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Heterotrophic Bacteria from Cultures of Autotrophic *Thiobacillus ferrooxidans*: Relationships as Studied by Means of Deoxyribonucleic Acid Homology

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From several presumably pure cultures of *Thiobacillus ferrooxidans*, we isolated a pair of stable phenotypes. One was a strict autotroph utilizing sulfur or ferrous iron as the energy source and unable to utilize glucose; the other phenotype was an acidophilic obligate heterotroph capable of utilizing glucose but not sulfur or ferrous iron. The acidophilic obligate heterotroph not only was encountered in cultures of *T. ferrooxidans*, but also was isolated with glucose-mineral salts medium, pH 2.0, directly from coal refuse. By means of deoxyribonucleic acid homology, we have demonstrated that the acidophilic heterotrophs are of a different genotype from *T. ferrooxidans*, not closely related to this species; we have shown also that the acidophilic obligate heterotrophs, regardless of their source of isolation, are related to each other. Therefore, cultures of *T. ferrooxidans* reported capable of utilizing organic compounds should be carefully examined for contamination. The acidophilic heterotrophs isolated by us are different from *T. acidophilis*, which is also associated with *T. ferrooxidans* but is facultative, utilizing both glucose and elemental sulfur as energy sources. Since they are so common and tenacious in *T. ferrooxidans* cultures, the heterotrophs must be associated with *T. ferrooxidans* in the natural habitat.

Thiobacillus ferrooxidans, a bacterial species capable of utilizing sulfur or ferrous iron as sources of energy, was discovered and named by Colmer and co-workers (4, 26), who considered it to be a strict autotroph. The first report that this species is capable of heterotrophic growth came from Lundgren's laboratory (17; C. C. Remsen and D. G. Lundgren, Bacteriol. Proc., p. 33, 1963). A culture of *T. ferrooxidans* was adapted to grow on glucose. This glucose-adapted culture lost the ability to utilize iron, but whether it also lost the ability to utilize elemental sulfur was not stated. A strain designation was not given; apparently this acidophilic heterotroph was derived from a *T. ferrooxidans* culture provided by Leathen (16). Later, Shafia and Wilkinson (23) adapted two strains of *T. ferrooxidans* (of six strains tested) to utilize glucose as the energy source. These two acidophilic strains also became obligate heterotrophs, losing the ability to utilize iron. Their ability to utilize sulfur was not examined. One heterotroph was derived from *T. ferrooxidans* isolated in Bingham Canyon, Utah; the other heterotroph was derived from *T. ferrooxidans* acquired from Lundgren. Tabita and Lundgren (25) derived an obligate heterotroph from Leathen's *T. ferrooxidans* strain TM, extending their earlier work to show that several different organic compounds could be used as energy sources. Shafia et al. (22) compared *T. ferrooxidans* (grown on ferrous iron) with the acidophilic heterotroph (grown on glucose) by serological methods. The autotroph and the heterotroph were antigenically different. Zavarzin (30) isolated acidophilic heterotrophs directly from an acidic swamp and also from a culture of *T. ferrooxidans* acquired from this swamp. The heterotroph populated the swamp at a much higher density than did *T. ferrooxidans*. Guay and Silver (8) isolated an acidophilic heterotroph from Leathen's *T. ferrooxidans* strain TM. This heterotroph was facultative; it could utilize both glucose and elemental sulfur but not iron as the sole source of energy, and they named it *Thiobacillus acidophilus*. Since the earlier workers did not establish whether their heterotrophs were able to utilize sulfur as the energy source, it is not possible to conclude whether their isolates were the same as *T. acidophilus*. Guay et al. (9) compared the base composition of DNA extracted from *T. ferrooxidans* grown on a variety of mineral sulfides. They found that the guanine plus cytosine mol% of the DNA varied from 54.2 to 60.2, depending upon the mineral substrate,

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and therefore concluded that *T. ferrooxidans* cultures may not comprise a distinct species. Tuovinen and Nicholas (28) studied the conversion of *T. ferrooxidans* from autotrophy to heterotrophy, and they concluded that heterotroph strain KG-4, derived from a Bingham Canyon (Utah) *T. ferrooxidans* (23), was impure as to biotype. Mackintosh (18) observed that "when *T. ferrooxidans*, whether isolated in a liquid culture or received from a culture collection, is plated out—at least two types of colonies are seen," and concluded, therefore, that *T. ferrooxidans* cultures may be contaminated. In our laboratory, the same conclusion was reached (12; B. W. Jarvis, M.S. thesis, University of Missouri, Columbia, 1979). Microscopic examination of some allegedly pure *T. ferrooxidans* cultures revealed more than one morphological cell type. In one instance, small motile rods were discernible among larger, nonmotile rods. Two biotypes were separated: a motile obligate heterotroph incapable of utilizing either ferrous iron or elemental sulfur as the source of energy, and a nonmotile strict autotroph indistinguishable from the *T. ferrooxidans* of Colmer et al. (4, 26).

There is a continuing interest in the acidophilic heterotrophs (7, 24, 27) with regard to their origin in *T. ferrooxidans* cultures, their activities with *T. ferrooxidans* in a natural habitat, and their role in the microbial desulfurization of coal. It is important, therefore, to characterize more thoroughly these bacteria and especially to establish unequivocally their genetic relationship to *T. ferrooxidans*. The successful extension of DNA homology analyses to autotrophic bacteria described for the first time herein makes this now possible. The present research compares a collection of acidophilic heterotrophs with each other and with *T. ferrooxidans*. The collection comprises strains donated by other investigators, additional strains derived from *T. ferrooxidans* in the laboratory of A.P.H., and strains isolated directly from acidic coal spoils.

MATERIALS AND METHODS

Bacteria, growth methods, and media. Table 1 lists the strains with their pedigree, and also lists the energy source on which they were grown for DNA isolation. For DNA extraction, the cells were grown in 10 liters of medium in a 5-gallon (ca. 19-liter) carboy with aeration by the passage of cotton-filtered, compressed air through a sintered-glass sparger. Growth studies were carried out either in a 100-ml volume of medium in 500-ml cotton-plugged Erlenmeyer flasks or in a 10-ml volume of medium in metal-capped test tubes (1.7 mm ID by 150 mm long). The flasks were incubated on a rotary shaker at 150 rpm, and the test tubes were incubated on a slant board which tilted them 20° above horizontal. Incubation was always at 26°C. Cell populations were determined with a Petroff-Hausser bacterial counting chamber and phase-contrast microscope. Viable cell counts were determined in serially diluted samples, using suitable agar medium in petri dishes (10 mm by 90 mm in diameter). The acidophilic heterotrophs were cultivated in a medium of the following composition (per liter): 2 g of $(NH)_4SO_4$, 0.1 g of KCl, 0.01 g of $Ca(NO_3)_2$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 1 g of D-mannitol, and 0.1 g of dehydrated Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), with the pH adjusted to 3.4 ± 0.2 with H_2SO_4. This medium was autoclaved at 15 lb/in^2 for 15 min. When glucose was used in lieu of mannitol, it was autoclaved separately and added aseptically to the other sterile ingredients. These two media will be designated mannitol medium and glucose medium, respectively. Assays for viable cells were with mannitol medium solidified with agar, 12 g/liter, pH adjusted to 4.5; the agar and other ingredients were prepared in separate vessels double strength, autoclaved, cooled to 45°C, and then mixed together. Sulfur-utilizing bacteria were cultivated in the above liquid medium with the omission of the organic ingredients. Colloidal sulfur S-597 (Fisher Scientific Co., St. Louis, Mo.) permitted fastest growth and highest cell yields of the several forms of commercial sulfur tested. For cultivation in a 100-ml volume of medium in flasks, 1 g of colloidal sulfur was added to each flask. Sterilization was at 105°C for 1 h on 2 successive days. For cultivation in a 10-liter volume of medium in carboys, 30 g of sulfur was sterilized separately (as above) in 1 liter of water with all the acid needed to adjust the final pH to 3.4; the other ingredients (9 liters) were autoclaved in the carboy for 2 h at 15 lb/in^2. After cooling, the sulfur emulsion was aseptically decanted into the carboy. No contamination was encountered in either flasks or carboys; heating at 105°C for 1 h on successive days combined with the low pH was adequate for sterilization. This medium will be designated sulfur medium. Iron-utilizing bacteria were cultivated also in the liquid medium already formulated with the omission of the organic components, but with 44 g of $FeSO_4 \cdot 7H_2O$ per liter, and the pH was adjusted to 3.2. This medium, designated iron medium, was sterilized by filtration through a membrane filter (type HA, Millipore Corp., Bedford, Mass.). *Thiobacillus thioparus* was cultivated in thiosulfate medium of the following composition (per liter): 10 g of $Na_2S_2O_3 \cdot 5H_2O$, 4 g of KH_2PO_4, 4 g of K_2HPO_4, 1 g of NH_4Cl, and 0.4 g of $MgCl_2 \cdot 6H_2O$, with 0.5 ml of trace element solution (29) added. The pH was 6.7.

Isolation, purification, and maintenance of cultures. Acidophilic heterotrophs were isolated from coal strip-mine spoils and from an artificial coal spoil maintained in the laboratory (10). One gram of soil was added to 100 ml of glucose medium in flasks and incubated for several days on the rotary shaker. Where molds were troublesome, the pH of the medium was reduced to 2.0. The acidophilic heterotrophs have a pH minimum for growth of around 1.8, almost as low a pH as permits growth of the *T. ferrooxidans* strains. Material from the flask was seeded on agar plates of glucose medium (or mannitol medium); single colonies were picked, and purity was ensured by streaking fresh

TABLE 1. *Bacterial strains*

Strain	Derivation and pedigree	DNA acquired from culture grown on:
L_p	Purified Fe^{2+}-utilizing autotroph isolated from *T. ferrooxidans* received from P. R. Dugan. (We designate the culture as received as strain L.) Dugan acquired this culture from D. G. Lundgren; it was isolated by W. W. Leathen.	Sulfur[a]
Lhet2	Purified glucose-utilizing heterotroph isolated from the *T. ferrooxidans* culture received from P. R. Dugan, above.	Glucose
13598	*T. ferrooxidans* ATCC 13598 used as received (unpurified) from the American Type Culture Collection. Was deposited by J. V. Beck, who isolated it from effluent of a 50-year-old leaching dump of the Kennecott Co., copper mine in Bingham Canyon, Utah. This dump is identified as "Markham" by J. V. Beck (1).	Sulfur[a]
13598het	Purified glucose-utilizing heterotroph isolated from *T. ferrooxidans* ATCC 13598, above.	Glucose
m-5	Purified glucose-utilizing heterotroph isolated from an artificial coal spoil (9). Source was a coal mine spoil in Calloway County, Mo., since soil from this source served as inoculum for the artificial spoil.	Glucose
Dhetl	Purified glucose-utilizing heterotroph isolated from acidic soil from a coal strip mine in Randolph County, Mo.	Glucose
$KG\text{-}4_p$	Purified glucose-utilizing heterotroph isolated from strain KG-4 received in glucose medium from O. H. Tuovinen. KG-4 is the heterotroph isolated by Shafia & Wilkinson (23) from a Bingham Canyon strain of *T. ferrooxidans*.	Glucose
7_p	Purified glucose-utilizing heterotroph isolated from *T. acidophilus* no. 7 received in glucose medium from O. H. Tuovinen. Isolated by Guay and Silver from *T. ferrooxidans* strain TM (8).	Glucose
2T10	Used as received (unpurified) from O. H. Tuovinen, who sent it to us in iron medium. This strain was derived by O. H. Tuovinen from *T. acidophilus* no. 7, above. Therefore, it may be anticipated that our 7_p and glucose-grown strain 2T10 are the same, or similar, organisms.	Glucose
23646_p	Purified isolate from *T. thioparus* ATCC 23646.	Thiosulfate

[a] Fe^{2+}-utilizing *T. ferrooxidans* autotrophs were cultivated on sulfur in lieu of Fe^{2+} because iron interferes with DNA extraction.

plates. The colonies required 10 days at 26°C to attain a 0.5-mm diameter. The heterotrophs were stored in the refrigerator on agar slants of mannitol medium and transferred to fresh agar medium every month or less. These heterotrophs maintain their characters and have never formed rusty discoloration in iron medium (which betrays the growth of autotrophic *T. ferrooxidans*) even after incubation for 6 weeks. Acidophilic heterotrophs were isolated also from cultures of *T. ferrooxidans* (Table 1). *T. ferrooxidans* was seeded onto glucose medium or onto the ISP medium of Manning (19). This latter medium contains ferrous sulfate with other mineral salts at pH 2.6. It was useful to detect heterotrophic contamination of *T. ferrooxidans* cultures; the autotroph forms distinctive rusty-red colonies, whereas the heterotroph forms a yellowish growth. (On iron-free agars the heterotrophs form white to pinkish growth.) The heterotrophs from *T. ferrooxidans* cultures were purified as already described.

Purification of *T. ferrooxidans* was undertaken as follows. The cell population was determined microscopically, using a Petroff-Hausser counting chamber. Then a series of 10-fold serial dilutions in 10 ml of iron medium in test tubes was prepared so that the highest dilution would receive no cells. Our assumption, apparently correct, was that in iron medium the cells of autotrophic *T. ferrooxidans* outnumber the cells of the heterotroph. After incubation on the slant board for several weeks, the highest dilution tube which showed growth, noted as rusty discoloration of the medium due to ferric hydroxide precipitation, was

examined microscopically to determine the cell population therein, and a new series of dilutions was prepared from it. The highest dilution again showing growth was selected for assay and serial dilution. After at least three such selections via dilutions, the *T. ferrooxidans* culture was presumed pure if no heterotrophs could be detected upon heavy seeding in glucose medium. Stock cultures of *T. ferrooxidans* were maintained in a 10-ml volume of iron medium in test tubes held in the refrigerator for 1 month or less between transfers. Stock cultures of *T. thioparus* were maintained on thiosulfate agar slants.

DNA isolation and homology. The bacteria were grown in a suitable liquid medium (Table 1) and harvested during late exponential growth from a sufficient volume, usually 10 liters, to collect 10^{12} cells. The cells were suspended in approximately 40 ml of EDTA buffer (20). The cells were lysed by adding sodium lauryl sulfate to 1%, and the DNA was then isolated according to the method of Marmur (20). The purified DNA was dissolved in 0.1× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and stored at −20°C.

Tritium-labeled DNA was prepared by an in vitro nick translation procedure similar to that of Chelm and Hallick (3). The DNA was nicked with DNase I (Sigma Chemical Co., St. Louis, Mo.), followed by reconstruction with DNA polymerase I (Bethesda Research Laboratories, Rockville, Md.) in the presence of the deoxyriboside triphosphates; the deoxythymidine triphosphate was tritium labeled in the methyl group of the thymidine moiety (New England Nuclear Corp., Boston, Mass.). The radiolabeled DNA was purified on a P-100 acrylamide column (Bio-Rad Laboratories, Richmond, Calif.). Specific radioactivities of 1×10^5 cpm per μg of DNA were obtained.

The fragment lengths of both labeled and unlabeled DNA preparations were reduced to approximately 400 nucleosides by means of sonication with a Biosonic instrument (Bronwill Scientific Inc., Rochester, N.Y.). Fragment lengths were monitored by the alkaline agarose electrophoresis technique of McDonell et al. (21). The DNA preparations were denatured by heating in a glycol bath at 101°C for 5 min and then rapidly cooled in an ice bath.

DNA homology experiments were done by a variation (13) of the S1 nuclease procedure described by Crosa et al. (6). The reassociation mixture in 0.2 ml microphials (in triplicate) consisted of 10 μl of labeled DNA (ca. 2 μg/ml), 50 μl of unlabeled DNA (400 μg/ml), and 50 μl of 0.88 M NaCl–10^{-3} M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES, pH 7.0). The mixture was incubated at 65°C for 24 h and then was treated with S1 nuclease (Calbiochem, San Diego, Calif.) as described by Johnson et al. (13). S1 enzyme with our DNA digests at least 93% of the single-stranded DNA, whereas 95% of the double-stranded DNA escapes digestion. The reassociated (double-stranded) DNA was precipitated with cold 10% trichloroacetic acid, collected on membrane filters (type HA, Millipore), and washed with trichloroacetic acid; the filters were dried, and radioactivity was measured with a Beckman LS-230 liquid scintillation system for 10 min.

Labeled DNA reassociating with its homologous unlabeled DNA yields the maximum number of S1-resistant, trichloroacetic acid-precipitable duplexes, giving the highest counts-per-minute value. This value minus the self-reassociated (no unlabeled DNA present during reassociation) counts-per-minute value represents the net homologous reassociation value for reference DNA. The net reassociation values resulting from the duplexes formed between labeled reference DNA and heterologous unlabeled DNA preparations divided by the net homologous value and then multiplied by 100 give the percent DNA homology value (Tables 2 and 3). In one experiment, for example, from an input of 3,950 cpm of labeled DNA added to each reaction vessel, 3,720 cpm were recovered upon reassociation with unlabeled homologous DNA. The self-reassociation cpm value was 450. Thus, the difference, 3,270 cpm, represents the net reassociation value for reference DNA, 100% homology.

RESULTS

Table 2 summarizes results with autotrophic *T. ferrooxidans* strain L_p supplying labeled DNA. Labeled DNA reassociated to the greatest extent with its homologous unlabeled DNA, and this amount was designated 100% homology. DNA from *T. ferrooxidans* strain 13598 had 28 ± 4% homology with strain L_p. The two strains were from different sources (Table 1). (The degree of genomic relatedness among strains of iron oxidizers currently assigned the binomial *T. ferrooxidans* remains to be determined through DNA homology and is presently under investi-

TABLE 2. *Percent DNA homology between autotrophic T. ferrooxidans strain L_p and other bacteria*

Unlabeled, single-stranded DNA from:	% Homology to *T. ferrooxidans* strain L_p (labeled single-stranded DNA)
T. ferrooxidans strain L_p	100
T. ferrooxidans strain 13598	28 ± 4
T. thioparus strain 23646	0
Lhet2 (heterotroph)	0
KG-4_p (heterotroph)	0
T. acidophilus strain 7_p	0

TABLE 3. *Percent DNA homology between heterotroph strain Lhet2 and other bacteria*

Unlabeled, single-stranded DNA from:	% Homology to strain Lhet2 (labeled, single-stranded DNA)
Lhet2 (heterotroph)	100
13598het (heterotroph)	100 ± 3
m-5 (heterotroph)	100 ± 4
Dhet1 (heterotroph)	91 ± 4
KG-4_p (heterotroph)	34 ± 4
T. acidophilus strain 7_p	14 ± 5
T. acidophilus strain 2T10	13
T. ferrooxidans strain L_p	0

gation in A.P.H.'s laboratory. Several North and South American *T. ferrooxidans* strains show 90 to 100% homology with *T. ferrooxidans* strain L_p, whereas others, like strain 13598, show a low homology with strain L_p.) *T. thioparus* had 0% homology with *T. ferrooxidans* strain L_p. Similarly, heterotroph Lhet2 had 0% homology with *T. ferrooxidans* L_p, thus demonstrating unequivocally that this heterotroph and autotroph are not closely related. (These two pair of bacteria were derived from a common, presumably pure culture of *T. ferrooxidans* [Table 1].) This result was confirmed in the reciprocal experiment where labeled DNA from the heterotroph was used (Table 3). *T. ferrooxidans* L_p showed 0% homology with Lhet2. But most interesting, a high order of homology occurred between Lhet2 and several acidophilic heterotrophs isolated from other sources. However, relatedness with *T. acidophilus* was of a low magnitude, 14 ± 5% homology. *T. acidophilus* differed from the other heterotrophs in several striking phenotype characters (Table 4). Obviously, more than one kind of heterotroph was isolated from cultures of *T. ferrooxidans*.

DISCUSSION

In the past, it had been assumed that prolonged cultivation of a mixed bacterial population in an enrichment culture containing a unique energy source (e.g., ferrous iron) would, through selective enrichment via serial transfers, render the culture pure as to physiological cell type. That cultures of *T. ferrooxidans*, for example, might harbor heterotrophic contaminants growing on organic compounds provided by the autotroph, though sometimes considered (17), was not always amply appreciated, so early investigators concluded that *T. ferrooxidans* could grow not only autotrophically with ferrous iron but also heterotrophically with glucose as the energy source. Indeed, some observations supported this view. Tabita and Lundgren (25) could detect no heterotrophic bacteria by streaking a *T. ferrooxidans* culture onto glucose agar, yet through stepwise replacement of iron with glucose they isolated a glucose-utilizing strain that eventually became obligately heterotrophic, losing the ability to oxidize ferrous iron. It might be claimed, of course, that the contaminant was too sparse in their *T. ferrooxidans* to be detected by direct streaking, and that their replacement of iron with glucose served to enrich for the contaminant. On the other hand, Zavarzin (30) found that his acidophilic heterotrophs had a requirement for ferrous iron, but not as the energy source. We have observed that the addition of heterotroph Lhet2 to *T. ferrooxidans* strain L_p in iron medium does not permit the heterotroph to be reliably isolated therefrom; one day it can, on another day it cannot, as though fluctuations occur in a heterotroph population similar to that observed and analyzed in mixed populations of *Enterobacter aerogenes* (11).

The five obligate heterotrophs (Table 4) grew to a low population in iron and sulfur media flasks on the rotary shaker. Washed cells from glucose medium were inoculated at 10^3 per ml in these media; cell populations increased, and the bacteria maintained themselves at approximately 10^7 per ml even after three serial transfers (Table 4). After 1 month, no lowering of pH was noted in sulfur medium with low buffer content; utilization of sulfur therefore was contraindicated. Likewise, no discoloration of iron

TABLE 4. *Phenotypic characters of five obligate heterotrophs and two species of Thiobacillus*

Strain	Morphology and motility in glucose medium[a]	Microscopic cell count/ml in:[b]			pH change in sulfur medium[c]	Optimal pH range in glucose medium
		Glucose medium	Iron medium	Sulfur medium		
Lhet2	Small rods, motile	$\sim 10^8$	$\sim 10^7$	$\sim 10^7$	No change	2.0–5.2
13598het	Coccoid rods, nonmotile	$\sim 10^8$	$\sim 10^7$	$\sim 10^7$	No change	2.4–5.2
m-5	Small rods, motile	$\sim 10^8$	$\sim 10^7$	$\sim 10^7$	No change	2.4–5.2
Dhet1	Small rods, motile	$\sim 10^8$	$\sim 10^7$	$\sim 10^7$	No change	2.2–5.5
KG-4_p	Coccoid rods, nonmotile	$\sim 10^8$	$\sim 10^7$	$\sim 10^7$	No change	2.2–4.5
7_p	*T. acidophilus*, small rods, nonmotile	10^9	$\sim 10^7$	2×10^8	Reduced to 1.8	2.0–4.5
L_p	*T. ferrooxidans*, small rods, nonmotile	No growth	3×10^8	2×10^8	Reduced to 1.8	No growth

[a] Motile strains show no motility when grown at the limits of their pH range. Old cultures lose motility.

[b] Plate counts of heterotrophs on mannitol agar are the same or slightly less than the microscopic count.

[c] For this determination, the K_2HPO_4 in the medium was reduced to 0.25 g/liter so that the medium would be more sensitive to acid formation. The initial pH was 3.7 and did not change over the 4-week period of incubation in flasks.

medium due to formation of ferric hydroxide was observed, nor was there any diminution in ferrous iron detectable by titration of Fe^{2+} with ceric ammonium sulfate (14). Since nitrite and nitrate were never detected in the medium (5), the utilization of NH_4^+ as the source of energy was unlikely. In fact, omitting any single ingredient (glucose, sulfur, ferrous sulfate, or ammonium sulfate) allowed these bacteria to attain a population of approximately 10^7 per ml through each of three serial transfers. Although these heterotrophs may be able to sustain themselves through several generations by using intracellular energy reserves, this cannot account for growth through three serial transfers, representing more than 20 cell generations. Rather, it is possible that these soil microorganisms, good scavengers, utilize trace amounts of organic matter from the atmosphere of the incubation chamber or as impurities in the inorganic ingredients in the medium. (In these experiments we used glass double-distilled water, since demineralized water may contain organic matter.)

The five obligate heterotrophs differ from the facultative heterotroph, *T. acidophilus*, which utilizes either glucose or sulfur as the source of energy and which grows to a much higher cell population in glucose medium (Table 4). Low homology between Lhet2 and *T. acidophilus* DNA (Table 3) confirms the view that Lhet2 is a different species.

Heterotrophs with a growth range below pH 3 are rare (15). However, Belly and Brock (2) isolated yellow-pigmented, nonsporeforming heterotrophs growing at pH 2.5 in coal refuse. We also have isolated yellow-pigmented heterotrophic bacteria from coal spoils and as contaminants in *T. ferrooxidans* (pH 3) and other thiobacillus cultures. However, our yellow-pigmented heterotrophs are not obligately acidophilic, but grow better at pH 6.7, and they are less common as contaminants in thiobacillus cultures than are the obligate acidophilic heterotrophs described here. The latter may grow, though poorly, in liquid medium at pH 1.8, and all grow at pH 2.0, but not at pH 6.7. These bacteria develop sparsely on glucose agar with white or pinkish growth and form no endospores, and some strains are motile.

That one pair, the heterotroph Lhet2 and the autotroph L_p, is shown to be not closely related does not prove that every such pair is unrelated, but the implication is clear, and the burden of proof of relatedness now rests on the one who makes such a claim. Therefore, to conserve hypotheses, we must conclude that all obligate heterotrophs in *T. ferrooxidans* cultures are apt to be contaminants, though interesting ones. Since they are so common and tenacious with *T. ferrooxidans*, perhaps they play a role with this species in its natural habitat. Perhaps they cleanse the microenvironment of organic compounds detrimental to *T. ferrooxidans* or, when in coal refuse, attack the organic sulfur in coal, as *T. ferrooxidans* attacks the pyritic sulfur, and thereby aid in the desulfurization process.

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