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Genomic and Physiological Diversity Amongst Strains of *Thiobacillus ferrooxidans*, and Genomic Comparison with *Thiobacillus thiooxidans*

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Abstract. Twenty-three strains of *Thiobacillus ferrooxidans* of known pedigree were examined. Thirteen strains survived 65° C for 5 min and 7 of these for 10 min, but sporulation was never observed. All strains grew between 25° C and 35° C and some strains grew at 5° and 40° C. They were genomically diverse, comprising 7 DNA homology groups, and the GC content varied from 55–65 mol%. Correlation between genomic group and growth temperature was noted. All strains grew on ferrous sulfate as energy source, but some failed to utilize elemental sulfur. Acidified thiosulfate supported growth of most of the strains examined but it was judged to be a poor substrate upon which to base taxonomic conclusions because of decomposition of thiosulfate in acid. Six strains of *Thiobacillus thiooxidans* showed negligible genomic affinity to *T. ferrooxidans*, and they comprised 2 DNA homology groups and their GC content varied from 52–62 mol%. Anomalies due to contaminants in cultures of *T. ferrooxidans* were resolved, and the contaminants were identified.

Key words: Acidophilic thiobacilli – Genomic diversity – Physiological diversity

Thiobacillus ferrooxidans was discovered in acid drainage from bituminous coal mines by Colmer and co-workers (Colmer and Hinkle 1947; Colmer et al. 1950; Temple and Colmer 1951). Its role in forming acid from pyritic coal was investigated by Leathen et al. (1953), and its importance in the solubilization of copper was described by Beck and others (Bryner et al. 1954; Beck 1967; Duncan et al. 1964). This species continues to draw interest because of its environmental impact and its commercial value. Microbial processes that employ *T. ferrooxidans* include: the volatilization of mercury from mercury compounds in solution (Olson et al. 1980), the possible decontamination of industrial slag wastes (Ebner 1978), the potential for controlled desulfurization of coal (Dugan and Apel 1978), as well as the current commercial leaching of copper (Brierley 1980; Groudev et al. 1978) and uranium (Brierley 1978) from low-grade ore. Reclaimation of copper from mine refuse has been systematically studied in Eastern Europe (Groudev 1980a, b).

The observation that commonly identifies *T. ferrooxidans* is growth as pure culture in ferrous sulfate medium where ferrous iron serves as the source of energy. Tacit in this observation are 4 additional attributes: obligate aerobiosis, since ample oxygen must be supplied; mesophilism, since the culture is incubated between ambient and 32° C; autotrophism, because the culture ingredients are solely mineral salts, including the ammonium ion as source of nitrogen; and acidophilism, since high acidity ($pH \leq 4.5$) is necessary to prevent autooxidation of Fe^{2+}. These bacteria are Gram-negative and rod-shaped. The utilization of sulfur or some compound of sulfur as energy source in lieu of Fe^{2+} confirms the genus in the binominal for this species. Current knowledge of *T. ferrooxidans*, however, is based on a study of relatively few strains. Unlike heterotrophic bacteria, the acidophilic thiobacilli are difficult to acquire in pure culture and are troublesome to maintain. As a consequence, large collections of this species have not been utilized in the past.

The present research begins a systematic study of *T. ferrooxidans* through comparison of numerous strains of stated pedigree, from diverse locales and habitats. Deoxyribonucleic acid (DNA) analyses were used to define genomic groups within this species, and physiological tests were performed to correlate phenotype (biotype) with the particular genomic group. There is advantage when the comparative method is undertaken by a single individual. Minor flaws of technique, even if they should occur, do a minimum of mischief. Comparisons are most precise under such a circumstance. If all conditions are held constant throughout the study, the flaw itself becomes a constant, thus *differences* between strains are nevertheless valid. Hopefully, the collection which has been amassed, especially after it is more thoroughly studied, will provide other laboratories a useful selection of genotypes and phenotypes, and a choice of strain best suited for particular application.

Materials and Methods

Media. Basal medium contained (%w/v): $(NH_4)_2SO_4$, 0.2; K_2HPO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.05; KCl, 0.01; and $Ca(NO_3)_2$, 0.001. Stock cultures of *Thiobacillus ferrooxidans* were maintained in basal medium containing 4% (w/v) $FeSO_4 \cdot 7H_2O$ adjusted to pH 3 with dilute H_2SO_4. Small volumes of ferrous sulfate medium, 1 l or less, were sterilized by filtration through a membrane filter (type HA, Millipore Filter Corp., Bedford, MA, USA). The medium was dispensed either in 10 ml aliquots into sterile test tubes (20 mm diam × 150 mm) or in 100 ml aliquots into sterile 500 ml Erlenmeyer flasks. Cultures in test tubes were incubated on a slant-board to increase the surface area of the medium. Flasks were incubated on a rotary shaker. The ferrous sulfate medium was employed also for routine cultivation, to exa-

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mine morphology, to observe motility, and to determine heat-resistance and growth temperatures. However, to acquire sufficient mass of bacteria for DNA extraction 10 l of culture were required. $FeSO_4 \cdot 7H_2O$ (400 g) was dissolved in 1 l dilute H_2SO_4 and was sterilized by filtration, whereas the basal medium was prepared in 9 l of dilute H_2SO_4 and was sterilized in a 23 l carboy by autoclaving 1 h at 121° C. The sterile (filtered) ferrous sulfate solution was decanted aspectically into the 9 l of basal medium in the carboy after the latter was cooled to 30° C. The quantity of H_2SO_4 in the two solutions was calculated so that the pH would become 1.6 after they were mixed together. The low pH prevents the precipitation of ferric ion arising during bacterial growth (Olli Tuovinen, personal communication). This is important because the hydrated ferric oxide (limonite) which otherwise forms will damage the bearings in the continuous-flow centrifuge employed to collect the bacteria (Sorvall KSB-R Continous Flow System, DePont Co., Newton, CT, USA).

Thiobacillus thiooxidans was cultivated in sulfur medium. This was prepared by adding 0.5 % powdered sulfur (w/v) to basal medium adjusted to pH 3.5 with dilute H_2SO_4. Acidic sulfur medium in test tubes or in flasks was sterilized by heating to 105° C for 1 h on two successive days. To prepare 10 l of sulfur medium, 9 l of acidic basal medium in a 23 l carboy was autoclaved as already described, then cooled to 30° C, and 1 l of acidic powdered sulfur emulsion already sterilized by intermittent heating at 105° C was added aseptically. Fastest growth was obtained using Fisher S-597 colloidal sulfur (Fisher Scientific Co., St. Louis, MO, USA), although this product contains reducing capacity, as detected using phenol-sulfuric acid colorimetric analysis (Dubois et al. 1956), indicating presence of an impurity. Other forms of powdered sulfur: flowers of sulfur, sublimed sulfur, and precipitated sulfur (Fisher Scientific Co., St. Louis, MO, USA), do not permit as rapid growth as the colloidal sulfur. The sulfur medium was used also to cultivate some strains of *T. ferrooxidans*. Residual powdered sulfur was allowed to settle before the cultures were centrifuged, and that which remained suspended in the medium did not interfere with continuous-flow centrifugation. For testing for the presence of heterotrophic contaminants glucose a medium was used. It contained 0.1 % anhydrous glucose (w/v) in basal medium at pH 3. The glucose was prepared as a 1 % solution (w/v) lacking acid, was autoclaved 15 min at 121° C, then was added to cooled, acidified basal medium. Thiosulfate medium contained 1 % $Na_2S_2O_3 \cdot 5H_2O$ (w/v) in basal medium at pH 7. It was dispensed in 100 ml portions into 500 ml Erlenmeyer flasks and was autoclaved 15 min at 121° C. When cool, sterile H_2SO_4 was added to adjust the solution to pH 4. The thiosulfate medium of Vishniac and Santer (1957) was also used, but with only half strength phosphate. The pH was adjusted after the medium at pH 7 was autoclaved 15 min at 121° C. In tetrathionate medium 1 % sodium tetrathionate (w/v, K & K Laboratories, Plainville, NY, USA) replaced thiosulfate. The ISP medium of Manning (1975), slightly modified, was employed to secure single colony isolates of *T. ferrooxidans*. It contained (% w/v): $(NH_4)_2SO_4$, 0.5; KCl, 0.02; $MgSO_4 \cdot 7H_2O$, 0.02; $Ca(NO_3)_2$, 0.001; $Fe_2SO_4 \cdot 7H_2O$, 3.5; agarose (FMC Corp., Rockland, ME, USA), 0.5; and sufficient H_2SO_4 to give a pH = 2.7. The agarose, lacking acid and prepared double-strength, was autoclaved 15 min at 121° C, then cooled to 45° C. The other ingredients, also prepared double-strength but containing the H_2SO_4, were sterilized by filtration, quickly warmed to 45° C, then decanted into the molten agarose. This solution was poured aseptically into sterile Petri dishes (15 mm deep × 90 mm diam) to give a solidified medium 10 mm thick.

Measurement of Bacterial Growth. Growth of *T. ferrooxidans* in ferrous sulfate medium was detected visually. The colorless or pale blue medium changed to the color of rust as Fe^{2+} was oxdized to Fe^{3+}. Growth was confirmed by microscopic assay using a Petroff-Hausser bacteria counting chamber. Viable cell assays were determined by the Most-Probable-Number technique using triplicate tubes of ferrous sulfate medium at each dilution. Heat resistance was determined as follows. Duplicate test tubes containing 10 ml of ferrous sulfate medium were placed in a 65° C constant-temperature water-bath. After the medium had attained 65° C, approximately 1 ml of fresh culture was added to each tube. After 5 min one tube was cooled under the tap and placed in the 30° C incubator; after 10 min the second tube was cooled and incubated. Survival is a function of the number of cells added; therefore, care was taken that each inoculum was approximately the same, between 5×10^7 and 1×10^8 cells. Growth of the bacteria in sulfur medium was detected by microscopic assay and verified by pH measurement. The sulfuric acid formed from the oxidation of sulfur caused lowering of pH concomitant with growth.

Deoxyribonucleic Acid Analyses. The bacteria were centrifuged from 10 l of medium during late exponential growth to obtain 10^{12} cells. The cell pellets from ferrous sulfate medium were washed with basal medium at pH 1.6 to remove residual iron which otherwise interferes with DNA extraction. The cells were lysed with 1 % sodium lauryl sulfate, and the DNA was extracted and was purified according to the method of Marmur (1961). The purified DNA was dissolved in 0.1 × SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) at a concentration of approximately 400 µg/ml. The DNA solutions were stored frozen at −24° C.

The guanine plus cytosine (GC) content of the DNA was determined by the DNA melting point (Tm) method in 0.1 × SSC; a Gilford thermoprogrammer and a multiple-sample recorder with a Beckman model DU spectrophotometer were used. In determining Tm, the diluted DNAs were dialyzed at the same time and together with DNA from *Escherichia coli* strain B as reference DNA. The best estimate for the GC content of *E. coli* B DNA is 51 ± 0.25 mol % (Mandel, personal communication; Seidler and Mandel 1971). Using this value and the experimental Tm for *E. coli* B DNA, the results were normalized to *E. coli* B DNA by means of the equation provided by Mandel and Marmur (1968).

The method to determine DNA homology was described previously (Harrison et al. 1980; Johnson et al. 1980). Briefly, the method was as follows: A reference strain was selected, its DNA was radio-lebeled, then DNA denatured by heating so that it existed solely as single-strands. It was mixed with unlabeled DNA, also denatured to form single-strands. The degree of reassociation to form double-strands between reference (labeled) DNA and test (unlabeled) DNA is proportional to their genomic, presumably genetic, similarity. The reassociation reaction was carried out at 67° C. Deoxyribonuclease S1 (Calbiochem, San Diego, CA, USA) was added to digest any remaining single-stranded DNA. Then the reassociated (double-stranded) DNA was precipitated with cold trochloroacetic acid (TCA) and was collected on a membrane filter, and was radio-assayed.

Labeled DNA reassociating with its homologous unlabeled DNA yields the maximum number of S1-resistant, TCA-precipitable double strands, giving the highest counts-per-minute value. This value minus the self-reassociated (no unlabeled DNA present during reassociation) counts-per-minute value represents to net homologous reassociation value for reference DNA. In like manner, labeled DNA reassociating with heterologous DNA yields S1-resistant, TCA-precipitable double-strands at the same or lower counts-per-minute value than obtained with homologous DNA, depending upon the degree of relatedness to homologous DNA. This value minus the self-reassociation counts-per-minute value represents the net heterologous reassociation value. Finally,

$$\frac{\text{net heterologous reassociation counts-per-minute}}{\text{net homologous reassociation counts-per-minute}} \times 100 = \% \text{ homology.}$$

Each homology value (Table 3) is the arithmetic mean obtained from 2, sometimes as many as 6, replicate determinations.

Since autotrophic *Thiobacillus* incorporates organic compounds poorly, in vivo radio-labeling was impractical. Moreover, the large volumes of culture make in vivo radio-labeling hazardous. Therefore, in vitro radio-labeling was employed. The DNA was "nicked" with DNase I (Sigma Chemical Co., St. Louis, MO, USA), then was reconstructed with polymerase I (Bethesda Research Laboratories, Rockville, MD, USA) in the presence of the deoxyribosides triphosphates, the deoxythymidine triphosphate being tritium-labeled. This technique has been described elsewhere (Harrison et al. 1980).

Cultures. Twenty-three strains of *T. ferrooxidans* from various habitats are examined in this report, Table 1. For comparison, 6 strains of *T. thiooxidans* are included, Table 2.

Results and Discussion

The DNA homology results are summarized in Table 3 together with the GC content of the DNA and the energy source of the culture from which the DNA was acquired. For strains with low homology, reproducibility of results in replicate experiments was in the range of 5% homology. Accordingly, homology values of 5% or less have been recorded as zero. This avoids a claim of precision that is unattainable and it simplifies visual comprehension of the table. Reproducibility of GC content was well within 1 mol%. GC contents that diverge by more than 2 mol% are considered significant, and strains with a GC content that differ by more than this cannot be closely related, thus should show low homology. On the other hand, strains with identical GC content are not necessarily closely related. Homology reflects the ordering of the bases along the DNA strand, not gross GC content, thus strains with identical GC content may show low homology. But strains with high DNA homology should have very similar GC content. The results in Table 3 meet these expectations.

About 25% of the cultures as received were contaminated and had to be purified. Some contaminants, for example, yeast, were observed directly by microscopic examination. Most contaminants, however, were more difficult to detect. *T. ferrooxidans* cultures that appeared to adapt to heterotrophic growth on glucose were, in reality, autotrophic *T. ferrooxidans* contaminated with a heterotrophic species. The transitions by *T. ferrooxidans* between autotrophy and heterotrophy described by Tuovinen et al. (1978) were undoubtedly due to employment of impure cultures, which the authors, themselves, recognized as a possibility. Heterotrophic contaminants were isolated from *T. ferrooxidans* and were described (Harrison et al. 1980), and they hace since been named *Acidophilium cryptum* (Harrison 1981). It could not use as energy sources ferrous sulfate, elemental sulfur, or the decomposition products in acidified thiosulfate medium, either in the presence or in the absence of glucose, but it used glucose and many other organic compounds. Growth in glucose was, in fact, inhibited by acidic thiosulfate. It grew in *T. ferrooxidans* cultures by utilizing organic impurities in the medium and organic matter produced by autotrophic *T. ferrooxidans*. Perhaps some of the acidophilic heterotrophs examined by Wichlacz and Unz (1981) from mine drainage are this species. However, a different species, *Thiobacillus acidophilus* (Guay and Silver 1975), was the first to be detected as a contaminant in *T. ferrooxidans*. It does not use ferrous sulfate as an energy source , but it is able to use elemental sulfur or glucose; thus it is a facultative autotroph. *T. acidophilus* was not encountered in the present study. *A. cryptum* and *T. acidophilus* are different in several other ways (Harrison et al. 1980; Harrison 1981).

More insidious than heterotrophic contamination were mixtures of two strains of the same species. Co-existence of two different strains in a presumably pure culture of *T. ferrooxidans* was detected when DNA acquired from a sulfur-grown culture was compared with DNA from a culture grown on ferrous sulfate. Apparently, the individual strains attained different relative populations in the two media, because the two DNAs had different GC contents and yielded anomalous homology results. (One culture contaminated with *T. thiooxidans* was similarly detected.) Earlier, Guay et al. (1976) reached a similar conclusion when a culture of *T. ferrooxidans* yielded different GC contents on different metallic sulfides as energy sources. Pure cultures show no such aberrations as shown by comparing the sulfur-grown with the iron-grown cultures of *T. ferrooxidans* WVa, ATCC 19859, and PH in Table 3. Purification of suspect cultures was carried out by single colony isolation on ISP medium. Cultures that failed to form colonies on ISP medium were purified by dilution (Harrison et al. 1980). All results recorded herein are from pure cultures. All strains were obligately autotrophic and aerobic, Gram-negative, non-sporulating rods of varying length and proportion; some were motile, and though all utilized ferrous sulfate as source of energy, some failed to grow in serial passage in sulfur medium, but one of these (D-26) grew in acidified thiosulfate, Table 4.

The first 6 strains in Table 3 (DSM 504 through DSM 612) grew in sulfur medium but not in ferrous sulfate medium and were appropriately labeled by the donors as *T. thiooxidans*. The habitat is given in Table 2. All other strains grew in ferrous sulfate, and the donors had labeled them *T. ferrooxidans*. The habitat for these is listed in Table 1. Observe in Table 3 that the 6 strains of *T. thiooxidans* show negligible homology with the 23 strains labeled *T. ferrooxidans*. This correlates with the phenotypic distinctiveness of these two species (Hutchinson et al. 1966), but additional strains of *T. thiooxidans* should be examined to substantiate genomic

Table 1. Strains of *Thiobacillus ferrooxidans*

Strain	Received from	Habitat
BU-1	Stoyan Groudev, Sofia, Bulgaria	Acid mine drainage water, Gramatikovo copper mine, south-eastern Bulgaria
BU-2	Stoyan Groudev, Sofia, Bulgaria	A natural variant of BU-1. Single colony isolation by Stoyan Groudev
BU-3	Stoyan Groudev, Sofia, Bulgaria	Acid mine drainage water, Vlaikov vrah copper mine, central Bulgaria
BU-5	Stoyan Groudev, Sofia, Bulgaria	Acid mine drainage water, Vlaikov vrah lowgrade copper ore dump, central Bulgaria
Lp	P.R. Dugan, Columbus, Ohio, USA	Coal mine, western Pennsylvania, USA. Isolated by W. W. Leathen. Single colony isolation in author's laboratory
D-26	Davor Cotorás, Santiago, Chile	Copper mine; Mina Disputada de Las Condes, Chile
C-52	Davor Cotorás, Santiago, Chile	Copper mine; Mina Sta. Rita, Combarbalá, Chile
A-6	Davor Cottorás, Santiago, Chile	Copper mine; Mina Vista Hermosa, Andacollo, Chile
A-4	Davor Cotorás, Santiago, Chile	Copper mine; Mina Rincón, Andacollo, Chile
BA-4	G. J. Olson, National Bureau of Standards, Washington, D.C., USA	Settling lond, Belle Ayr coal mine, Gilette, Wyoming, USA
DECp	Olli Tuovinen, Columbus, Ohio, USA	Sulfur spring, New Mexico, USA. Single colony isolation in author's laboratory
AGN	Olli Tuovinen, Columbus, Ohio, USA	Uranium mine, Agnew Lake, Ontario, Canada
11Fe	G. P. Shimizu	Ningyo-Toge uranium deposit, Japan. Isolated by Tomizuka
TIO	G. P. Shimizu	Water from Tioga river, coal mining area, Blossburg, Pennsylvania, USA
ATCC 19859	American Type Culture Collection, Rockville, Maryland, USA	Acid copper leaching water, British Columbia, Canada. NCIB 9490; deposited by P. C. Trussell
ATCC 13598p	American Type Culture Collection, Rockville, Maryland, USA	Fifty-year-old copper leaching dump, Bingham canyon, Utah, USA. Deposited by J. V. Beck. Single colony isolation in author's laboratory
ATCC 13728	American Type Culture Collection, Rockville, Maryland, USA	Deposited by J. A. Sutton
DSM 583	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG	Coal mining area, western Pennsylvania, USA. Isolated by W. W. Leathen. Deposited by D. P. Kelly
F221	Klaus Bosecker, Hannover, FRG	Uranium mine water, Forstau, Austria
WVa	Bruce Jarvis, author's laboratory	Coal mine reclaimation site, Preston county, West Virginia, USA. Purifired by Bruce Jarvis
PH	Bruce Jarvis, author's laboratory	Six-year-old coal refuse, Bevier coal seam, Prairie Hill strip mine, Randolph county, Missouri, USA. Purified by Bruce Jarvis
m-l	Bruce Jarvis, author's laboratory	Forty-year-old coal strip mine refuse, Bevier coal seam, Calloway county, Missouri, USA. Purified by Bruce Jarvis
SOC	L. E. Murr, Socorro, New Mexico, USA	Copper ore leachate. New Mexico, USA

distinction. All except strain DSM 612 comprise a single DNA homology group, showing from 85% to 100% homology to reference DNA from strain DSM 504. Strain DSM 612 shows also a different GC content, 62 mol% versus 52–53 mol% for the other 5 strains. Since the heterotrophic contaminants have high GC contents, 63–64 mol% for *T. acidophilus* (Guay and Silver 1975) and 68–70 mol% for *A. cryptum* (Harrison 1981), contamination was suspected. But contamination was not detected, either in this laboratory or by Arkesteyn and DeBout (1980). Therefore, the phenotype presently designated *T. thiooxidans* includes a strain of vastly different GC content than the 52–53 mol% reported by Vishniac (1974) and Jackson et al. (1968). Additional study is needed to determine if, indeed, strain DSM 612 is a new species.

The BU-series of *T. ferrooxidans* (Table 3) are unique. They have a lower GC content than other *T. ferrooxidans* and they comprise a single homology group, which will be arbitrarily labeled DNA Homology Group No. 1. The next 6 strains (Lp through BA-4) comprise a second distinct *T. ferrooxidans* homology group which will be designated DNA Homology Group No. 2. Reference DNAs were prepared

Table 2. Strains of *Thiobacillus thiooxidans*

Strain	Received from	Habitat
DSM 504	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG	Soil; New Jersey, USA. Isolated by Waksman and Joffe, ATCC 8085; deposited by R. L. Starkey
DSM 622	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG	Pont water, Göttingen, FRG. Isolated by H. Hippe
DSM 612	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG	Acidic sulfate soil. Mijdrecht, Netherlands. Isolated by H. Hippe
ATCC 19377	American Type Culture Collection, Rockville, Maryland, USA	Libyan sulfur-producing lake. NCIB 8343; deposited by K. R. Butler and J. R. Postgate
Ram 8T	Klaus Bosecker, Hannover, FRG	Water from a 900-year-old copper and zinc mine, Harz Mountains, Rammelsberg, Goslar, FRG
T219	Klaus Bosecker, Hannover, FRG	Water from a uranium mine, Forstau, Austria

Table 3. DNA homologies among strains of *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans*

Species	Strain	Energy source[a]	GC[b] (mol%)	Percent homology to											Homology group
				DSM 504	BU-3	Lp	WVa	D-26	DECp	A-4	ATCC 19859	ATCC 13598p	PH	m-l	
				S	Fe	Fe	S	Fe	Fe	Fe	Fe	Fe	S	Fe	
T. thiooxidans	DSM 504	S	53	100	0	10	14	11	0	0	13	0	0	0	
	DSM 622	S	53	85	0		17	10	0		0	11	7	0	
	ATCC 19377	S	52	87	0	9	10		0	13	9	12	0	0	
	Ram 8T	S	53	100	0		19		0		0	23	0	0	
	T219	S	53	89	10		12		0		0	23	0	0	
	DSM 612	S	62	11	0	20	18	12	0	0	0	19	12	0	
T. ferrooxidans	BU-3	Fe	53	0	100	0	0	7	6	8	14	12	0	6	
	BU-1	Fe	53	7	89	0	0	14	11	0	13	0	0	0	No. 1
	BU-2	Fe	53	0	89	0	0				16	8	0	9	
	BU-5	Fe	53	0	87	0	0				12	10	0	0	
	Lp	Fe	57		0	100	78	86	25	37	31	43	27	0	
	WVa	Fe	57	0	0	64	100	73	20	25	26	30	19	0	
	WVa	S	57		0	70	100	74	18	28	27	32	14	0	
	D-26	Fe	57	7	0	72	88	100	38	31	41	31	32	0	No. 2
	C-52	S	56	10	0	77	83	77	28	28	37	23	30	0	
	A-6	Fe	57	18	0	79	82	82	26	38	36	46	16	0	
	A-6	S	57		0	87	96	85	30	42	40	45	19	0	
	BA-4	Fe	57		0	66	90	91	20	31	24	43	22	0	
	DECp	Fe	57	0	0	36	38	31	100	100	58	73	13	0	
	A-4	S	58	15	0	38	17	36	99	100	65	58	19	6	No. 3b
	11Fe	Fe	57		0	47	33	46	70	90	60	69	17	7	
	TIOp	Fe	57	10	0	22	33	31	56	75	59	66	23	9	
	ATCC 19859	Fe	58	11	0	24	39	31	47	67	100	86	35	0	
	ATCC 19859	S	58		0	22	28	29	40	66	100	88	38	0	
	ATCC 13598p	Fe	59	17	0	28	45	31	51	57	88	100	0	0	No. 3a
	ATCC 13728	Fe	59		0	27	46	19	38	68	87	97	15	9	
	DSM 583	Fe	58		0	34	18	30	52	72	97	84	11	0	
	F221	S	59		0	26	44	45	41	70	85	90	21	8	
	PH	S	59	13	0	26	35	23	10	24	19	30	100	0	No. 4
	PH	Fe	59		0	20	33	20	14	20	16	27	98	0	
	SOC	S	59	14	0	38	55	34	57	65	60	61	8	0	No. 5
	AGN	Fe	62	33	10	28	52	32	21	9	24	13	0	0	No. 6
	m-l	Fe	65	0	8	8	0	20	10	6	0	19	6	100	No. 7

[a] S = Cultivated on sulfur medium. Fe = Cultivated on ferrous sulfate medium

[b] Determined by the Tm method. For values determined by other methods, see text

Table 4. Phenotype traits of *Thiobacillus ferrooxidans*

Strain	Growth at[a]				Survival at 65°C for[b]		Motility[c]	Serial growth in elemental sulfur[d]	Serial growth in thiosulfate decomposition products[e]	DNA homology group
	5°C	30°C	35°C	40°C	5 min	10 min				
BU-1, BU-2, BU-3, BU-5	−	++	++	−	+	+	++	−		No. 1
Lp	+	++	+	−	+	+	−	+		No. 2
WVa	+	++	++	−	−	−	−	+	+	
D-26	−	++	+	−	+	+	+	−	+	
C-52	−	++	+	−	+	−	−	+		
A-6	−	++	+	−	+	−	±	+		
BA-4	−	++	+	−	−	−	+	+		
DECp	−	++	++	±	−	−	−	+		No. 3b
A-4	+	++	++	−	+	−	+	+		
11Fe	−	++	++	−	+	−	−	+		
TIOp	+	++	++	−	−	−	+	+		
ATCC 19859	+	++	++	+	−	−	−	+	+	No. 3a
ATCC 13598p	+	++	++	+	−	−	−	+		
ATCC 13728	−	++	++	+	+	+	−	+		
DSM 583	+	++	++	++	+	−	−	+	+	
F221	−	++	++	+	+	−	−	+		
PH	−	++	++	±	−	−	+	+		No. 4
SOC	−	++	++	−	−	−	−	+		No. 5
AGN	−	++	++	−	−	−	+	+		No. 6
m-1	−	++	++	±	−	−	−	−		No. 7

[a] Tested in 10 ml ferrous sulfate medium, ph 3, in slanted test tubes. ++ = Good (normal) growth; + = slower growth than at 30°C; ± = variable or very poor growth; − = no growth after 2 weeks at 30°C, 35°, and 40°C, and after 5 weeks at 5°C. The − cultures at 5°C may have shown growth if incubated longer, and growth responses may be different under different conditions of aeration and pH

[b] Tested in 10 ml ferrous sulfate, and incubated at 30°C for 3 weeks

[c] Observed in ferrous sulfate medium at 30°C. Strain BU-1 is always motile in young culture. Strain A-6 is motile rarely. The other strains fall between these extremes of performance

[d] Tested in 100 ml sulfur medium in shake flasks at 30°C

[e] Tested in 100 ml modified Vishniac's (1957) thiosulfate medium, pH 3.0 to 5.8, in shake flasks at 30°C. See Discussion

from three strains: Lp, WVa, and D-26. In all but two instances, the homologies fall between 70% and 100%. (Reference DNA from strain Lp was less reactive, generally, than reference DNA from strain WVa and strain D-26). Observe that the reciprocal homologies to the BU-series are zero in most instances. Strains DECp, A-4, 11Fe, and TIOp comprise another homology group. However, these four strains have genomic affinities with the next group of five strains: ATCC 19859, ATCC 13598p, ATCC 13728, DSM 583, and F221. The latter group will be designated DNA Homology Group No. 3a, whereas the former group of four strains (DECp through TIOp) will be designated DNA Homology Group No. 3b. Homology Groups No. 3a and No. 3b manifest closer genomic affinity than occur between them and other homology groups, or between any other combination. Strain PH shows negligible homology to the other 22 strains. Likewise, strains SOC and AGN appear each to be unique. Strain m-l also manifests negligible homology with other *T. ferrooxidans*. Thus the 23 strains of *T. ferrooxidans* diverge into 7 different homology groups, and they show a wide range of GC content. It was important to confirm this striking divergence in GC content by other means. Accordingly, M. Mandel, employing the CsCl buoyant density technique, examined some strains. He found the GC for strain m-1 to be 65.3 mol %, and for strain BU-1 to be 57.1 mol % (personal communication). Since the values of GC content for BU-1 were significantly different by the two methods (Tm and buoyant density), a chemical analysis was undertaken. After enzymatic hydrolysis, the deoxynucleosides were subjected to reverse-phase, high-pressure, liquid chromotography. The mol % GC was determined to be 55.6 (C. W. Gehrke, K. C. Kue, and R. A. McCune; personal communication). Therefore, the best estimate of the GC content of *T. ferrooxidans* is 55−65 mol %, far greater than previously recorded (Vishniac 1974; Jackson et al. 1968). A trace of 5-methyl-deoxycytidine was detected in BU-1 DNA, but the reason for the divergent GC contents determined for BU-1 by the Tm and buoyant density methods remains a mystery. (The three different analytical methods of course employed the same homogenous batch of BU-1 DNA).

The wide GC spectrum and the several DNA homology groups demonstrated for *T. ferrooxidans* betray a high degree of genomic diversity within this species as presently defined. But it is not possible to conclude whether different organisms from different lines-of-descent converged and independently acquired the ability to utilize ferrous iron or, conversely, whether a single organism once acquired this ability and then diverged from the ancestral genotype. Comparison of several unrelated genera illustrates the degree of speciation assigned to them, genera with the same degree of GC divergence as *T. ferrooxidans*, approximately 11 mol%. *Pseudomonas* has a GC range of 12 mol% and comprises 16 species (Duodoroff and Palleroni 1974), *Staphylococcus* has a GC range of 11 mol% and comprises 3 species (Baird-Parker 1974), and *Streptococcus* with a GC range of 10 mol% contains 21 species (Deibel and Seeley 1974).

Too few phenotypic characters have been tallied to permit the homology groups to be identified solely by means of physiological tests. Some correlations, however, already have been noted. The strains of Homology Group No. 3a grew at 40° C, and most strains of Homology Group No. 2 grew somewhat faster at 30° than 35° C, Table 4. Strains in the homology groups at the extremes of the GC spectrum, the BU-series and strain m-1, failed to utilize elemental sulfur, sublimed sulfur and precipitated sulfur as well as colloidal sulfur. Strain D-26 also failed to utilize elemental sulfur, but perhaps it will do so when some pecularity of pH or temperature is met. It does utilize some compound of sulfur as energy source because it grew in acidified thiosulfate, Table 4. At the present time the BU-series and m-1 have failed to grow in thiosulfate, but they are still under investigation with particular attention to the valuable paper by Tuovinen and Kelly (1974) which describes culture conditions that influence adaptation to acidified thiosulfate. There appear to be pH preferences at the strain level, however, that affect adjustment by an iron grown culture to grow in acidified thiosulfate. Tuovinen and Kelly (1974) found that pH 3.5–4.8 was optimum for their strains, and Landesman et al. (1966) using washed cell suspensions found this same range to be optimum for respiration by their strain. Likewise, in the present investigation strains ATCC 19859 and WVa adapted quicker to thiosulfate at pH 4 than at pH 5.8, but strain D-26 adapted much faster at pH 5.8 than at pH 4. Indeed, there have been probably more disagreements in the literature concerning thiosulfate utilization by *T. ferrooxidans* than over any other trait of this microorganism. An iron-oxidizer isolated from the Bingham Canyon copper mine drainage water by Unz and Lundgren (1961) could not be maintained through serial passage in thiosulfate medium, and subculturing Leathen's strain TM "in thiosulfate medium without loss of activity was difficult and undependable." These investigators sought to confirm thiosulfate utilization by means of serial passage which is, of course, necessary in order that this, or any other, substrate is concluded to be a suitable energy source. In manometric studies Silverman and Lundgren (1959) found that strain TM did not oxidize thiosulfate, confirming the growth results with this strain by Leathen et al. (1956). On solidified thiosulfate medium the agar can add impurities and results may be ambiguous. Early in the present investigation several strains formed tiny colonies on acidic thiosulfate agar but the colonies developed almost as well on agar lacking thiosulfate, and the strains were discovered to be contaminated with heterotrophic bacteria. The different conclusions expressed in the literature may reflect genetic differences at the strain level. More important, however, is the intrinsic instability of the thiosulfate ion in mild acid.

If thiosulfate medium containing 1% $Na_2S_2O_3 \cdot 5H_2O$ (w/v) is acidified at 25° C to bring the pH below 5.5, decomposition always takes place to yield visible colloidal sulfur and a sulfurous odor reminiscent of H_2S within several minutes to a quarter hour at pH 3–4 and within an hour or two at the higher pH. The decomposition may be overlooked if the medium is autoclaved soon after preparation because the gaseous products are mostly lost and the colloid is changed to an inconspicuous floc as a result of the heating. In the present investigation the best results in thiosulfate were with the medium of Vishniac and Santer (1957) with half strength phosphate, sterilized at pH 7 where thiosulfate is stable. When cool, the medium was adjusted to the desired pH with sterile H_2SO_4. The maximum quantity of sulfur in all its forms is retained in the medium.

Colloid chemists routinely have used acidified thiosulfate as a means of acquiring colloidal sulfur (Zaiser and LaMer 1948). "The products obtained when a dilute solution of thiosulfate is acidified are very complex and depend on the conditions –" (Lyons and Nickless 1968); "– thiosulfate decomposes when slightly acidified to form sulfite, free sulfur, and polythionate" (Huff 1970). The products are of constantly changing proportions, and include: elemental sulfur (Both S_6 and S_8), H_2S, SO_2, and various polythionic acids (Davis 1958; Schmidt 1972). In the observations by sulfur chemists we find an explanation for the divergent conclusions by microbiologists. A series of enzymatic adaptations will occur as a *Thiobacillus* sequentially utilizes the several decomposition products. Indeed, this can account for the common, and seemingly contradictory observation that thiosulfate-grown cells enter a long lag before they begin to grow when transferred to fresh thiosulfate. Growth in acidic thiosulfate may constitute presumptive evidence of sulfur utilization, but the form of sulfur being utilized is ambiguous, and it is unlikely to be solely thiosulfate.

It will be important to determine whether the BU-series and m-1 utilize sulfur. If they do not, they lie beyond the definition of *Thiobacillus*. Leathen et al. (1956) coined the name *Ferrobacillus* for certain iron-oxidizing bacteria clained to lack adaptation to sulfur, but Kelly and Tuovinen (1972) demonstrated that the claim was invalid and favored *Thiobacillus*. Additional information is necessary to characterize the very high and the very low GC-containing strains that presently bear the binomial *T. ferrooxidans*. It seems unlikely that organisms that diverge by as much as 11 mol% GC can constitute one, and the same, species.

Determination of homology between DNA and ribosomal RNA can aid *Thiobacillus* classification by defining more distant relationships, as it has succeeded so well in doing for the genus *Pseudomonas* (Palleroni et al. 1973). When a conflict arises, however, one must be willing to decide whether genomic or phenotypic differences are to take precedence. If bacterial classification is intended to reflect phylogeny as its nomenclatural trappings imply, then genomic differences must be accommodated in the classification.

An overview of the results demonstrates that the DNA homology groups are ubiquitous. Aside from the Bulgarian isolates, the homology groups do not correlate with geography. As with other bacteria, it is the micro-environment which selects for a given genotype, and the micro-environment suitable for each genotype apparently is available worldwide. It would be interesting, possibly useful, to

know which homology group preferentially fluorishes under which special kind of micro-environment. Correlation of physiological traits with homology group suggests the properties of the respective micro-environments. An extension of this research should aid our understanding the role of *T. ferrooxidans* in its natural habitat and may lead to selection of strains best suited for a particular kind of mineral leaching or desulfurization process.

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