JOURNAL OF BACTERIOLOGY, Sept. 1988, p. 3915–3923
0021-9193/88/093915-09$02.00/0

Vol. 170, No. 9

Phenotypic Switching of *Thiobacillus ferrooxidans*

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Received 1 April 1988/Accepted 9 June 1988

Two solid medium formulations, designated 100:10 and 10:10, were developed for the growth of *Thiobacillus ferrooxidans*. The new media contain a mixture of both ferrous iron and thiosulfate as available energy sources, permitting the detection of colony morphology variants that arise spontaneously in a wild-type population. Several morphological and physiological characteristics of a class of *T. ferrooxidans* variants, termed LSC for large spreading colony, are described. LSC variants lack the ability to oxidize iron but retain the capacity to utilize thiosulfate or tetrathionate as energy sources. An LSC colony spreads on the surface of solid 100:10 medium as a monolayer of cells in a fashion resembling that of certain swarming or gliding bacteria. The LSC variant reverts to a parental wild type at frequencies that vary in different independently arising isolates. The identity of the LSC variant as a derivative of the parental wild-type *T. ferrooxidans* was established by Southern blot hybridization.

Thiobacillus ferrooxidans is a gram-negative, acidophilic, obligate autotrophic bacterium capable of using iron or various reduced sulfur compounds as a sole energy source (2, 6). There is considerable interest in developing a better understanding of the biochemistry and genetics of this microorganism, both for reasons of fundamental interest in its unusual metabolism and because it has been implicated as one of the most important microorganisms involved in metal solubilization in mining operations.

It has previously been shown that *T. ferrooxidans* is capable of forming colonies on solid media supplemented with inorganic salts and either iron at pH 2.4 (13, 38) or thiosulfate at pH 4.5 (21, 39). In this report, we describe two new solid medium formulations for the growth of *T. ferrooxidans*, both containing mixtures of thiosulfate and iron at pH 3.5. Wild-type cells form colonies on both these media at high efficiency. Of particular interest, however, is the appearance of an alternate colony morphology. This alternate colony morphology is inherited, indicating a genetic basis, but it can revert at high frequency to the parental morphology. The switch from the parental to the alternate morphology is accompanied by a reversible loss of iron oxidation capability. Phenotypic switching has been reported for several organisms such as *Pseudomonas cepacia* (8), *Thiobacillus versutus* (3), *Serratia marcescens* (22), and *Candida albicans* (30, 32) and may represent an important way for microorganisms to generate genetic and phenotypic diversity in the environment. It could also explain the ability of *T. ferrooxidans* to adapt to changing conditions such as lowered pH (41) or increased metal concentration (40).

MATERIALS AND METHODS

Bacterial strains. *T. ferrooxidans* ATCC 19859, ATCC 23270, and ATCC 33020 were obtained from the American Type Culture Collection (Rockville, Md.). *T. ferrooxidans* Pyrite and Penn Coal were obtained from Henry L. Ehrlich (Rensselaer Polytechnic Institute, Troy, N.Y.). *T. ferrooxidans* YS14 was isolated in our laboratory by John Lobos. All strains were clonally purified three times on solid medium (43) before use. Stock cultures were maintained in a minimal salts medium supplemented with ferrous iron (9K-$FeSO_4$) (29), hereafter termed iron medium, or in a basal salts (medium no. 5) supplemented with sodium thiosulfate (21), termed thiosulfate medium. Cell counts of liquid cultures or suspensions were determined microscopically with a Petroff-Hausser counting chamber and phase-contrast microscopy.

Media. Liquid 9K iron medium (pH 2.4) was prepared by the procedure of Tuovinen and Kelly (38) as modified by Yates and Holmes (43). This medium will be referred to as iron medium in the rest of the text.

Liquid thiosulfate medium (pH 4.5) was made by the procedure of Parker and Prisk (21).

Solid iron and thiosulfate media were made as described above with 0.5% agarose (type I, Low EEO; Sigma Chemical Co., St. Louis, Mo.) as the solidifying agent.

Solid 10:10 medium was made as follows (per liter). A 70-ml portion of modified 10× 9K salts (29, 43) adjusted to pH 3.5 was added to 655 ml of H_2O (solution A). Agarose (5 g; type I, Low EEO; Sigma) (no substitutions) was added to 250 ml of H_2O (solution B). $Na_2S_2O_3 \cdot 5H_2O$ (0.5 g; Mallinckrodt, Inc., St. Louis, Mo.) was added to 10 ml of H_2O (solution C). $FeSO_4 \cdot 7H_2O$ (2.2 g) was added to 15 ml of H_2O, and the pH was adjusted to 2.2 with H_2SO_4 (solution D). Solutions A and B were autoclaved separately and cooled to 60°C in a water bath. Solutions C and D were filter sterilized separately. Solutions A, B, C, and D were mixed. The 10× modified 9K salts were made as follows. Dissolve the following in 3 liters of H_2O in the following order: 150 g of $(NH_4)_2SO_4$, 5 g of KCl, 2.5 g of K_2HPO_4, 25 g of $MgSO_4 \cdot 7H_2O$, and 0.72 g of $Ca(NO_3)_2 \cdot 4H_2O$. Add 50 ml of 10 N H_2SO_4. Store at 4°C.

Solid 100:10 medium was made as described for medium 10:10 except that solution C contained 5 g of $Na_2S_2O_3 \cdot 5H_2O$ in 100 ml and the volume of H_2O in solution A was decreased to 565 ml. Corresponding liquid media of mixed iron and thiosulfate were prepared by omitting the agarose. Solid tetrathionate-ferrous iron media were prepared as described above for media 10/10 and 100/10 except that the 9K salts and the tetrathionate solution (4 g in 100 ml) were adjusted to pH 2.5. Solid ferric iron 100:10 and 10:10 media were made exactly as described above with equimolar amounts of ferric iron as ferric sulfate in place of the ferrous sulfate.

DNA preparation. DNA was prepared as described by Yates and Holmes (43).

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Southern blots and nick translations. DNA was digested with restriction endonucleases (Bethesda Research Laboratories, Inc., Bethesda, Md.) according to the recommendations of the manufacturer. DNA fragments were separated in 0.8% agarose gels (ultrapure grade; Bethesda Research Laboratories) in 0.4 M Tris (pH 8.0)–0.005 M sodium acetate–0.001 M EDTA. Southern blots (33) were performed with Gene Screen hydridization transfer membranes by the method outlined in the Gene Screen Manual provided by the manufacturer (New England Nuclear Corp., Boston, Mass.). *T. ferrooxidans* probe pTf32 (43) was labeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.), using a nick translation kit (Bethesda Research Laboratories) following the directions of the manufacturer.

Assay for ferrous iron-oxidizing activity. A standard curve of ferric iron concentration versus A_{410} was prepared with $FeCl_3 \cdot 6H_2O$ in 0.01 N H_2SO_4 (26). Sterilized 9K salts minus ammonium sulfate (9K-N) (pH 4.0) were used to wash and dilute mid-log-phase cultures in preparation for the ferrous iron oxidation assay. Iron- or thiosulfate-grown cultures were filtered and washed twice in 0.33 volume of 9K-N. After a third filtration, the cells were suspended in 3 ml of 9K-N and centrifuged for 5 min in an Eppendorf centrifuge. The pellets were gently resuspended in 9K-N, without disturbing the visible iron- or sulfur-containing precipitate under the cell pellet. Washes were repeated until iron or sulfur salts were not visible in the pellet. Cells were then diluted in 9K-N to approximately 2×10^8 cells per ml. Wild-type cells in 9K-N supplemented with ferrous iron (0.22%) and thiosulfate (0.05%) were able to oxidize ferrous iron but did not multiply. Samples of the washed cells were diluted in 9K salts (pH 4) and plated on solid media to verify viability. To test ferrous iron-oxidizing activity, we supplemented washed cells with ferrous iron ($Fe_2SO_4 \cdot 7H_2O$, 0.22%) with or without thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$, 0.05%) and incubated them at 30°C in a rotary shaker for the specified times. At the end of incubation, cells were pelleted in an Eppendorf centrifuge for 5 min. Samples (1 ml) of the supernatant were pipetted into 2 ml of 0.01 N H_2SO_4, and 0.9 ml of concentrated HCl was added. The A_{410} was read on a Perkin-Elmer spectrophotometer. Soluble ferric iron was calculated from the standard curve.

Microscopic observations of LSC variant growing in slide chambers. Slide chambers were prepared as described previously (12, 23) except that medium 100:10 was used for the thin agarose layer. An approximately 1-mm-thick layer of medium 100:10 was pipetted onto 25-mm-square cover slips. The agarose was trimmed to 15 mm square and inoculated with a toothpick. The inoculated cover slips were immediately inverted over the prepared microscope slides and sealed in place. Sealed chambers were incubated at 30°C. Slide chambers were observed at 24-h intervals with a Leitz Laborlux 12 microscope mounted with a camera. Photographs were taken with Kodak Panatomic × 35 mm film.

Time-lapse photomicroscopy. Time-lapse photomicroscopy was done with a Panasonic NV-8050 time-lapse recorder and an M50 microscope (Wild Heerbrugg).

Electron microscopy. Samples for electron microscopy were taken from the surfaces of static liquid cultures or from colonies on agarose media. When collecting samples from liquid cultures, we used wide-bore pipettes without suction to minimize breakage of flagella that might be present. A few drops of culture were transferred from the wide-bore pipette to a glass cover slip. Samples were obtained from colonies growing on agarose plates by placing a drop of 9K salts, adjusted to the appropriate pH, directly on the colony. Carbon-coated grids were floated, coated side down, on the culture liquid on a cover slip or on the salts solution covering a colony. After the desired amount of time, the grid was removed, blotted, and stained with 1% (wt/vol) uranyl acetate.

In addition to the cultures of *T. ferrooxidans* ATCC 19859 and the LSC variants, *Pseudomonas putida* obtained from Frank Mondello (General Electric Research and Development, Schenectady, N.Y.) was used as a flagellum-positive control. *P. putida* was suspended in a drop of water from a colony on a nutrient agar plate. The grids were examined and photographed under a Hitachi electron microscope.

RESULTS

New *T. ferrooxidans* medium formulations. Mixed media were prepared containing both ferrous iron and thiosulfate in various concentrations, using agarose as a solidifying agent. Two of these media, reported here as 10:10 and 100:10, were selected as most useful in allowing the development of various phenotypes within a *T. ferrooxidans* population. Medium 100:10 contains 100% of the thiosulfate concentration found in standard thiosulfate medium (21) and 10% of the ferrous iron concentration found in standard 9K iron medium (29) and is formulated at an initial pH of 3.5. Medium 10:10 is similar to 100:10 but contains only 10% the thiosulfate concentration. A complete description of the preparation of these media is given in Materials and Methods.

Isolation of LSC variant. *T. ferrooxidans* ATCC 19859 grown for several years with monthly transfers in liquid iron medium (pH 2.4) was plated onto 100:10 solid medium. Colonies formed with a plating efficiency of 10 to 15%. Colonies were initially clear, but in about 14 days they developed alternating cream- and rust-colored concentric rings (Fig. 1A). After about 3 weeks, the colonies turned pale yellow and often developed 1- to 3-mm clear halos or lobes. The reason for the development of the alternating rings is not known.

Clear or cloudy white lobes, fans, or snakelike extensions of secondary growth began to develop from many colonies 2 or more weeks after plating (Fig. 1B). This secondary growth continued to expand in a few cases (0 to 3% of the total colonies) and eventually spread over the entire surface of a 10-cm petri dish. The presence of wild-type colonies on the same plate appeared to inhibit the advance of the spreading colonies. The spreading colonies grew around and past the small (1- to 5-mm) wild-type colonies, leaving 1- to 2-mm zones of inhibition around them.

Cells were scraped from one of these spreading regions, diluted in 9K salts (pH 3.5), and spread on solid 100:10 medium. Colonies appeared after 6 days with a 10 to 25% plating efficiency. The colonies were clear and at low plating densities (about 10 colonies per plate) spread rapidly to cover the entire agarose surface. Clonal purification was repeated three times. Because of the ability of this variant to spread rapidly on medium 100:10, we termed it LSC (large spreading colony).

Growth of LSC on solid medium 100:10. LSC was transferred with a toothpick from 100:10 medium into the center of a 15-cm petri dish containing 100:10 medium. A clear colony developed from the site of inoculation that was roughly circular in shape with a smooth edge, although colonies with crenellated edges occurred occasionally. As the colony spread over the surface of the agarose, it maintained its circular shape (Fig. 1C). The rate of increase of the

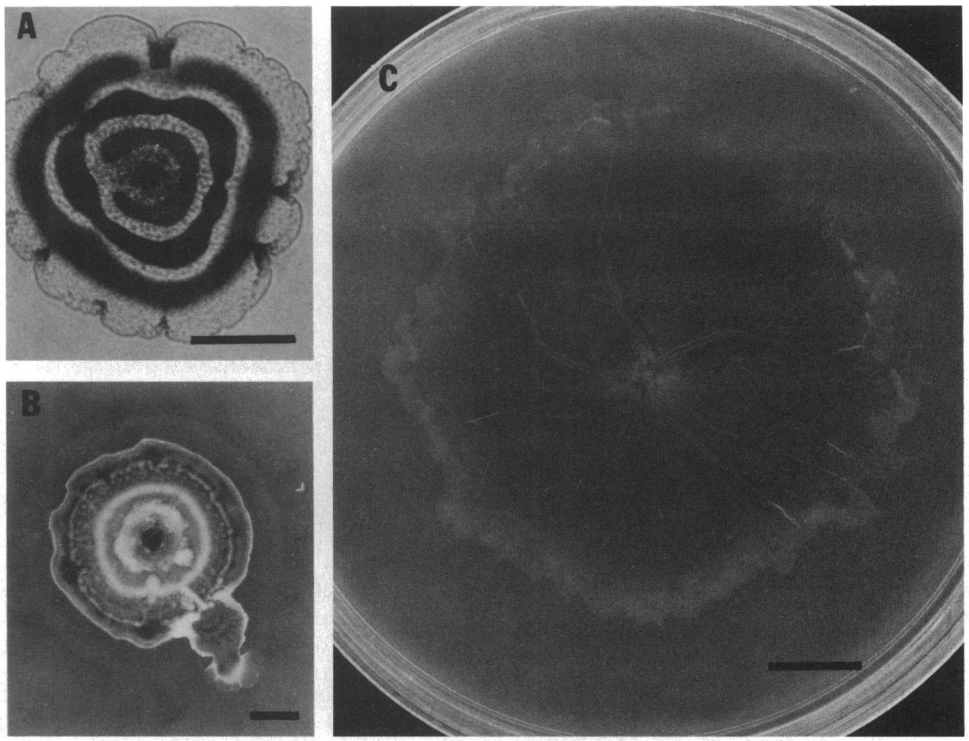

FIG. 1. Colonies of *T. ferrooxidans* ATCC 19859 on medium 100:10. (A) Wild-type colony exhibiting alternating rust- and cream-colored rings. (B) Wild-type colony with fan-shaped secondary growth. (C) Spreading growth of LSC. This colony will continue to expand and cover the agarose surface. (A and B) Bar, 1 mm. (C) Bar, 1 cm.

colonies was measured in 10 colonies grown at 20, 30, or 37°C. The maximal rate of increase in colony radius once the colony initiated expansion was similar at all temperatures and reached 16 μm/min. However, the time for initiation of expansion of the colony (lag phase) differed at the various temperatures and was 4 to 7 days at 30°C, 11 to 16 days at 37°C, and 13 to 16 days at 20°C.

Beginning about 4 days after inoculation with a toothpick, the older part of the colony, toward the center, turned from clear to milky white (Fig. 1C). Eventually, the whole colony turned milky white and later gradually turned clear again with a reticulate pattern in relief. During the clearing stage, circular areas resembling bacteriophage plaques were observed. There were 10^9 to 10^{10} cells in the colony by the time it completely covered the surface of a 10-cm petri dish. Using an estimate of 0.5 μm^2 for the area occupied by a single cell, it can be calculated that such a colony consists of no more than a monolayer of cells.

The flowing effect of the LSC colony observed with time-lapse photomicroscopy suggested that fluid motion was responsible for enlarging the colony and that cells were passively carried out with the flow. To test this possibility, we inoculated medium 100:10 plates in the center with toothpicks. Plates were then incubated flat or vertically. There was no difference in symmetry in colonies from plates incubated vertically as compared with the plates incubated flat. Therefore, we conclude that either the cells are moving by some active process or else they are passively carried by the outward flow of a liquid which can expand upward against gravity, perhaps by capillary flow.

Observations of LSC variant growing in slide chambers. Colony growth of LSC was observed on medium 100:10 prepared on a microscope cover slip and sealed in a slide chamber. The edge of the colony was smooth or scalloped up to 48 h after inoculation. The colony edge subsequently developed dendritic and very irregular extensions occupied by a monolayer of cells with extensive space between individual cells (Fig. 2). The space between cells was reduced as more cells moved into the dendritic extensions or as cells divided (Fig. 2B). The leading edge of the colony moved at an average rate of 0.83 μm/min during this 5-h period. The small size of the agarose sheet inside slide chambers (15 by 15 by 1 mm) as compared with a 10-cm-diameter petri dish limited slide chamber measurements to the early phases of colony expansion. This value is consistent with the initial expansion rate of 1 μm/min observed with LSC on petri plates.

Confirmation that LSC variant is *T. ferrooxidans* ATCC 19859. A crucial issue is whether the LSC variant is derived from *T. ferrooxidans* ATCC 19859 or whether it is a contaminant of the culture. To address this question, we prepared DNA from four independently arising LSC variants. The DNA was cleaved with *Eco*RI, Southern blotted, and probed with the recombinant plasmid pTf32 containing a cloned DNA segment derived from the parental wild-type *T. ferrooxidans* ATCC 19859 (43). The pattern of hybridization of the probe to DNA prepared from wild-type or LSC cells was identical. Two examples of the pattern of hybridization of LSC DNA and a wild-type control are shown in Fig. 3. Therefore, we conclude that the LSC variants are derived from the parental strain of *T. ferrooxidans* ATCC 19859. In contrast, 15 other strains of *T. ferrooxidans* and two strains of *Thiobacillus thiooxidans* from various culture collections gave different patterns of hybridization with this probe and two strains of *Thiobacillus acidophilus*, *Acidophilium organovorum*, *Acidophilium cryptum*, *Thiobacillus novellus*, and

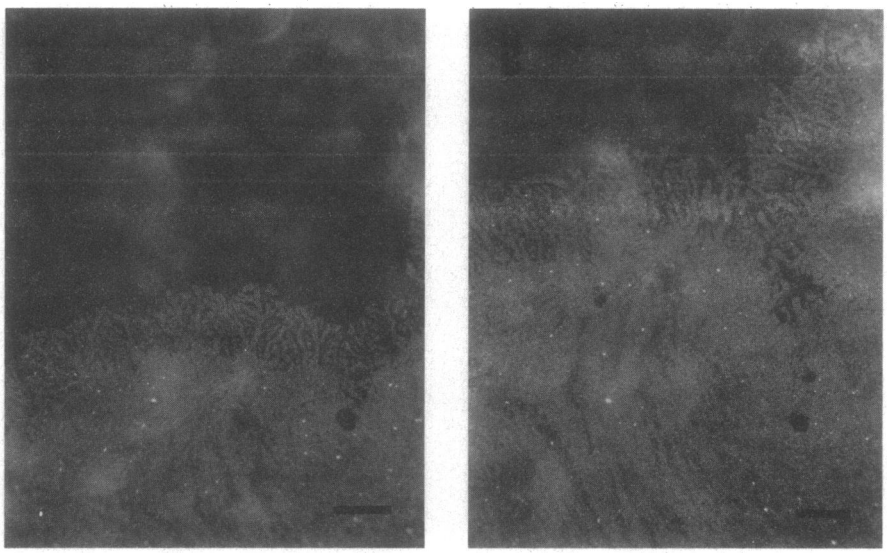

FIG. 2. *T. ferrooxidans* LSC variant growing on medium 100:10 in a slide chamber at 30°C as described in Materials and Methods. The photographs were taken at ×100 magnification 3 days after inoculation with a toothpick. The photograph in panel B was taken 5 h after the photograph in panel A. Bar, 10 mm.

Thiobacillus thioparus showed no hybridization with the probe (data not shown).

Growth on LSC on medium 10:10. A colony of LSC grown on solid 100:10 medium was picked, diluted in 9K salts (pH 3.5), and spread on solid 10:10 medium at a density of 10 to 20 cells per plate. Colonies arose at a plating efficiency of 50 to 100%. Clear round colonies were visible after 5 to 7 days at 30°C. At this cell density, colonies reached a diameter of approximately 10 mm in 2 weeks. After 2 weeks of incubation, rusty spots began to appear within the clear colonies. The number of spots visible to the eye was counted in 33 1-month-old colonies. A range of 20 to 101 spots per colony was found with an average of 52 spots per colony. This isolate is shown in Fig. 4A, and an enlargement is shown in the insert. It is apparent that many more spots are visible in the magnified photograph than are visible with the naked eye. We propose that these rusty spots arise by reversion of LSC to the wild-type phenotype.

Colonies derived from cells taken from independently arising LSC isolates with few (1 to 20) rusty spots developed few rusty spots. Colonies derived from cells taken from colonies with an intermediate number (20 to 100) or many (100 to 300) rusty spots also developed approximately the same number of such spots as their parental colonies.

Rusty spots could be isolated from LSC colonies by microdissection or by suspension of the entire colony in 9K

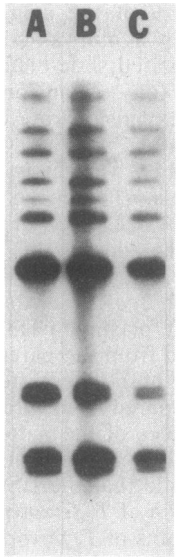

FIG. 3. Southern blot of *Eco*RI-digested genomic DNA hybridized with a multiple-copy probe (pTf32) isolated from *T. ferrooxidans* ATCC 19859 (43). Lanes: 1, wild-type *T. ferrooxidans* ATCC 19859; 2, LSC isolate 1; 3, LSC isolate 2.

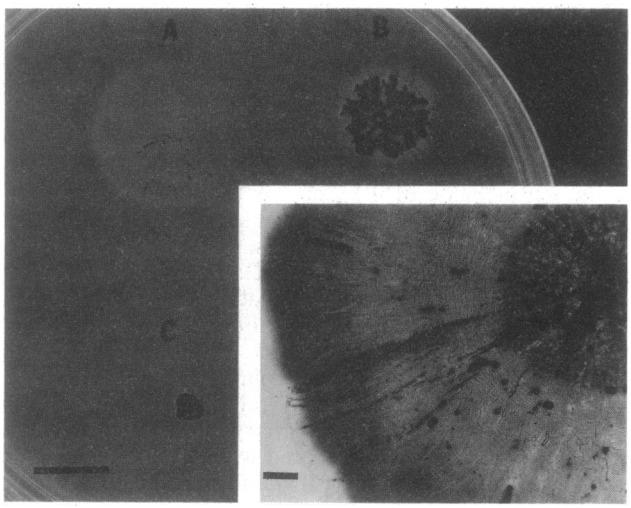

FIG. 4. LSC and wild-type *T. ferrooxidans* growing on medium 10:10. (A and B) LSC isolates showing different numbers of rusty spots within the colonies. (C) Wild-type colony of *T. ferrooxidans* ATCC 19859. All colonies were transferred to the agarose with a toothpick. Bar, 1 cm. Insert: Enlargement of section of colony A. Bar, 1 mm.

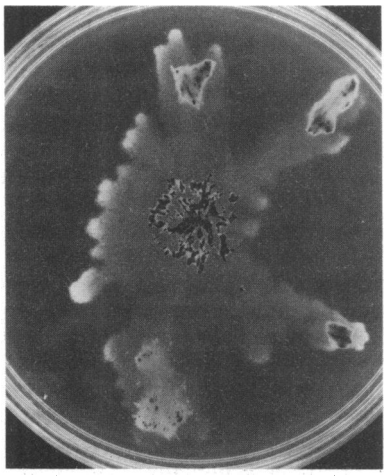

FIG. 5. LSC isolate growing on medium 10:10 in an 10-cm petri plate illustrating an irregular LSC colony isolate.

salts (pH 1.5) (low pH 9K). Repeated washing and settling in 9K salts yielded intact rusty spots free from associated LSC-type cells. The spots were then partially disrupted into constituent cells by homogenization in 9K salts (pH 1.5) and inoculated in liquid or solid 9K iron medium, solid 100:10 medium, or solid 10:10 medium. The resulting growth or colony morphology was typical of the wild-type phenotype. We conclude that the rusty spots are derived from LSC cells that have reverted to the wild-type phenotype.

A group of 35 colonies contained an average of 4×10^7 cells and 70 rusty spots per colony. If it is assumed that each rusty spot arose as the result of a single independent event, an average of 70 reversions arose per 4×10^7 cells or per 26 cell generations. This is likely to be an underestimate since additional reversions to wild type occurring late in colony growth would result in spots too small to be detected. The reversion rate was estimated for colonies containing an intermediate number of rusty spots. The rate would be proportionately greater or lower for LSC colonies exhibiting fewer spots (e.g., 1 to 10) or more spots (e.g., 300) per colony.

In addition to the rusty spots, we observed yellow and white spots within many LSC colonies that did not turn rusty red on prolonged incubation. The number of such spots per colony was proportional to the number of rusty spots. No further analysis of the yellow and white spots was done. It should be noted that in Fig. 4, rusty, white, and yellow spots all appear dark. The insert shown in Fig. 4 illustrates a terraced surface of scalloped concentric rings seen on some colonies.

With regard to colony diameter and shape, some isolates remained small (approximately 5 mm in diameter) even when there was only one colony per plate. A few isolates reached diameters of 50 to 70 mm. These larger isolates also had a tendency to form more irregular colonies (Fig. 5), while the smaller (less than 30-mm diameter) isolates were usually round or only slightly irregular.

In comparison, wild-type colonies of *T. ferrooxidans* remained uniformly small (1 to 3 mm) after 4 weeks of growth at 30°C and became iridescent white or yellow or rusty brown after 14 days of incubation (Fig. 4C). They arose at a plating efficiency of 50 to 100%.

Growth of LSC variant in liquid thiosulfate medium. The LSC variant was readily transferred to liquid thiosulfate medium from solid medium 100:10 and reached cell densities of 10^9 cells per ml after 10 days of incubation at 30°C in a rotary shaker. This is comparable to the growth rate and cell density achieved by thiosulfate-adapted parental *T. ferrooxidans* ATCC 19859 under these conditions. LSC was maintained in liquid thiosulfate medium for up to 6 months and retained its variant phenotype when subsequently plated on medium 10:10 or 100:10.

Enrichment of LSC variants in thiosulfate medium. Wild-type *T. ferrooxidans* ATCC 19859 grown in liquid iron medium (pH 2.4) was washed with 9K salts and transferred to liquid thiosulfate medium (pH 4.5). Cells were passaged in liquid thiosulfate medium by serial transfers every 5 days. At the end of 2 months, cells were plated onto 100:10 medium with a plating efficiency of 50%. A total of 2 to 5% of the resulting colonies exhibited the LSC phenotype. Growth in liquid thiosulfate medium can therefore be used to enrich a culture for LSC-type variants, but it is not possible to determine whether these variants are arising independently.

Growth of LSC on solid ferrous iron-tetrathionate media. We questioned whether the unusual colony morphology exhibited by the LSC variants was restricted to 100:10 and 10:10 media. To address this issue, we prepared mixed media containing ferrous iron and sodium tetrathionate at concentrations comparable to those in 100:10 and 10:10 as described in Materials and Methods. Iron-tetrathionate media were prepared at a lower pH (2.5) than iron-thiosulfate media (pH 3.5). This provided the opportunity to observe growth characteristics of the LSC variant on media containing both iron and another form of reduced sulfur and at a pH similar to that used in iron media. The characteristic colony morphologies of the LSC variant on 100:10 and 10:10 media were reproduced on the equivalent tetrathionate 100:10 and 10:10 media. The colony phenotype of the LSC variant is, therefore, not strongly pH dependent, nor is it only expressed when thiosulfate is present as an energy source.

Growth of LSC on solid ferric iron-thiosulfate media. We presumed that owing to the relatively high pH (3.5) of media 10:10 and 100:10, a higher proportion of the ferrous iron would be air oxidized to ferric iron than at the lower pH of iron medium (2.2). The influence of ferric iron on colony morphology was therefore of interest. In addition, several groups reported ferric iron reduction during growth of *Thiobacillus* species on sulfur compounds (1, 16, 34). Sulfite inhibition of *Thiobacillus* growth on sulfur compounds was relieved by ferric iron (36). This is a possible explanation for the improved plating efficiency of *T. ferrooxidans* on the mixed media relative to that on standard thiosulfate medium. It was possible that an important characteristic of these media was the simultaneous presence of an inorganic source of sulfur and ferric iron, as well as the presence of ferrous iron for the organisms preferentially growing on this energy source.

Media 10:10 and 100:10 were prepared as usual except that equimolar amounts of ferric iron were substituted for the ferrous iron. Colonies of wild-type *T. ferrooxidans* and the LSC variant were transferred to these media with toothpicks. The wild-type strain grew very poorly and remained in the area touched with the toothpick. The LSC variant formed clear or golden colonies at a rate of growth similar to that found on 10:10 ferrous iron medium. However, no rusty inclusions or spots formed. This is consistent with the interpretation that the spots arise from growth of revertants that utilize ferrous iron.

On medium 100:10 prepared with ferric iron, wild-type cells formed clear colonies with a slight metallic sheen. After

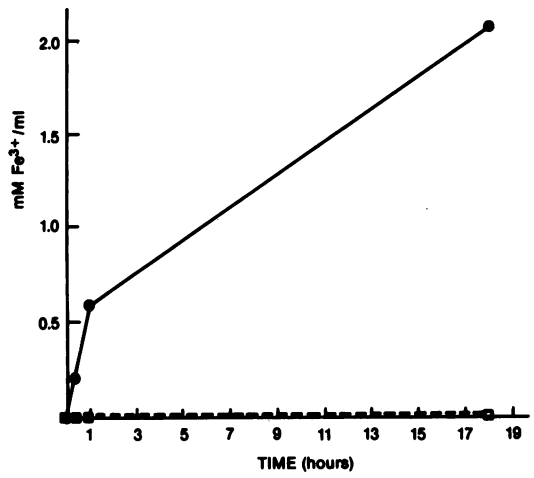

FIG. 6. Oxidation of ferrous iron by intact cells of wild-type *T. ferrooxidans* ATCC 19859 (———) and the LSC isolate shown in Fig. 4A (– – – –).

continued incubation, the colonies turned slightly golden orange. In contrast, LSC variants spread over the surface of the ferric iron 100:10 medium in a fashion similar to that observed on ferrous iron 100:10 except that the lag phase before colony spreading began was reduced on the ferric iron medium.

Iron oxidation by *T. ferrooxidans* ATCC 19859 and LSC variant. To evaluate the ability of the LSC variant to oxidize ferrous iron in either the presence or absence of a reduced sulfur compound, we performed whole-cell iron oxidation assays essentially by the method of Schnaitman et al. (26).

All assays were run at an initial concentration of ferrous iron (0.22% $FeSO_4 \cdot 7H_2O$) equal to that in media 10:10 and 100:10. Cells harvested from liquid thiosulfate cultures of both the parental and LSC strains were used in these assays. Cells were also plated on solid media to verify viability and to determine whether there were wild-type cells present in the LSC cultures. Both wild-type and LSC cultures had 100% viability on medium 10:10, and less than 1% of the cells in the LSC culture were of the parental phenotype. The results of a representative iron oxidation assay are shown in Fig. 6. *T. ferrooxidans* ATCC 19859 was able to oxidize ferrous to ferric iron without a discernable lag at a rate comparable to that reported previously (17). In contrast, LSC cultures could not oxidize ferrous to ferric iron. These assays were also done in the presence of thiosulfate to test the possibility that the LSC phenotype could use ferrous iron only in the presence of a source of reduced inorganic sulfur. The presence of thiosulfate caused a slight reduction in the rate of ferrous iron oxidation by the wild-type cells and did not promote the oxidation of ferrous iron by the LSC variant.

Electron microscopy of LSC. LSC or wild-type cells were prepared for electron microscopy from static liquid thiosulfate or from cells lifted directly from the surface of 100:10 plates. These techniques have been shown to minimize physical loss of flagella in other bacterial strains. No flagella were observed in any of the preparations, whereas *P. putida* prepared for electron microscopy in a similar way had polar flagella.

Isolation of LSC-type variants from other strains of *T. ferrooxidans*. To increase the probability of finding LSC-type variants in other strains (besides ATCC 19859), we grew *T. ferrooxidans* ATCC 23270, ATCC 33020, Pyrite, Penn Coal, and YS14 in liquid thiosulfate medium. After 3 weeks of growth (subcultured weekly) in liquid thiosulfate, cells were plated onto solid media 10:10 and 100:10. LSC-type variants which maintained the phenotype after subculture were isolated from all five strains. LSC-type variants were therefore found in all six *T. ferrooxidans* strains examined.

DISCUSSION

In this report, we describe the isolation and characterization of a phenotypic variant (termed LSC) of *T. ferrooxidans* ATCC 19859. The LSC variant manifests itself on media containing a mixture of thiosulfate and ferrous iron. LSC is not found in the standard iron medium routinely used for growth of *T. ferrooxidans* because it lacks the ability to oxidize iron and, although it is found in standard thiosulfate medium, its phenotype is difficult to distinguish from that of the wild type under these conditions. However, on mixed media of thiosulfate and ferrous iron, LSC has a colony morphology that is readily distinguishable from that of the wild type. The nature of the LSC colony morphology on the mixed media depends on the ratio of thiosulfate to ferrous iron. On solid medium (100:10) containing a higher ratio of thiosulfate to ferrous iron, LSC spreads over the surface of an agarose plate as a monolayer of cells. On solid medium (10:10) containing a lower ratio of thiosulfate to ferrous iron, it forms large clear colonies with inclusions of rusty spots. We propose that the rusty spots represent revertants to the wild-type phenotype. Revertants also arise on 100:10 as can be demonstrated by plating cells from 100:10 medium onto standard iron medium. However, they do not appear as visible rusty inclusions in the colonies, perhaps owing to the movement of the surrounding cells sweeping the revertants along with the flow, preventing the revertants from forming distinct inclusions. Differences in motility of the cell types or different cell surface characteristics may be responsible for the absence of sectoring in these colonies.

The spreading growth of LSC on medium 100:10 is similar to that of colonies formed by swarming, swimming, or gliding gram-negative organisms (7, 11, 14, 23). However, the following evidence is more consistent with gliding as the mechanism of colony expansion. First, the rate of LSC colony expansion (up to 16 μm/min) is within the range of gliding organisms but is less than that reported for swarming or swimming bacteria (11). Second, swarming or swimming bacteria typically have flagella, which the LSC variants lack. Third, the streaming observed in time-lapse photomicroscopy resembles that seen with gliding organisms. The description of the growth of the outer edge of an LSC colony, observed by low-power microscopy, is characteristic of swarming or gliding bacteria (7, 14). Unfortunately, the molecular mechanism(s) of gliding remains to be determined (5, 23), as does the mechanism of spreading colony development in the LSC variant. It is of course possible that LSC growth on medium 100:10 represents a previously unreported mode of motility or that it is an entirely passive form of colony spreading.

Since the LSC variant lacks the ability to oxidize iron, why is the presence of ferrous iron important for the appearance of the variant colony morphologies? We propose that it is actually the presence of ferric iron that is important. The ferric iron arises by air oxidation of ferrous iron in the mixed thiosulfate-iron media. We suggest that the ferric iron is involved in the chemical or biological oxidation of sulfite, an intermediate in the oxidation of thiosulfate (1, 16, 20, 34,

35), removing the inhibitory effect of the sulfite on growth (35, 36).

The variant arises from ATCC 19859 on mixed medium plates at frequencies that exceed normal rates for spontaneous mutation, and it reverts to the wild type at frequencies that significantly exceed normal reversion rates. This raises two important issues.

First, one must be certain that the proposed LSC variant is not just a contaminating microorganism in an iron-grown culture of *T. ferrooxidans* and that the apparent reversion to wild type is not merely due to an analogous contamination of the variant with residual *T. ferrooxidans*. This issue is particularly germane in the light of recent findings that *T. ferrooxidans* cultures are frequently contaminated with acidophilic heterotrophic bacteria (10, 19). To address this issue, we used a cloned family 2 DNA probe (pTf32) from the parental *T. ferrooxidans* ATCC 19859 and compared the pattern of hybridization by Southern blot hybridization with that of DNA prepared from the parental *T. ferrooxidans* and also from independently arising LSC variants and revertants to wild type. In all cases, the pattern of hybridization was identical. Such congruency is not found when DNA is compared between different species of *Thiobacillus* or even between different strains of *T. ferrooxidans* (44). This provides the strongest evidence available that the LSC variant is derived from the parental wild-type *T. ferrooxidans* and that the wild-type revertant derives, in turn, from the LSC variant.

The second issue concerns the mechanism by which the LSC variant arises from the wild type and by which it can revert to the wild type at frequencies that exceed normal rates expected for mutations. We have previously shown that the parental strain of *T. ferrooxidans* ATCC 19859 contains two families of repeated DNA sequences (43). Repeated DNA sequences have also been reported in the archaebacterium *Halobacterium halobium* (25), *Caulobacter crescentus* (42), *P. cepacia* (18), and *Streptomyces* species (24). We have recently observed that the repeated DNA sequences of *T. ferrooxidans*, particularly family 1 repeats, are mobile (J. Schrader, J. Yates, and D. S. Holmes, manuscript in preparation), and preliminary evidence indicates that specific changes in the position of these mobile elements are associated with the formation of the LSC variant (D. S. Holmes, J. R. Yates, and J. A. Schrader, *in* D. Kelly and F. Norris, ed., *Biohydrometallurgy 87*, in press). These specific changes return to a wild-type pattern in the wild-type revertant. We have also sequenced one of the families of mobile repeated DNA sequences (J. Yates, R. Cunningham, and D. S. Holmes, Proc. Natl. Acad. Sci. USA, in press) and have demonstrated that it has all the characteristics of an insertion sequence (IS element), namely, a size of about 1.4 kilobases, three open reading frames, terminal inverted repeats, and a target site duplication. These reasons strongly implicate the transposition of an IS element(s) as the causative mechanism giving rise to the LSC variant and its subsequent reversion.

It is not known whether this transposition is entirely random or whether it is genetically programmed in the sense that phase variation in *Salmonella* species (28) or antigen-switching in *Neisseria gonorrhoeae* (9) is the result of specific genetic switching. However, the relationship between loss of iron-oxidizing activity and altered colony morphology in these variants caused us to speculate that these two changes are causally related.

The phenotypic switching described in this report is not restricted to *T. ferrooxidans* ATCC 19859. We observed the LSC variant phenotype on solid media 100:10 and 10:10 with all five other *T. ferrooxidans* strains tested. This raises the possibility that LSC-type variants provide a selective advantage for the maintenance of *T. ferrooxidans* in its natural environment. Some variable phenotypes, for example, *N. gonorrhoeae* pilus variation (15, 37), are reflected by changes in colony morphology. In addition, colony heterogeneity has also been reported in long-term chemostat cultures of *T. versutus* (3), and high-frequency, reversible colony morphology switching has been described in *Candida albicans* (30–32). This apparent instability within the bacterial population may introduce flexibility in response to changing environmental conditions (27). The ability of some members of a clonal population to survive under conditions that prevent growth of the majority of the population would enhance survival of the species.

The visualization on the mixed media of variants lacking the ability to oxidize iron should facilitate the isolation of natural and artificially induced iron oxidation mutants of *T. ferrooxidans*. However, our efforts to detect chemically induced iron oxidation mutants were thwarted by the occurrence of LSC-type variants which exceeded in number mutants that might have arisen from the mutagenesis. We observed examples in which cells lacked the ability to oxidize iron and did not revert to wild type like the typical LSC variant. However, we do not know whether these arose as a result of chemically induced mutations or whether they were a form of LSC variant that lacked the ability to revert, perhaps as a result of an error in the transposition of an IS element. It should be noted that the presumptive iron-oxidizing mutants of *T. ferrooxidans* isolated by Cox and Boxer (4) exhibited slow or delayed growth on iron medium. This is characteristic of the typical LSC phenotype in which reversion to wild type gives rise to delayed growth on iron plates. It is possible, therefore, that some or all of the presumptive iron oxidation mutants of Cox and Boxer (4) are actually naturally occurring LSC-type variants. This would also explain the high frequency of occurrence of the presumptive mutants in their experiments.

The ubiquitous and high-frequency occurrence of LSC variants in *T. ferrooxidans* might also help to explain the commonly observed phenomenon that a proportion of cells grown in thiosulfate frequently lose their ability to grow on iron (39). This would be expected if some of the thiosulfate-grown cells were LSC variants.

Another frequently observed phenomenon is the low plating efficiency of iron-grown *T. ferrooxidans* on thiosulfate plates (39). Our estimate of one colony formed per 17,000 iron-grown cells plated confirms this observation. It is possible that genetic switching, perhaps by a mechanism similar to that which results in the LSC variant, is involved in generating cells capable of utilizing thiosulfate. Alternatively, induction of appropriate biochemical pathways might be involved.

The LSC variant was not the only type of colony morphology variant observed on medium 10:10 spread with *T. ferrooxidans* ATCC 19859. For example, there were colonies which had an LSC phenotype on medium 10:10 but did not grow readily on either iron or thiosulfate medium alone. Certain clones may, therefore, grow better when both iron and thiosulfate are present. Another colony type formed small (1 mm or less) flat white or yellow iridescent colonies on medium 10:10 and grew readily on iron medium and slowly if at all on thiosulfate medium. These examples are suggestive of the diversity of colony morphology variants that arise in clonally purified cultures of this strain.

Preparation of mixed media containing different combinations of substrates might result in isolation of other colony morphology variants. For example, media compounded to reflect the conditions present in available ores could facilitate the rapid isolation of suitable clones for optimal metal extraction. Isolation of mutants and variants with differences in iron and sulfur metabolism will make it possible to dissect the energy metabolism of *T. ferrooxidans* and to develop genetic systems to optimize exploitation of this valuable organism.

ACKNOWLEDGMENTS

This work was supported in part by grants CPE-8420881 and MSM-8215220 from the National Science Foundation.

We thank J. Yates and J. Bergeron for stimulating discussions, I. Giaever for the use of the time-lapse photomicroscope, and E. Uzgiris and William P. Schrader for assistance with the microscopy.

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