of thiol compounds on growth of *T. denticola* and H_2S production under aerobic conditions. (A) Effects of substrates for cystalysin and GGT as well as other compounds on *T. denticola* growth. (B) Positive correlation of H_2S production and growth with dose dependence in the presence of L-cysteine in the culture. Error bars indicate standard deviations.

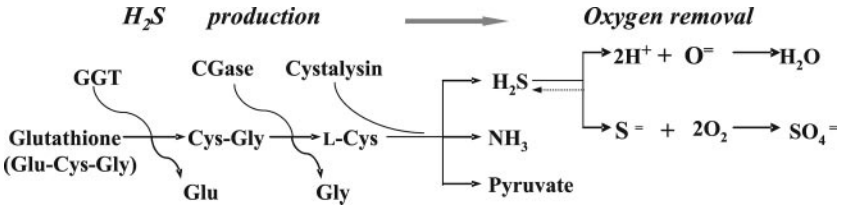

FIG. 7. Proposed model for H_2S production in *T. denticola* and creation of an anaerobic microenvironment in aerobic cultures. Our model entails the presence of an H_2S-producing enzyme pathway which is critical in the development of a local microanaerobic environment for *T. denticola* and the promotion of the subsequent conditional growth of the organism under aerobic conditions.

cess of screening several S-containing compounds, peptides, and amino acids, L-cysteine and glutathione appeared to be the main sources for H_2S production in *T. denticola* culture medium under aerobic conditions and the major supplements for supporting the growth of *T. denticola* (Fig. 6). Based upon previous publications, all growth media for *T. denticola*, including TYGVS, MTYGS, GM-1, and PTY broth (8, 18, 31, 47), contained L-cysteine as one of the major supplements. *T. denticola* can utilize this substrate to produce H_2S (10), which reduces O_2 and creates a microanaerobic environment to facilitate the growth of the spirochete under aerobic conditions. We have previously described a stepwise enzyme pathway for H_2S production. Three enzymes, GGT, CGase, and cystalysin, were found to be involved in converting any of the three substrates glutathione, Cys-Gly, and L-cysteine into H_2S (8, 10, 11). Therefore, we propose that the three-enzyme pathway is likely to be an important player in the coupling of H_2S production to O_2 removal in mediating the survival of *T. denticola* under aerobic conditions.

Taking all of the present findings together, we propose a model for H_2S production involving a stepwise enzyme pathway in *T. denticola* which produces a microanaerobic environment for promoting the growth of the organism (Fig. 7). To this end, culture media containing one of the three substrates, i.e., glutathione, Cys-Gly, or L-cysteine, can be converted by *T. denticola* cells in a stepwise manner to the end product H_2S. When acted on under certain conditions, H_2S releases $2H^+$ and sulfide (S^{2-}). The strong reducing reagent H_2S will subsequently react with dissolved O_2 and produce H_2O and sulfate (50). Therefore, such changes in the medium will provide protective conditions supporting the survival and growth of *T. denticola* under aerobic conditions. The culture depth and the size of the inocula are two additional factors which favor the creation of such a microanaerobic environment.

To better explain the capacity of *T. denticola* to grow under aerobic conditions, oxygen metabolism and the oxidative enzymes, including NAD (NADH) oxidase (5), oxidoreductase, peptidohydrolase (47), and peptidases as well as other enzymes (31), have been previously studied. Thus far, no specific oxidative enzymes had been found to be capable of removing dissolved O_2 from the culture medium. In this study, our results have demonstrated that in *T. denticola*, GGT and cystalysin activities were increased under aerobic conditions compared to anaerobic conditions (data not shown). The mechanism for the up-regulation of these enzymatic systems is not clear and remains to be studied.

Some current views suggest that anaerobes may have some ability to metabolize oxygen, but these organisms usually lack sufficient defenses against the toxic metabolites of oxygen such as H_2O_2, $O_2 \cdot^-$, or $OH \cdot$ (5, 29). *T. denticola* 35405 was shown to possess a moderate oxygen metabolic activity based mainly on NADH oxidases, and it also produces NADH peroxidase and superoxide dismutase, which are known to be protective against oxidative damage (5, 26). These protective enzymes, even if they may contribute to survival in aerobic environments, do not appear to be primarily involved in *T. denticola* growth under aerobic conditions for the following reasons. First, our experiments showed that only those S-containing compounds capable of producing H_2S can support *T. denticola* growth under aerobic conditions. This indirectly demonstrated that the ability to metabolize oxygen is not a likely possibility for the organism to sustain aerobic growth. Second, the gaseous environment had no remarkable effect on the catalase, peroxidase, and superoxide dismutase activities (47). Third, it has been shown previously that fresh clinical isolates of anaerobic bacteria can tolerate oxygen exposure for about 8 h at room temperature (48), which suggests that oxygen tolerance based on the function of superoxide dismutase is not sufficient to support growth under aerobic conditions. Our findings have also shown that deeper broth cultures with higher H_2S versus lower O_2 levels favor cell proliferation and are in agreement with earlier clinical observations (15, 16, 21, 25, 26, 30). It was reported that H_2S is a major metabolic end product detected in deep periodontal pockets which is produced by resident periodontopathic microbiota (8, 41).

At least five genera of anaerobic oral microorganisms commonly found in the deepest portion of these pockets have the capacity to form H_2S, and the most potent producers of H_2S were *T. denticola* and the black-pigmented species *Porphyromonas gingivalis*, *Bacteroides intermedius*, *Bacteroides loescheii*, and *Porphyromonas endodontalis* (39, 41). Deep pockets also were found to have a lower pO_2 than moderate pockets and to contain higher proportions of strictly anaerobic spirochetes such as *T. denticola* (26). We speculate that the mechanisms by which these prokaryotes create this environmental niche in periodontal pockets may be similar to the coupling of H_2S production with removal of dissolved O_2 as demonstrated in this study. Therefore, these findings suggest that spirochetes may play a significant role in maintaining the anaerobic environment at diseased sites in periodontitis and also suggest that local oxygen therapy may serve as a novel treatment for necrotizing periodontal disease (17).

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