THE EVOLUTION OF HYDROGEN SULFIDE BY *GLUCONOBACTER* SPECIES

by

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
in
Microbiology

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January 18, 1995

Blacksburg, Virginia

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(ABSTRACT)

Previous studies demonstrate that members of the strictly aerobic genus *Gluconobacter* produce detectable quantities of hydrogen sulfide (H_2S) when incubated in SYP medium (5% sorbitol, 1% yeast extract, and 1% peptone) containing thiosulfate. This finding is puzzling, since the microbial evolution of H_2S is characteristic of anaerobic or facultative bacteria. The goal of this research was to determine the physiological role of H_2S evolution for the gluconobacters. A methylene blue method was used to quantify the amount of H_2S evolved from cultures grown aerobically for 3 days at 28°C. Five of the six tested strains of gluconobacter evolved from 6 to 68 μg of H_2S. Strains which grew to a higher density (> 300 μg cell protein/ml) evolved between 10 and 68 μg of H_2S. Strains which grew to a lesser extent (< 140 μg cell protein/ml) evolved no more than 6 μg of H_2S. Uninoculated SYP medium containing 1% thiosulfate showed no evidence of H_2S evolution; however, sterile SYP medium with decreasing concentrations of yeast extract and peptone showed increasing amounts of H_2S evolved. When SYP medium was exhausted by gluconobacter growth for 72 hours, then supplemented with thiosulfate, filter sterilized, and incubated for 3 days

at 28°C, these sterile solutions evolved over 400 μg of H_2S. A drop in pH, similar to that which occurs during gluconobacter growth, is not sufficient to evolve H_2S. My results to date suggest that H_2S evolution results not from gluconobacter metabolism, but rather from spontaneous decomposition of thiosulfate and the depletion of media components during growth.

Dedicated to
Richard V. and Monica L. Swartwood

ACKNOWLEDGMENTS

First and foremost, I thank the members of my committee: Dr. G. William Claus, Dr. James G. Ferry, and Dr. Noel R. Krieg for their insightful ideas. Without those ideas one mystery of the gluconobacters might have remained a mystery. I especially thank Dr. Claus for his support, concern, and encouragement when my negative controls became positive and the bottom seemed to fall out of my project.

I thank Lori Brookman for her friendship, advice, and mutual commiseration.

I thank Jonathan Scherrer for his belief in my abilities and his constant love during the turmoil of graduate school.

Lastly, I thank my family, for their love, support, and encouragement. Without them, I never would have accomplished all that I have.

I acknowledge the Sigma Xi Society for partial funding of my research.

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INTRODUCTION

The genus *Gluconobacter* is composed of gram negative, heterotrophic, strictly aerobic, short rods (12). They are characterized by: (i) their inability to completely oxidize ethanol, acetic acid, and other substrates to carbon dioxide and water (3); (ii) the lack of a complete Tricarboxylic Acid (TCA) cycle (17); and (iii) their ability to carry out a limited (one- or two-step) oxidation of hydroxyl-containing organic compounds and to quantitatively excrete the oxidized products into the media (13). Additional information on the habitat, morphology, and physiology of the gluconobacters is given in Appendix A.

Early phenotypic studies lead to the proposal of a single gluconobacter species called *G. oxydans* (16, 23). However, DNA/DNA homology studies performed by Yamada et al. (36) and Micales et al. (26) found two and three distinct homology groups, respectively. Differentiating phenotypic characteristics for the three homology groups of Micales et al. (26) were later characterized as: (i) differences in growth on arabitol as the primary carbon source; (ii) differences in growth on ribotol as the primary carbon source; and (iii) growth after three passages and incubation for 24 hours in nicotinate-deficient media (24). The species names proposed for the three homology groups were *G. oxydans*, *G. frateurii*, and *G. asaii* (24).

In conducting phenotypic tests to differentiate among the three gluconobacter homology groups, Mason and Claus (24) reported that eight of eight *Gluconobacter* strains show hydrogen sulfide production

when incubated aerobically in a chemically complex medium containing sodium thiosulfate. However, the strictly aerobic physiology of the gluconobacters does not lend itself to an easy explanation of this phenomenon. The production of hydrogen sulfide has traditionally been attributed to only two types of bacteria: those which reduce thiosulfate during anaerobic bacterial respiration, and those which degrade sulfur containing organic compounds. Additional information on bacterial thiosulfate metabolism is located in Appendix B.

The purpose of this research is to better understand the physiology of hydrogen sulfide production in the *Gluconobacter* species.

MATERIALS AND METHODS

Bacterial strains. To examine the possible connection between the quantity of H_2S evolved and the three species of the genus *Gluconobacter* (24), I chose the following six stains: ATCC strain 19357, ATCC strain 23561a (both are *G. oxydans*); IFO strain 3264, IFO strain 3254 (both are *G. frateurii*); IFO strain 3276a, IFO strain 3297a (both are *G. asaii*).

Culture maintenance. Working cultures were maintained as unfrozen liquid suspensions in 66% glycerol stored at -15°C. For long term storage, cells were suspended in 15% glycerol and stored in liquid nitrogen (34).

Chemicals and solutions. Sodium sulfate, sodium sulfite, and sodium tetrathionate were obtained from Sigma Chemical Company (St. Louis, MO). Sodium thiosulfate was obtained from Fisher Scientific (Pittsburgh, PA). All medium components were obtained from Difco Laboratories (Detroit, MI). All solutions were prepared as percent weight per volume (w/v) in distilled-deionized water (dd H_2O) unless otherwise noted. Standard 5% sorbitol broth contained 5% sorbitol, 1% peptone, and 1% yeast extract (SYP). Standard 5% sorbitol agar contained the same quantities of sorbitol, peptone, and yeast extract plus 2% agar.

Subcultures. A 0.02-ml portion of a working culture was used to inoculate five or six ml of SYP medium in 25-ml test tubes (inside diameter = 1.5 mm). Cultures were incubated at 28°C and aerated by rotating them at 27 rpm on a roller-drum apparatus (model TC-5; New

Brunswick Scientific Co., Inc.). These culture tubes were used as cuvettes to determine the optical density at 620 nm (OD_{620}) using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc.). When the culture OD_{620} reached 0.8, it was used as an inoculum for growth experiments.

Growth comparisons. Fifty milliliters of SYP medium with or without additives were placed in 500-ml growth flasks, sterilized by autoclaving, and inoculated with 0.02 ml of a working culture. Flasks were incubated at 28°C with shaking at 200 rpm. Growth was determined by measuring the optical densities at 620 nm (OD_{620}) on a Milton Roy Spectronic 1201 using a quartz cuvette with a 1-cm path length.

Total protein. SYP medium plus thiosulfate was separately inoculated with 0.5 ml of a subculture of one of six strains of gluconobacter. After incubating at 28°C for 3 days, 1 ml samples of these bacterial suspensions were separately diluted with 3 ml of dd H_2O, centrifuged for 10 min at 3500 x G, and the cell pellets resuspended in 1N NaOH. The resulting alkaline-treated suspensions were heated in a 80°C water bath for 60 min to destroy the cell and hydrolyze the cellular proteins. One-half ml of each hydrolyzed sample was transferred to a clean test tube and diluted with 0.5 ml dd H_2O. Total protein in each hydrolyzed sample was determined by the Lowry method (18).

A 1 ml sample of BSA solution (10 mg/ml) was added to 1 ml of 2N NaOH and heated in a 80°C water bath for 60 minutes. The heated BSA was diluted with 4 ml of dd H_20 and the resulting BSA solution (1000 μg

protein/ml) was used to make solutions containing between 0 and 500 µg of protein. These samples were treated as described above for the hydrolyzed samples. A standard curve was prepared to correlate the total protein in each sample with absorbance of the treated sample at 500 nm.

Evolved hydrogen sulfide detection and quantitation. H_2S was qualitatively detected by placing lead-acetate-saturated-filter paper (31) in the lids of inverted petri plates during incubation.

Quantitative determination of H_2S was accomplished by covering the mouth of a 25-ml Erlenmeyer flask with a conically shaped Whatman 42 (4.25 cm diameter) filter paper that was saturated with 1M zinc-acetate (27). The mouth of the flask was then covered with aluminum foil and the flask was incubated at 28°C for 3 days. After incubation, the zinc-acetate-saturated-filter paper was transferred to a 50-ml beaker containing 22 ml of dd H_2O. Two and one half ml of p-aminodimethyl aniline sulfate and 0.5 ml of ferric ammonium sulfate (33) was added to the beaker. The beaker was gently swirled to mix the reagents and then incubated at room temperature for about five minutes to allow for methylene blue formation (27). The resulting color was measured spectrophotometrically at 670 nm using a Milton Roy Spectronic 1201 spectrophotometer.

A standard curve was prepared using known amounts of evolved H_2S to correlate µg of H_2S with the absorbance of methylene blue at 670 nm as described by Morra and Dick (27) with the following exception. The contents of the center well were transferred to a 50 ml beaker

containing 22 ml of dd H_2O and treated as described in the preceding paragraph.

All evolved H_2S determinations were made from 5-ml volumes contained in 25-ml Erlenmeyer flasks. For evolved sulfide determinations from cultures, 4.5 ml of SYP medium with and without additives in 25-Erlenmeyer flasks was inoculated with 0.5 ml of subculture. Unless otherwise noted, these flasks were statically incubated in the air at 28°C for 3 days. All determinations of H_2S evolution were performed at least three separate times with four replicates each time.

Detection of soluble sulfide. The quantity of dissolved sulfide was determined by adapting the method of Rand, et al. (30). One-half milliliter samples were transferred to 20-ml screw-cap glass test tubes (inside diameter = 9 mm) containing 0.2 ml 2M zinc acetate and 0.2 ml 6N NaOH. These test tubes were filled with dd H_2O, capped with threaded Teflon lids, and refrigerated for 72 h to allow the precipitate to settle. The supernatant fluid was then decanted, and the pellet was dissolved in 0.2 ml ferric ammonium sulfate and 0.5 ml p-aminodimethyl aniline sulfate. These tubes were filled with dd H_2O, recapped, inverted several times to mix the reagents, then left at room temperature for about five minutes to allow for methylene blue formation. The resulting color was measured spectrophotometrically at 670 nm using a Milton Roy Spectronic 1201 spectrophotometer.

A known amount of $Na_2 \cdot 9H_20$ was dissolved in SYP medium to prepare a solution containing 10 μg sulfide/ml. Different volumes of this

solution were used as samples and treated as described in the preceding paragraph. A standard curve was prepared to correlate micrograms of the known amount of soluble sulfide to the absorbance of methylene blue at 670 nm.

RESULTS

Hydrogen sulfide (H_2S) evolution by the gluconobacters. To determine if H_2S was evolved from gluconobacters in a measurable and consistent manner, the following experiments were performed three times with four replicates each time. *Gluconobacter oxydans* ATCC strain 19357 was inoculated into standard 5% sorbitol (SYP) medium either with or without 1% sodium thiosulfate and incubated at 28°C for 3 days. Sterile uninoculated controls were incubated in the same manner. After incubation, no evolved H_2S was detected from either the uninoculated medium or the *G. oxydans* culture incubated in the absence of thiosulfate. However, when incubated in the presence of thiosulfate, ATCC strain 19357 evolved 68.5 with a standard deviation of 4.6 μg of H_2S. Five additional strains representing the three gluconobacters species were incubated under identical conditions. Four of the five cultures evolved H_2S but only in the presence of sodium thiosulfate. These results indicated that (i) the gluconobacters were responsible for the evolution of H_2S, and (ii) the presence of thiosulfate is necessary for H_2S evolution.

Degradation of cysteine. To determine if cysteine served as a source of the H_2S, the following experiment was performed three trials with four replicates each time. The same strains representing the three species of gluconobacters were separately inoculated into SYP medium plus 0.02% cysteine and incubated at 28°C for 3 days. After incubation,

no H_2S evolution was detected for any of these strains. These results indicated that cysteine was not the source of the H_2S.

Assimilatory reduction of thiosulfate. During assimilatory reduction, thiosulfate is reduced independently of anaerobic respiration to form the sulfide ion and the sulfide ion is used by the cell in the synthesis of cysteine. If the gluconobacters reduce thiosulfate for this purpose, then cysteine must be limiting to either the rate or extent of growth in the SYP medium. The possible reduction of thiosulfate for biosynthesis was examined by incubating *G. oxydans* ATCC strain 19357 in SYP medium either with or without cysteine. The inclusion of cysteine had neither a positive nor a negative effect on growth as compared to the SYP medium (Figure 1). These results indicated that neither the rate nor extent of growth of gluconobacters was limited by cysteine. Therefore, it seems unlikely that the H_2S evolution is a consequence of excessive sulfide formation during assimilatory thiosulfate reduction.

In addition, the inclusion of the thiosulfate had an inhibitory effect on growth (Figure 1) which was contrary to the predicted result. If the absence of sulfide ions or sulfur-containing organic compounds, such as cysteine, was limiting to either the rate or extent of growth of the gluconobacters, then the inclusion of thiosulfate should have a positive effect on either the rate or extent of growth.

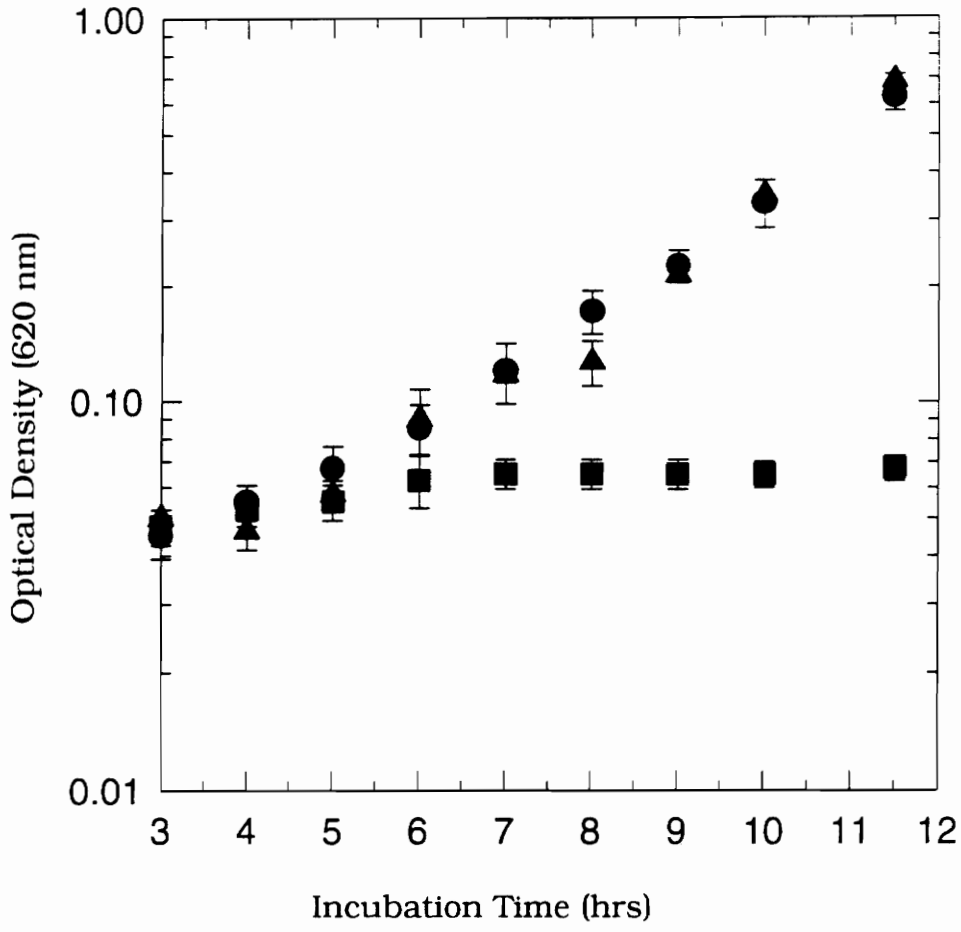

Figure 1. Growth of *Gluconobacter oxydans* ATCC strain 19357 in Standard 5% sorbitol medium (●), standard 5% sorbitol medium with 0.02% cysteine (▲), and standard 5% sorbitol medium with 1% sodium thiosulfate (■). Growth flasks containing 50 ml of medium were inoculated with 0.5 ml of a prepared culture and incubated at 28°C with shaking as described in the Materials and Methods. Each point represents the mean of two trials with two replicates for each trial. The standard deviation of all the replicates is indicated by the error bars at each point.

Anaerobic respiration. Reduction of inorganic sulfur compounds during anaerobic respiration by anaerobic and facultative bacteria characteristically leads to the evolution of H_2S. To examine the possibility of anaerobic growth among the gluconobacters, solid SYP media separately containing various reduced sulfur sources were prepared and streaked with the same strains representing the three species of gluconobacters and concurrently incubated under both anaerobic and aerobic conditions. After 10 days incubation at 28°C, all plates incubated under anaerobic conditions showed no evidence of colony formation (Table 1). However, colonies were evident on plates incubated under aerobic conditions except for those containing 1% sodium sulfite. These results indicated that the gluconobacters are not capable of sufficient anaerobic respiration using thiosulfate as a terminal electron acceptor to support growth.

Although the gluconobacters were incapable of anaerobic growth using thiosulfate, it was conceivable that they could still maintain themselves by reducing thiosulfate. Therefore, I wished to examine if the gluconobacters were capable of reducing thiosulfate to H_2S under anaerobic conditions. Solid SYP medium containing thiosulfate was prepared and streaked with same strains representing the three species of gluconobacters. A sterile piece of lead-acetate-saturated filter paper was placed in the lids of the inverted plates to detect evolved H_2S. These plates were then incubated as described in Table 1 and showed no evidence of colony formation under anaerobic conditions (Table 2). However, H_2S was evolved under both aerobic and anaerobic conditions

Table 1. Test for aerobic and anaerobic growth on solid media with various reduced sulfur compounds.[a]

Species	Strain	Aerobic growth			Anaerobic growth		
		$SO_4^=$	$SO_3^=$	$S_2O_4^=$	$SO_4^=$	$SO_3^=$	$S_2O_4^=$
G. oxydans	ATCC 23651	+	-	+	-	-	-
	ATCC 19357	+	-	+	-	-	-
G. frateurii	IFO 3264	+	-	+	-	-	-
	ATCC 15178	+	-	+	-	-	-
G. asaii	IFO 3276a	+	-	+	-	-	-
	IFO 3297a	+	-	+	-	-	-
Uninoculated		-	-	-	-	-	-

[a] All cultures were incubated on plates containing standard 5% sorbitol medium with 2.0% agar and 1% of either sulfate, sulfite, or tetrathionate at 28°C for 10 days. Growth was defined as visible colonies as examined under 30X on a dissecting microscope. Growth = +; no growth = -. Results are representative of three trials with two replicates each for each trial.

Table 2. Test for aerobic and anaerobic growth and hydrogen sulfide evolution on solid media containing thiosulfate.

Species	Strain	Aerobic		Anaerobic	
		growth [a]	H_2S [b]	growth [a]	H_2S [b]
G. oxydans	ATCC 23651	+	+	-	+
	ATCC 19357	+	+	-	+
G. frateurii	IFO 3264	+	+	-	+
	ATCC 15178	+	+	-	+
G. asaii	IFO 3276a	+	+	-	+
	IFO 3297a	+	+	-	+
Uninoculated		-	+	-	+

[a] All cultures were incubated on plates containing standard 5% sorbitol medium with 2.0% agar and 1% sodium thiosulfate at 28°C for 10 days. Growth = +; no growth = -. Results are representative of three trials with two replicates each.

[b] H_2S was qualitatively detected by the discoloration of lead-acetate-saturated filter paper as described in the Materials and Methods. H_2S evolution = +; no evolution = -. Data represent three trials with two replicates for each trial.

(Table 2). Since H_2S evolution occurred under both aerobic and anaerobic conditions, it seemed very unlikely that H_2S evolved as a result of anaerobic respiration using thiosulfate. Instead it seemed possible that H_2S production was caused by an abiotic process.

Abiotic reduction of thiosulfate. The following experiment was performed, to determine if the presence of cells was required for H_2S evolution. Cultures of *G. oxydans* ATCC strain 19357 were prepared as described in the Materials and Methods. When the cultures reached an OD_{620} of 0.8, 5 and 10 ml samples of the bacterial suspensions were centrifuged and the cells resuspended in sterile solutions of either 5% sorbitol, dd H_2O, or 1% sodium thiosulfate. Each suspension was separately transferred to a sterile flask and incubated in the presence of lead-acetate-saturated filter paper at 28°C for 3 days to qualitatively detect H_2S evolution. Sterile solutions were also incubated in the same manner. Within 24 hours, H_2S evolution was detected from all the sodium thiosulfate solutions including the sterile control. These results indicated that H_2S evolution from thiosulfate resulted from an abiotic decomposition of the thiosulfate under these incubation conditions.

The Merck Index (35) states that thiosulfate decomposes at elevated temperatures to form sulfide. Based on this and the observation that sterile SYP medium with thiosulfate does not evolve H_2S (see Result p. 8), I hypothesized that sulfide is formed under these incubation conditions but that it reacts with SYP medium components thus remaining in solution until those components are removed. To test this hypothesis, SYP medium was diluted with dd H_2O or used alone to

prepare sterile solutions containing 0% to 100% SYP, and all were incubated at 28°C for 3 days. The solutions having the least amount of medium components evolved the most H_2S (Table 3).

The quantity of soluble sulfide was also determined for each of the sterile solutions described above. The sterile solution containing the highest concentration of medium components had the highest quantity of soluble sulfide. As the concentration of the medium components decreased, the quantity of soluble sulfide decreased. These results suggested two things: first, that the sulfide was formed abiotically by the decomposition of thiosulfate and, second, that the sulfide was released as H_2S as the medium components were depleted. These results also suggested that the H_2S evolved from the gluconobacter cultures resulted from two things: first, the decomposition of thiosulfate to sulfide during incubation and, second the consumption of media components during growth of the gluconobacters.

The following experiments were performed to determine whether the sorbitol or some component of the yeast extract or peptone reacted with the sulfide to keep it in solution. Solutions containing varied concentrations of peptone and yeast extract or sorbitol were prepared as described in the Materials and Methods. These sterile solutions were incubated at 28°C for 3 days. There was no detectable H_2S evolution when the concentration of sorbitol was decreased from 5 to 0% (Table 4, row 7 - 12). However, when the peptone and yeast extract concentration decreased from 1 to 0% , H_2S was detected (Table 4, row 1 - 6).

Table 3. Hydrogen sulfide formation in sterile solutions containing 1% sodium thiosulfate and varying concentrations of media components.

Media Concentration (%) [a]	Evolved Sulfide (μg) [b]	Soluble Sulfide (μg) [b]
100	0	6.40 ± 1.11
80	0	4.00 ± 0.38
60	2.10 ± 0.76	4.80 ± 0.20
40	59.94 ± 2.77	0.28 ± 0.26
20	73.91 ± 0.70	0.05 ± 0.06
0	133.41 ± 11.19	0

[a] Standard 5% sorbitol medium was prepared as described in the Materials and Methods and then diluted with dd H_2O to make solutions containing decreasing amounts of the medium components. The flasks were incubated at 28°C for 3 days.

[b] Quantities of evolved and soluble sulfide were determined by the methylene blue method as described in the Materials and Methods. Each value represents the mean and standard deviation of three trials with four replicates each.

Table 4. Hydrogen sulfide evolution from sterile solutions containing 1% sodium thiosulfate and varying concentrations of peptone, yeast extract, and sorbitol.

Concentration (% w/v) [a]			Evolved H_2S (µg) [b]
Peptone	Yeast Extract	Sorbitol	
1.0	1.0	5.0	0
0.8	0.8	5.0	0
0.6	0.6	5.0	0
0.4	0.4	5.0	0
0.2	0.2	5.0	26.92 ± 0.07
0.0	0.0	5.0	84.99 ± 0.00
1.0	1.0	5.0	0
1.0	1.0	4.0	0
1.0	1.0	3.0	0
1.0	1.0	2.0	0
1.0	1.0	1.0	0
1.0	1.0	0.0	0

[a] Concentration of peptone and yeast extract and sorbitol in media prepared as described in the Materials and Methods and incubated as sterile solutions for 3 days at 28°C.

[b] Quantities of evolved H_2S were determined by the methylene blue method as described in the Materials and Methods. Each value represents the mean and standard deviation of 3 trials with four replicates for each trial.

These results indicated that some component of the peptone and yeast extract fraction of the medium was responsible for interacting with sulfide to keep it soluble.

Various gluconobacter-exhausted media were prepared to determine if growing cells depleted enough yeast extract and peptone to allow for H_2S evolution. *Gluconobacter oxydans* ATCC strain 19357 was separately incubated in SYP medium for 24, 48, and 72 hours at 28°C. After incubation, cells were removed by centrifugation and the supernatant fluid (spent medium) was supplemented with 1% sodium thiosulfate. These thiosulfate supplemented spent media were then filter-sterilized and incubated at 28°C for 72 h (Table 5). The 24-hr exhausted medium showed no H_2S evolution. The 48 hr exhausted medium showed evolution of 190 μg of H_2S. The 72 hr exhausted medium showed evolution of 412 μg of H_2S. These results indicated that the type strain of *G. oxydans* depleted enough medium components after 48 and 72 hrs incubation in SYP medium to allow for H_2S evolution. These results also show that a lesser amount of strain 19357 growth (24 h exhaustion) does not sufficiently deplete the medium to allow for H_2S evolution.

During growth in SYP medium, the gluconobacters produced acids that lowered the pH of the medium from 6.5 to 4.5 (Table 5). The absence of H_2S evolution from the 24 hr exhausted medium suggested that the pH drop did not cause the H_2S evolution. To further examine this possibility, the following experiment was performed with three trials with four replicates for each trial. Sterile SYP medium with 1% sodium

Table 5. Hydrogen sulfide evolution from sterile exhausted medium supplemented with 1% sodium thiosulfate.

Extent of Growth Medium Exhaustion (hrs) [a]	Evolved Sulfide (μg) [b]	pH [c]	
		Initial	Final
24	0	6.5	4.5
48	189.93 ± 0.02	6.5	4.5
72	412.83 ± 0.02	6.5	4.5

[a] Standard 5% sorbitol medium was inoculated with 0.02 ml of a working culture of *Gluconobacter oxydans* ATCC strain 19357 and incubated for 3 days at 28°C with shaking at 200 rpm for 24, 48, and 72 hours. After incubation the suspension was centrifuged to remove the cells, filter sterilized, and supplemented with thiosulfate, and incubated for 3 days at 28° as described in the Materials and Methods.

[b] Quantities of evolved sulfide were determined by the methylene blue method as described in the Materials and Methods. Each value represents the mean and standard deviation of three trials with four replicates for each trial. Uninoculated flasks incubated for 24, 48, and 72 hrs, centrifuged, supplemented with thiosulfate, and incubated under the same conditions, evolved no H_2S.

[c] Initial pH determinations were made prior to filter sterilizing the exhausted medium. Final pH determinations were made after the 3 day incubation.

thiosulfate was prepared, the pH adjusted to 4.5, and incubated for 3 days at 28°C as described in the Materials and Methods. After incubation, no evolved H_2S was detected, and the final pH was 4.5. From these results I concluded that the drop in pH, which occurs during gluconobacter growth in SYP medium, did not cause the H_2S evolution.

Preliminary results with the six gluconobacter strains demonstrated that different amounts of H_2S were evolved accompanying the growth of each culture. To determine if this was due to differences in the extent of gluconobacter growth (medium depletion) the following experiment was performed. The same strains representing the three species of gluconobacters were separately inoculated into SYP medium containing 1% sodium thiosulfate. After incubating at 28°C for 3 days, the total amount of growth in the flask was determined by measuring total cellular protein using the Lowry method as described in the Materials and Methods. The change in cellular protein during growth was determined by subtracting the protein in the inoculum from the protein of the bacterial suspension after cultures were incubated for 3 days at 28°C. Those strains with a total growth greater than 1500 µg of protein evolved between 10 and 143 µg of H_2S, whereas those strains with a total growth less than 700 µg of protein evolved no more than 6 µg of H_2S (Table 6). These results indicated the strains of gluconobacters that grew to a higher extent evolved more H_2S than those strains that grew to a lesser extent. Strains which grow to a higher cell density would be expected to consume more medium components, and, therefore release more of the abiotically formed sulfide as H_2S.

Table 6. Relationship between the extent of gluconobacter growth and the amount of evolved hydrogen sulfide.[a]

Gluconobacter Strain	Growth Extent (Δ µg protein) [b]	Evolved Sulfide (µg) [c]
ATCC 23651	1673 ± 13	143 ± 0
ATCC 15178	1653 ± 18	45 ± 1
ATCC 19357	1551 ± 18	10 ± 0
IFO 3276a	625 ± 9	6 ± 0
IFO 3264	417 ± 8	4 ± 0
IFO 3297a	384 ± 20	0

[a] 4.5 ml volumes of standard 5% sorbitol medium with 1% sodium thiosulfate in 25 ml Erlenmeyer flasks were separately inoculated with 0.5 ml of prepared subculture as described in the Materials and Methods. The flasks were incubated statically at 28°C for 3 days.

[b] Total protein was determined for the inoculum and resulting bacterial suspension, after 3 days incubation at 28°C, using the Lowry method as described in the Materials and Methods. The difference in the total protein between the inoculum and the final bacterial suspension is reported as the change (Δ) in total protein. Each value represents the mean and standard deviation of three trials with four replicates for each trial.

[c] Quantities of evolved sulfide were determine by the methylene blue method as described in the Materials and Methods. Each value represents the mean and standard deviation of three trials with four replicates for each trial.

DISCUSSION

Sources of evolved hydrogen sulfide (H_2S). Mason and Claus (24) reported that the gluconobacters produce H_2S when incubated in media containing sodium thiosulfate, and my initial results agreed with this finding. However, none of the traditional physiological reasons for H_2S evolution seemed to apply to the gluconobacters. In this investigation, I found no evidence for anaerobic respiration using reduced sulfur compounds, for the degradation of sulfur-containing organic compounds, nor for assimilatory thiosulfate reduction. The evolution of the H_2S occurred only when both gluconobacters and sodium thiosulfate were present in the medium. This suggested two things: first that thiosulfate was the source of the evolved H_2S; and second, that the H_2S evolution was a microbial process.

Decomposition of thiosulfate. Sodium thiosulfate decomposes in aqueous solutions at ordinary temperatures and more rapidly when heated (35). I found that a sterile aqueous sodium thiosulfate solution evolved H_2S when incubated at 28°C, but not when sodium thiosulfate was dissolved in sterile standard 5% sorbitol (SYP) medium (see Results, p. 8). These experiments suggested that the medium components either interacted with the thiosulfate to prevent its decomposition or interacted with the H_2S to keep it in solution. When I measured the levels of soluble sulfide in sterile growth media, my results indicated that soluble sulfide was formed during incubation (Table 3). As the concentration of the yeast extract and peptone decreased, H_2S evolution increased and

the quantity of soluble sulfide decreased (Table 3). Since these solutions were sterile, I concluded that soluble sulfide was formed by abiotic decomposition of the thiosulfate and that soluble sulfide was released as evolved H_2S as the medium components were depleted.

My next goal was to determine if the gluconobacters sufficiently depleted the medium components to allow for H_2S evolution. I assumed that the more the gluconobacters grew the more yeast extract and peptone were consumed; therefore I examined the relationship between cell density and H_2S evolution. I found that those strains of gluconobacter which grew to a higher cell density evolved more H_2S (Table 6). I also examined H_2S evolution from sterile exhausted medium and found that the amount of evolved H_2S increased as the length of the exhaustion increased (Table 5). From these results, I concluded that the consumption of the medium components by the gluconobacters allowed for the evolution of H_2S.

Hydrogen sulfide evolution was detected in sterile media only after reducing the peptone and yeast extract concentration to 0.2% (Table 4). From a chemical standpoint, this was not surprising, since both peptone and yeast extract contain a variety of compounds such as amino acids that carry positive charges. The negatively charged sulfide ion may form an ionic bond with these postively charged components of peptone and yeast extract. This resulting ionic relationship may then prevent formation and/or evolution of H_2S.

All of these experiments (Tables 3 to 6) reinforced the hypothesis that thiosulfate decomposes abiotically to form soluble sulfide; and H_2S

is released as growing cells consume peptone and/or yeast extract from the medium.

Use of H_2S evolution as a phenotypic test. Throughout the microbiological literature, detection of H_2S from media containing thiosulfate is equated with the microbial production of sulfide from thiosulfate (4, 7, 10, 31). Among the facultative and anaerobic bacteria, H_2S production is assumed to result from thiosulfate reduction during anaerobic respiration (4, 7). However, there are a number of bacteria that produce H_2S for no apparent physiological reason (4, 9). Barret and Clark (4) argued that the H_2S could result from the desulfurization of cysteine rather than the reduction of the thiosulfate. Since the typical testing medium for evolved H_2S detection contains varying amounts of peptone, which contains varying amounts of cysteine, positive results may be misinterpreted as indicating that the evolved H_2S is from thiosulfate. Additionally, different detection methods for evolved H_2S may produce conflicting results (4, 9). For example, the typical indicators (iron chloride, lead-acetate, and 5,5'-dithiobis-2-nitrobenzoic acid) have different sensitivities for sulfide (2, 4, 37). For example, H_2S from bacterial degradation of cysteine will produce a positive result with lead acetate as the indicator, but produces a negative result when iron chloride is used as the indicator (4).

My research suggests yet another explanation for H_2S evolution: that the evolved H_2S may result from the abiotic decomposition of thiosulfate followed by cellular consumption of medium components. Perhaps, abiotic decomposition of thiosulfate has been overlooked

because one of the standard methods for detecting H_2S uses a medium, Triple-Sugar-Iron (TSI) Agar, that contains peptone and yeast extract or pancreatic digest of casein and peptic digest of animal tissue (31). Early studies on H_2S evolution used a complex medium containing meat extract or peptone reinforced with an additional source of sulfur, usually thiosulfate or cysteine (9, 37). My results show the presence of the peptone and yeast extract in concentrations greater than 0.2% prevents H_2S evolution (Table 4) under sterile conditions. Therefore, uninoculated medium would show a negative result and inoculated medium, in which bacteria had consumed the medium components, might show a positive result. The method I used would have the same limitations as the standard method, since the medium contains both peptone and yeast extract in concentrations greater than 0.2%. The evolved H_2S would be more easily detected by the lead-acetate filter paper that I used, since the lead-acetate method is more sensitive than the ferric sulfate used in the TSI agar (9, 37).

The use of the ferrous iron or ferric iron within media to detect H_2S (31) may also effect the detection of H_2S. After heat sterilization and microbial growth, the ferrous iron may be oxidized to ferric iron depending on the redox potential of the media. Ferric iron is a poor indicator of H_2S. (37). Additionally, both ferrous iron and ferric iron may have toxic effects on the microbial culture (9) thereby limiting growth and H_2S evolution.

Due to the many variables intrinsic to the H_2S detection methods (such as differences in redox potential, concentrations of ferric and/or

ferrous iron, and the variability of the chemical makeup peptone and yeast extract) statements about H_2S producing capabilities of microorganisms are incomplete unless all experimental conditions are reported. These variables prevent an accurate comparison of results when different methods are used. Since H_2S formation may result from the abiotic decomposition of thiosulfate rather than microbial reduction of thiosulfate, the detection of H_2S cannot be equated with the microbial production of H_2S. The detection of H_2S as an indication of a physiological process must be used cautiously with proper controls to insure that the H_2S is not from abiotic decomposition of thiosulfate.

CONCLUSIONS

1. H_2S is evolved from gluconobacter cultures incubated both aerobically and anaerobically in standard 5% sorbitol (SYP) medium supplemented with sodium thiosulfate.

2. This evolved H_2S does not result from anaerobic respiration with thiosulfate, disproportionation of thiosulfate, cysteine degradation, or assimilatory thiosulfate reduction.

3. Soluble sulfide is formed during the abiotic decomposition of thiosulfate but remains soluble due to interaction with the medium components in concentrations greater than 0.2% (w/v).

4. Soluble sulfide is released as evolved H_2S when the concentration of the peptone and yeast extract in the medium is lowered below 0.2% (w/v).

5. Detection of evolved H_2S from bacterial cultures cannot be equated with the microbial H_2S production, since the H_2S may result from the abiotic decomposition of thiosulfate during incubation.

6. Theoretically, results of the TSI agar method and/or use of ferrous or ferric iron for detecting H_2S may be deceptive because of the role peptone, yeast extract, anaerobic conditions, redox potential, and/or ferrous or ferric iron play in the decomposition of thiosulfate and the release of H_2S.

7. The detection of H_2S as an indication of a physiological process must be used cautiously with proper controls to insure that H_2S evolution is not a consequence of abiotic decomposition and biotic release of thiosulfate.

RESEARCH SUGGESTIONS

1. My results indicate that compounds within the peptone and/or yeast extract interact with soluble sulfide spontaneously formed from thiosulfate. This interaction prevented the evolution of the soluble sulfide as hydrogen sulfide (H_2S) unless the yeast extract and peptone concentration was less that 0.2 %. Using a defined minimal medium, determine which compounds in the peptone and/or yeast extract interact with the sulfide ion to keep it in solution.

2. The standard method for examining microbial H_2S production uses solid or semi-solid agar medium supplemented with sodium thiosulfate. Since the medium is stabbed, the method works best for facultative and anaerobic bacteria. However, since this method inhibits the growth of most aerobic organisms, a positive result (the detection of H_2S) is interpreted as biological thiosulfate reduction. Using the H_2S detection method described in the Materials and Methods, examine other strictly aerobic bacteria for H_2S evolution to determine if H_2S evolution from thiosulfate decomposition is limited to *Gluconobacter* species or is widespread.

3. Since detection of evolved H_2S cannot be equated with microbial production of H_2S from the reduction of thiosulfate, another method for thiosulfate reduction needs to be developed. An assay system based on the presence on the enzyme thiosulfate reductase, rather than the presence of the end product, H_2S should be developed. This could be

accomplished by sequencing different samples of purified thiosulfate reductase and using unique segments to create a labeled probe for the enzyme. A wide variety of microorganisms, particularly those reported positive for H_2S production from thiosulfate, should be examined using this method for the presence of thiosulfate reductase.

4. Instead of using a defined medium to determine which components in the peptone and/or yeast extract interact with the sulfide, radioactive sulfide ($^{35}S^{=}$) could be used. After adding the ^{35}S labeled thiosulfate to standard medium, the medium might be fractionated into major categories (such as amino acids, lipids, carbohydrates), then one could determine which fraction(s) contained the radioactivity. This radioactive fraction(s) could then be further fractionated to determine the chemical nature of the radioactive components. However, if the relationship between the binding components and the sulfide ion is ionic, the fractionation procedure may disrupt this relationship, and the sulfide ion may be released as H_2S. Additionally, there seems to be no simple procedure to separate radioactivity from non-radioactivity especially when the chemical nature of the compounds is unknown.

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APPENDIX A:
GLUCONOBACTER CLASSIFICATION AND METABOLISM

Habitat and morphology. The genus *Gluconobacter* is composed of gram negative, heterotrophic, aerobic, short rods (12). They contain straight chain fatty acids, ubiquinone-10 as the main quinone, and polar multitrichous flagella when motile (12, 24). They are characterized by: (i) their inability to completely oxidize ethanol, acetic acid, and other substrates to carbon dioxide and water (3); (ii) the lack of a complete Tricarboxylic Acid (TCA) cycle (17); (iii) and their ability to rapidly carry out a limited oxidation of hydroxyl containing organic compounds and to quantitatively excrete the oxidized product into the media (13).

In nature, the gluconobacters may be found in a variety of habitats, such as flowers, fruits, honey, honey bees, and palm sap (3, 15, 22, 28). Industrially they are important in the production of Vitamin C, dihydroxyacetone, and beverages (1, 14). They have also been implicated in the production of ß-lactom antibiotics, as well as the spoilage of wine, beer, apples, pears, and pineapple (20, 32).

History of classification. The genus *Gluconobacter* was first described by Asai in 1935 (3). Strains isolated from fruit were capable of producing gluconic acid from glucose but grew poorly in ethanol-containing media. Although they were able to grow at pH 4.2 they could not assimilate or oxidize acetic acid. The genus *Acetobacter*, on the other hand, was described as being capable of oxidizing and assimilating acetic acid. Greenfield and Claus (17) later showed that the gluconobacters

were capable of assimilating acetic acid and using it in the synthesis of glutamate.

Loitsyanshaya et al. (23) used numerical techniques to examine 136 characteristics of fifteen strains of the genus *Gluconobacter* and forty-one strains of the genus *Acetobacter*. The evidence of a 90% similarity among all *Gluconobacter* strains led to the proposal of a single species called *G. oxydans*.

Gossele et al. (16) examined 177 phenotypic features of 98 *Gluconobacter* strains and concluded that too few differences existed among the strains to support the existence of more than one species. However, there was evidence for two distinct groups based upon the requirement for nicotinic acid. The two groups were referred to as Phenon A, which did not require nicotinic acid, and Phenon B, which did require nicotinic acid.

Simultaneously, Yamada et al. (36) and Micales et al. (26) performed DNA/DNA homology studies. Yamada's group examined twenty strains of gluconobacters and found evidence of two distinct homology groups. DNA homology was determined using the membrane-filter method of Komatsu and Kaneko. *G. oxydans* showed from 46 to 82% homology to the type strain ATCC 19357 and required nicotinic acid for growth. The second species, *G. cerinus* showed from 1 to 24% homology to the type strain and did not require nicotinic acid for growth. The two homology groups corresponded to Phenon B and Phenon A, respectively, of Gossele.

Micales et al. (26) examined 54 strains and concluded that there were three distinct homology groups. DNA homology values were determined using free solution S1 nuclease. Group I showed from 42 to 94% homology to the type strain, Group II showed from 16 to 26% homology to the type strain, and Group III showed from 13 to 23% homology to the type strain. There was only a 16% relatedness among the three groups. They concluded that there are at least three species represented among the *Gluconobacter* strains.

Mason and Claus (24) determined differentiating phenotypic characteristics for the three homology groups described by Micales et al (1984). Differences were noted in growth on arabitol or ribotol as the primary carbon source and growth after three passages and incubation for 24 hours in nicotinate-deficient media. In the rabitol/arabitol test, Group I grew to 0.5 optical density (OD_{620}) or less, Group II grew to 1.0 OD_{620} or greater, and Group III grew to 0.5 OD_{620} or less. In the nicotinate test, Group I grew to an OD_{620} of 0.5 or less, Group II grew to an OD_{620} of 1.0 or greater, and Group III grew to an OD_{620} of 1.0 or greater. The species name proposed were *G. oxydans*, *G. frateurii*, and *G. asaii* respectively.

Metabolism. According to Edwards and Claus (13), the *Gluconobacter* perform limited oxidation reactions in two different ways. In the first, a NAD(P)-independent dehydrogenase, bound to the plasma membrane, removes two hydrogens from the substrate. The hydrogens enter the electron transport system, and the oxidized product is released back into the medium. The second way involves the transport of the

substrate into the cell with subsequent oxidation by soluble NAD(P)-dependent hydrogenases that remove two hydrogens from the substrate, use the hydrogens to reduce $NAD(P)^+$, and the NAD(P)H then carries the hydrogens to the electron transport system. The oxidized substrate may then be further oxidized or excreted into the medium.

Further internal oxidation of the substrate probably occurs via the Hexose Monophosphate (HMP) pathway (8). In resting cells, an energy source such as glucose is oxidized through the HMP pathway to glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate is then oxidized to acetate in a series of reactions similar to the latter part of the Embden-Myerhoff-Parnus (EMP) pathway.

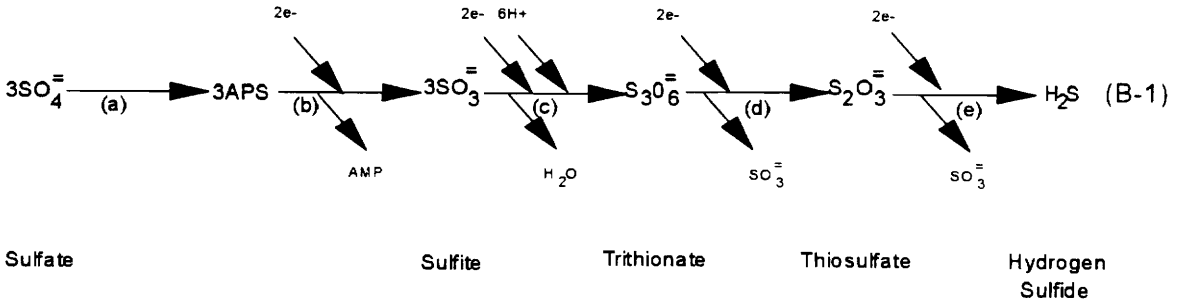
On the other hand, Kersters and DeLey (21) reported the presence of 2-keto-5-deoxy-6-phosgluconoate (KDPG) aldolase, which is a key enzyme in the ED pathway, in two gluconobacter strains. Therefore, it is possible that the ED pathway is responsible in addition to, or instead of, the EMP pathway for some of the internal oxidation reactions (13)

Gluconobacter strains are capable of growth in media containing only an oxidizable carbohydrate, mineral salts, three vitamins, and inorganic nitrogen (5). This growth indicates that all amino acids are being synthesized.

APPENDIX B:
BACTERIAL THIOSULFATE METABOLISM

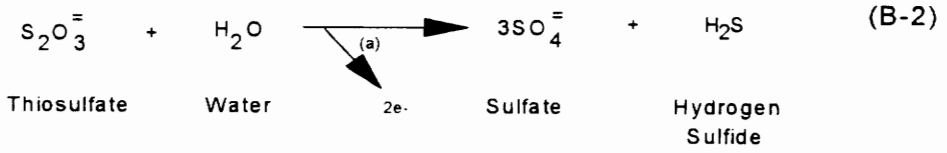
Sources of hydrogen sulfide. Hydrogen sulfide, a colorless, and toxic gas whose odor is reminiscent of rotten eggs, may be produced by many different types of bacteria. Hydrogen sulfide production has traditionally been attributed to two types of microbial activities: the anaerobic respiration using thiosulfate as a terminal electron acceptor, and the bacterial degradation of sulfur-containing organic compounds. Two additional pathways that may also lead to the bacterial production of hydrogen sulfide are disproportionation, and the reduction of thiosulfate for biosynthesis.

Terminal electron receptor. In anaerobic respiration, an inorganic compound, such as the sulfate or thiosulfate ion, is used as a terminal electron acceptor rather than oxygen (7). Anaerobic bacteria, such as *Desulfovibrio vulgaris*, and facultative anaerobes, such as *Escherichia coli*, are capable of using these ions and reducing them in a step-wise fashion to hydrogen sulfide. The sulfate ion is first activated to adenosine phosphosulfate (APS) and then reduced to sulfite, the sulfite to trithionate, the trithionate to thiosulfate, and the thiosulfate to hydrogen sulfide (Reaction B-1).

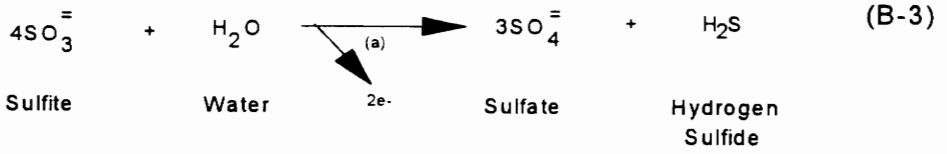


There are, in some interpretations of the pathway, two intermediates between the sulfite and trithionate (29). These steps are catalyzed by the enzymes, ATP sulfurylase (a), adenyl sulfate reductase (b), sulfite reductase (c), trithionate reductase (d), and thiosulfate reductase (e), respectively (4 ,29). The reduction of APS to sulfite (b) produces an $\Delta E'_0$ of 0.445 V and the reduction of trithionate to thiosulfate (d) produces an $\Delta E'_0$ of 0.745 V (29). There is enough of a difference in electron potential ($\Delta E'_0$), 0.17 V, in both reactions (b and d) to support the phosphorylation of one adenosine diphosphate (ADP) to form one molecule of adenosine triphosphate (ATP).

Disproportionation. This type of metabolism refers to the splitting of a compound into two new compounds, one of which is more reduced and the other more oxidized than the original compound (7). In a few sulfate-reducing bacteria, this is known to occur with both thiosulfate and sulfite (Reactions B-2 and B-3).



(B-2)



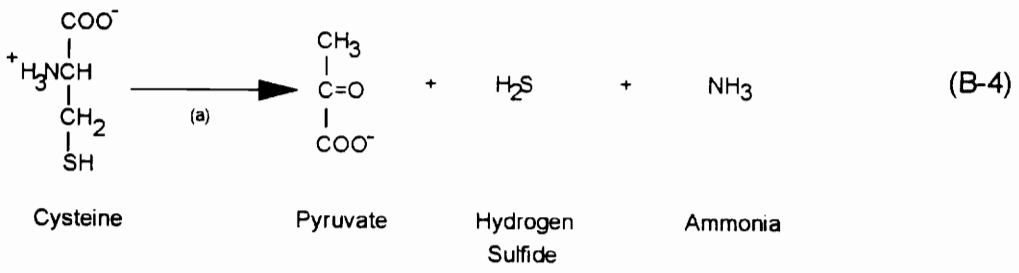
(B-3)

Both reductions are energy requiring. The disproportination of the thiosulfate ion requires the input of 27.6 kJ per molecule of hydrogen

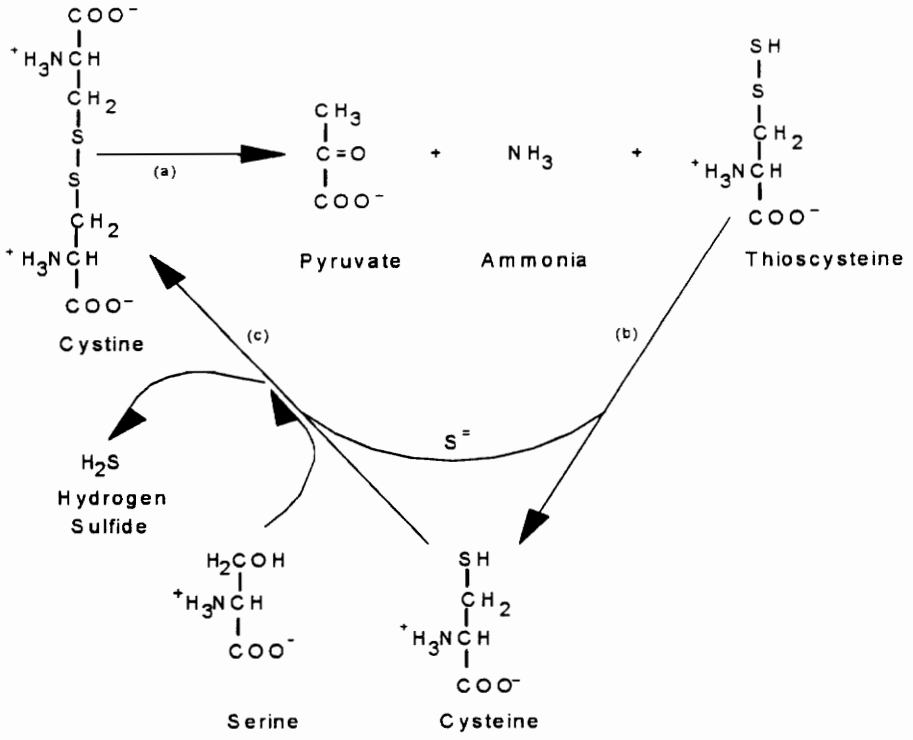
sulfide formed, and the disproportination of the sulfite ion requires the input of 241.3 kJ per molecule of hydrogen sulfide formed (7). The electrons from the oxidation of the thiosulfate and sulfite can be shunted to the electron transport system and be used to reduce sulfate and sulfite during anaerobic respiration. The reduction of sulfate supports ATP production (Reaction B-1, step b) and therefore balances the energy deficit created during the disproportination reactions. Disproportination seems only to occur when organic energy sources are low but there is a plentiful supply of reduced sulfur compounds available (7).

Degradation of organic sulfur compounds. Hydrogen sulfide may be produced during the degradation of certain organic sulfur-containing compounds. Although several sulfur-containing compounds are commonly present in complex laboratory media, cysteine is recognized as the primary source of hydrogen sulfide (2, 4). The degradation of cysteine may occur via two different pathways catalyzed by either cysteine desulfhydrase or cystathionine γ-lyase.

Cysteine desulfhydrase (Reaction B-4) catalyzes the breakdown of cysteine into pyruvate hydrogen sulfide, and ammonia (27).



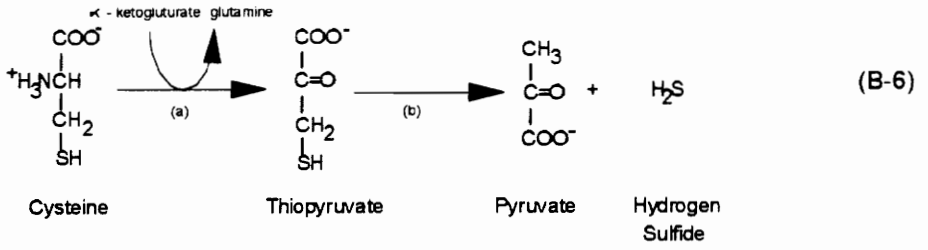
In contrast, cystathionine γ-lyase (Reaction B-5) catalyzes the breakdown of cystine to pyruvate, ammonia, and thiocysteine (6).



(B-5)

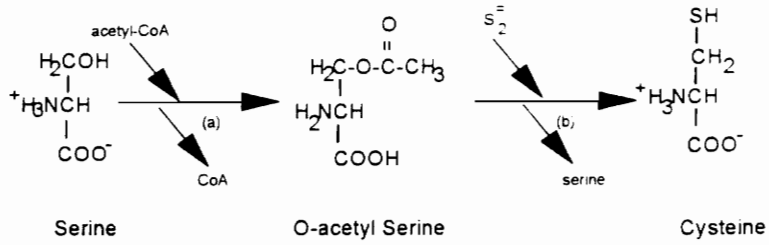
Thiocysteine is then desulfurated to cysteine. Free sulfur combines with the newly formed cysteine and a serine molecule to regenerate cystine and produce hydrogen sulfide.

The amino group of cysteine may also be transferred to α-ketogluturate, during transamination, to form thiopyruvate (6). Thiopyruvate then undergoes desulfuration to pyruvate and hydrogen sulfide (Reaction B-6).



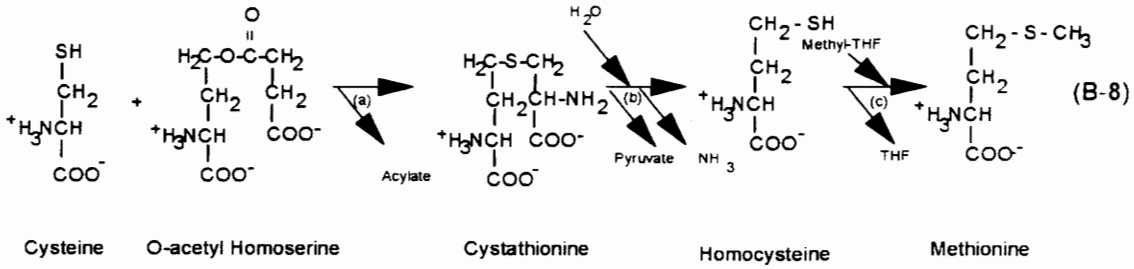
Regardless of the pathway used for cysteine degradation, the final products are pyruvate, hydrogen sulfide, and ammonia. Pyruvate may then enter the Tricarboxylic Acid Cycle (TCA) to be used as any energy source for resting cells (other than the gluconobacters), or pyruvate may be used as an intermediate during biosynthesis of new compounds.

Assimilatory reduction of thiosulfate for synthesis. The assimilatory reduction of thiosulfate may occur, independent of anaerobic respiration, to provide sulfide for the synthesis of sulfur containing compounds (6, 11, 25). In the synthesis of cysteine for example, serine is acetylated and then combined with sulfide to form cysteine (Reaction B-7).



(B-7)

These reactions are catalyzed by acetyl transferase (a) and serine sulfhydrase (b) (6, 19). Methionine is synthesized primarily from cysteine (11). Cysteine combines with O-acetyl homoserine to form cystathionine which is then hydrated and deaminated to form homocysteine. The homocysteine is then methylated to methionine (Reaction B-8).



(B-8)

Cystathionine γ-lyase (a), β-cystathionase (b), and homocysteine methyl transferase (c) catalyze these reactions (6, 19).

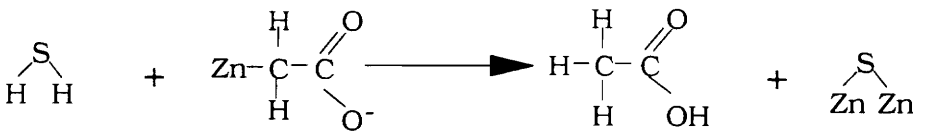
APPENDIX C:
THE QUANTITATION OF EVOLVED HYDROGEN SULFIDE (H_2S)

Trapping known quantities of H_2S. A standard curve correlating micrograms of evolved H_2S to the absorbance of methylene blue at 670 nm was prepared in the following way. A known quantity of $Na_2S \cdot 9H_2O$ was dissolved in an antioxidant buffer containing 8% NaOH, 32% sodium salicylate, and 7.2% acetic acid (27) to prepare a standard solution containing 10 μg of sulfide ion/ml of antioxidant buffer. Two-tenths, 0.5, 1.5, 2.5, 3.5, and 4.5 milliliter volumes of this standard solution were placed in separate 25 ml Erlenmeyer flasks with sidearms to prepare reaction mixtures containing 2, 5, 15, 25, 35, and 45 μg of sulfide ion. Enough antioxidant buffer was added to each flask to increase the final volume to 5 ml. A piece of Whatman 42 (4.25 cm diameter) filter paper was folded in quarters, molded into a cone, inverted, and placed so as to completely cover the mouth of the flask. A Pasteur pipette was used to saturate the filter paper with a 2M zinc acetate solution. A rubber stopper was placed over the open end of the sidearm, and the mouth of the flask was covered with foil.

Three ml of concentrated HCl was injected through the sidearm of the flask to convert the dissolved sodium sulfide to evolved H_2S. These flasks were incubated at room temperature for four hours to allow for trapping of the evolved H_2S on the filter paper (27).

Formation of methylene blue from trapped H_2S. After the 4 hr incubation of the HCl treated mixtures, the zinc-acetate saturated filter papers were separately transferred to 50 ml beakers each of which contained 22 ml of dd H_2O. Then, 2.5 ml of p-aminodimethylaniline sulfate solution and 0.5 ml of ferric ammonium sulfate solution were added to each beaker. The beakers were gently swirled to mix the reagents then incubated at room temperature for about 5 minutes to develop the methylene blue. The absorbance of the methylene blue in each beaker was measured spectrophotometrically at 670 nm. A typical graphical comparison of known amounts of sulfide ion initially present in each flask compared with the methylene-blue absorption is shown in Figure 2.

Chemistry of quantitative methylene blue formation. Evolved H_2S can be trapped on filter paper saturated with zinc acetate. The zinc acetate reacts with the H_2S to form acetic acid and zinc sulfide as shown in reaction C-1.



(C-1)

hydrogen sulfide zinc acetate acetic acid zinc sulfide

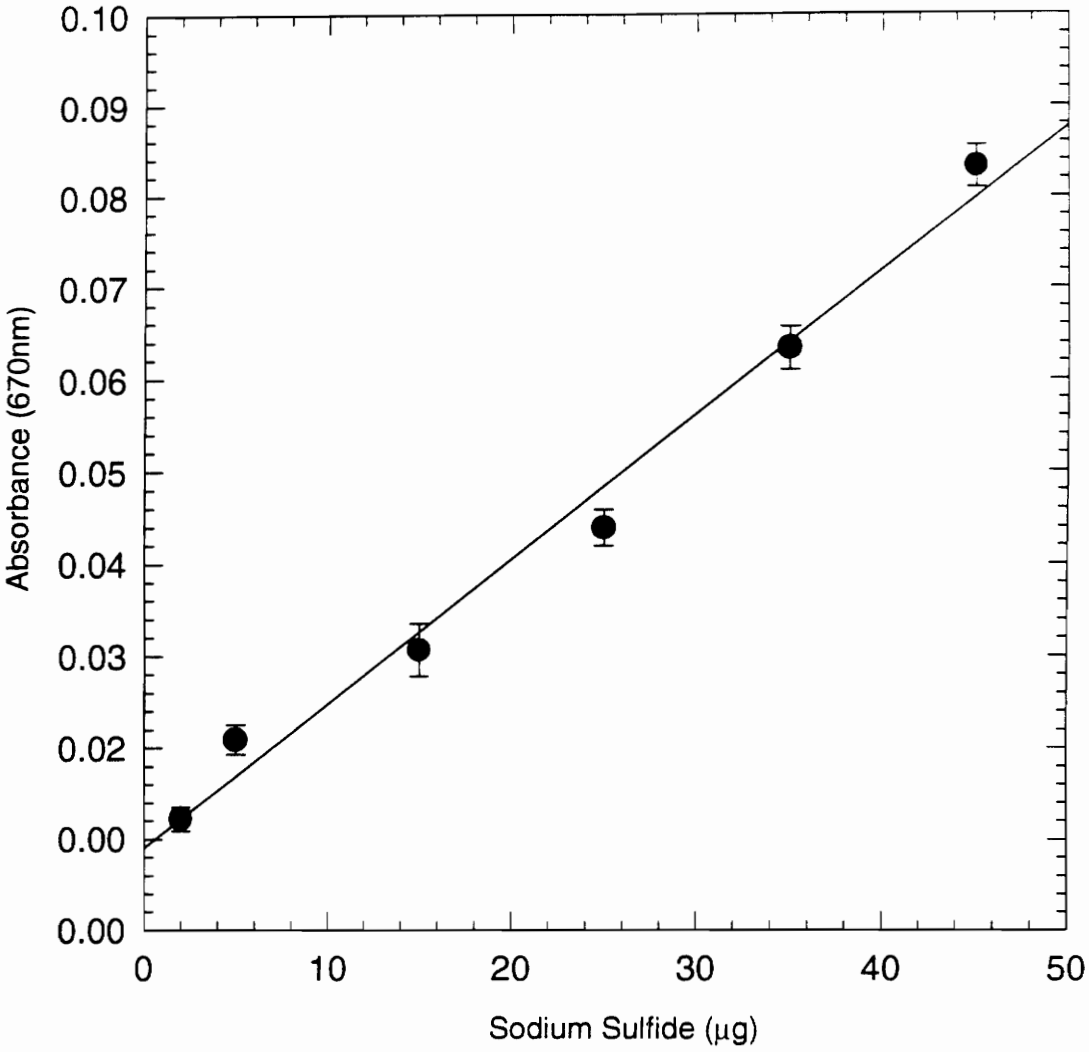
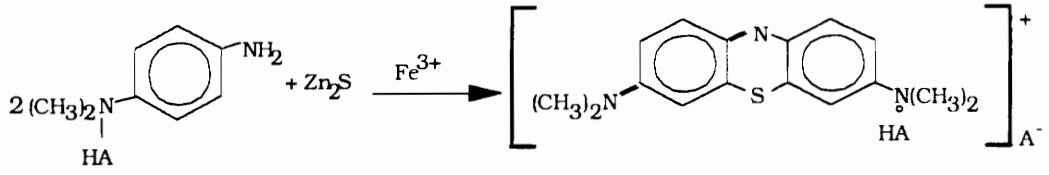


Figure 2. Relationship between known amounts of sulfide ion in standard solutions evolved as H_2S and the development of methylene blue. Known quantities of sodium sulfide were dissolved in anti-oxidant buffer and evolved as hydrogen sulfide by the addition of hydrochloric acid. The evolved sulfide was trapped on zinc-acetate-saturated filter paper and quantified through the use of the methylene blue method. Each data point represents the mean of three trials with three replicates for each trial. The standard deviation of all replicates is indicated by the error bars at each data point.

The p-aminodimethylaniline sulfate solution reacts with zinc sulfide in the presence of iron (present in these reaction mixtures in the form of ferric ammonium sulfate), to form methylene blue (27) as shown in reaction C-2.



(C-2)

p-aminodimethvaniline sulfate zinc sulfide methylene blue

The intensity of the methylene blue was measured spectrophotometrically at 670 nm as indicated by Tabatabai (1982). When I prepared methylene blue by separately reacting 50 µg and 100 µg of $Na_2 \bullet 9H_2O$ with p-aminodimethyl aniline sulfate and ferric ammonium sulfate, the resulting mixtures absorbed maximally at 666 nm and 665 nm, respectively.

The quantitation of H_2S by this method is indirect since only 60 to 70 % of $Na_2 \bullet 9H_2O$ is evolved as H_2S (33) and recovery rates vary from 88% to 93% (27) depending on the quantity of $Na_2 \bullet 9H_2O$ in solution. However, if the reaction conditions remain the same throughout all trials and replicates, then the amount of methylene produced is repeatable.

APPENDIX D:
SOLUBLE SULFIDE IN GROWTH MEDIA

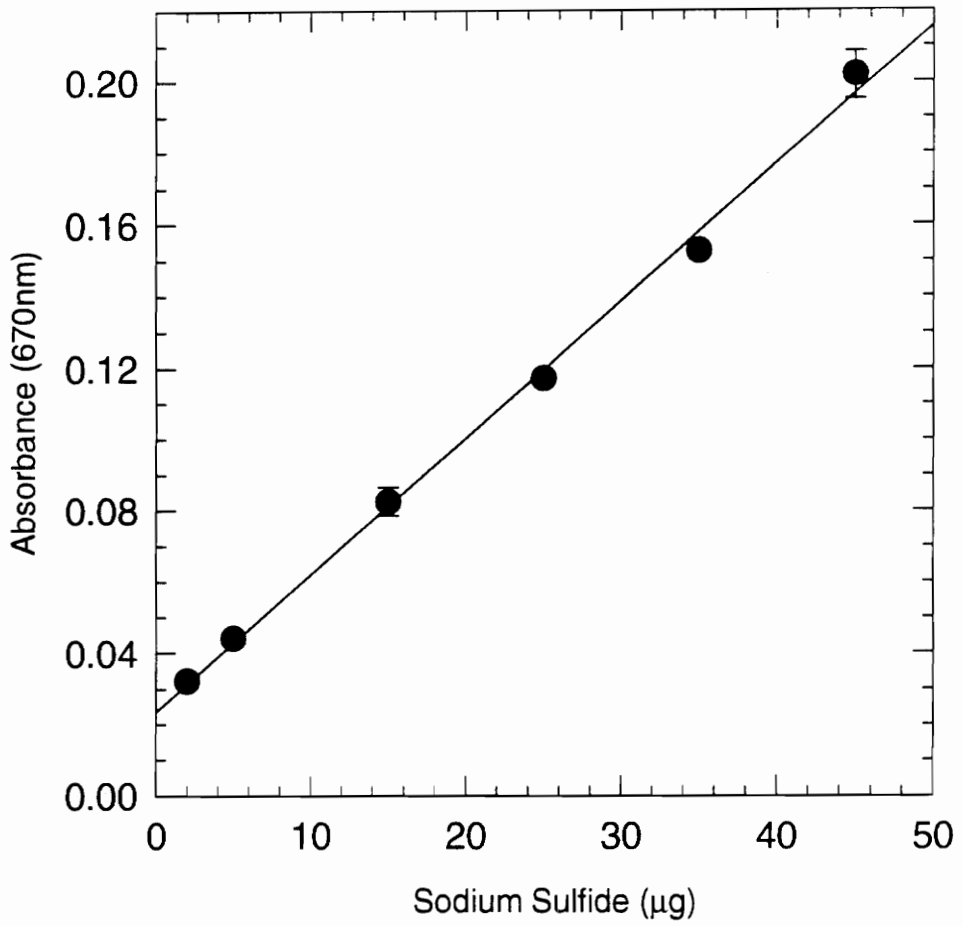


Figure 3. Standard curve for soluble sulfide in growth media. Known quantities of sodium sulfide were dissolved in standard 5% sorbitol media and the sulfide was precipitated through the addition of zinc acetate and sodium hydroxide. After the precipitate was dissolved, the amount of sulfide was determined through the use of the methylene blue method as described in the Materials and Methods. Each point represents the mean of two trials with four replicates for each trial. Standard deviation bars are shown for each data point, but often reside within the closed circle.

CURRICULUM VITAE

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1992 Bachelor of Science
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Research Interests:

Microbial Physiology

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Overall Rating - 3.50

Spring Semester 1994
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Abstracts Submitted

The Evolution of Hydrogen Sulfide by *Gluconobacter* species
American Society for Microbiology, 94th General Meeting
May 21 - 25, 1995
Washington, D. C.
Submitted: December, 1994

Seminars Given:

Discussion of a paper entitled "The effect of sulfate reduction on the thermophilic (55°) methane fermentation process"
Jointly presented with another graduate student
Graduate Microbiology and Immunology Seminar, VPI & SU
Fall, 1992

The Production of Hydrogen Sulfide from Thiosulfate by Bacteria
Graduate Microbiology and Immunology Seminar, VPI & SU
Fall 1993

The Evolution of Hydrogen Sulfide by *Gluconobacter* species
Graduate Microbiology and Immunology Seminar, VPI & SU
Fall 1994

Papers Presented:

10 Minute Talk: The Production of Hydrogen Sulfide from Thiosulfate by Gluconobacters
ASM, Virginia Branch Regional Meeting
November 1993

10 Minute Talk: The Evolution of Hydrogen Sulfide by *Gluconobacter* species
ASM, Virginia Branch Regional Meeting
December 1994

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"The Development of a Quantitative Assay for Hydrogen Sulfide Production"
Sigma Xi Grant-in-Aid of Research
Submitted Spring 1993 - Funded

Awards Received :

Instructional Fees Scholarship, Fall 1993
Instructional Fees Scholarship, Spring 1994

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93rd General Meeting
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May 16-20, 1993

American Society for Microbiology
Virginia Branch Regional Meeting
Lexington, Virginia
November 1993

American Society for Microbiology
94th General Meeting
Las Vegas, Nevada
May 23-27, 1994

American Society for Microbiology
Virginia Branch Regional Meeting
Richmond, Virginia
December 1994

Membership in Honorary Organizations:

Phi Sigma Biological Honor Society
Gamma Beta Phi Honor Society

Membership in Professional Organizations

Alpha Chi Sigma Professional Chemistry Fraternity
Alpha Phi Omega National Service Fraternity
American Society for Microbiology
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Undergraduate: Organic Chemistry and Lab, Analytical Chemistry and Lab, Cell Physiology, General Microbiology and Lab, Developmental Biology, Microbial Genetics, Virology, Immunology, Pathogenic Bacteriology, Food Microbiology

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Molecular Biology Lab