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Mixotrophic Growth of a *Thiobacillus ferrooxidans* Strain

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Mixotrophic growth of a *Thiobacillus ferrooxidans* strain is described. DNA moles percent guanine plus cytosine and homology determinations confirmed that the mixotrophically grown *T. ferrooxidans* cultures were not contaminated with heterotrophic *Acidiphilium* strains.

At present, there is a considerable degree of uncertainty regarding the ability of *Thiobacillus ferrooxidans* strains to grow mixotrophically. This is due to the very close association between an obligately heterotrophic bacterium, *Acidiphilium cryptum*, and *T. ferrooxidans* in supposedly pure cultures (1, 4). Recently, Sugio et al. (8) reported the isolation of a facultative iron-oxidizing *T. ferrooxidans* strain which was able to utilize glucose and grow on an organic medium without iron. However, the culture used by Sugio et al. (8) has been shown to be impure and was a mixture of heterotrophic *A. cryptum* (70 mol% guanine plus cytosine [G+C] content) and an obligately autotrophic *T. ferrooxidans* (57 mol% G+C) (A. Harrison, Division of Biological Sciences, University of Missouri, Columbia, Mo., personal communication). There is, therefore, no definite evidence of a heterotrophic or mixotrophic *T. ferrooxidans* strain.

We have been investigating various *T. ferrooxidans* strains from South Africa mines and describe the isolation of a *T. ferrooxidans* strain which is capable of mixotrophic growth. To avoid the pitfalls and problems of a contaminating heterotrophic *Acidiphilium* strain, we determined the moles percent (mol%) G+C and DNA homologies before and after mixotrophic growth and compared these with a heterotrophic *Acidiphilium* sp. control culture and an obligately autotrophic *T. ferrooxidans* control culture.

Three bacterial strains were utilized after isolation of individual colonies after several cycles of subculture on agar media. An obligately autotrophic *T. ferrooxidans* ATCC 19859 strain was isolated and grown on 9K medium (7) which contained ferrous iron as the sole source of energy. A mixotrophic *T. ferrooxidans* FD1 strain (Florence Devonian Ore, General Mining Co., South Africa) was isolated on 9K agar medium and grown in FeG medium (6) which contained glucose (5 g/liter) and iron (7 g/liter). The *T. ferrooxidans* FD1 strain was not able to grow in medium without iron and did not give rise to colonies on GYE medium (6) which contained glucose and yeast extract. The heterotrophic *Acidiphilium* Het FD2 strain was isolated from a sample of acid leach liquor (Florence Devonian Ore) by enrichment on GYE medium and identified as belonging to the genus *Acidiphilium* according to Harrison (2). This strain was unable to grow on or oxidize iron in pure culture in 9K iron medium.

The DNA mol% G+C and DNA homologies between the two *T. ferrooxidans* strains and the *Acidiphilium* strain were determined after growth on various media. The bacterial cultures were washed in 9K basal salts (pH 1.6) and suspended in SET buffer (50 mM Tris, 2 mM EDTA, 25% sucrose). After the bacterial solution was frozen at −20°C, predigested pronase was added to give a final concentration of 2 mg/ml. Sodium dodecyl sulfate was added to a final concentration of 1%, and the suspension was held at 4°C for 10 min. RNase was added to a final concentration of 50 μg/ml, and the mixture was incubated at 37°C for 10 to 15 min. The solution was dialyzed overnight and extracted several times with an equal volume of cold chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated with cold 95% ethanol, dissolved in 0.1× saline citrate solution (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and dialyzed overnight against 0.1× SSC. The DNA base composition was calculated from the thermal denaturation profiles of the DNA, using a Beckman Du-8 spectrophotometer equipped with a Tm Compuset module. The formula of Mandel and Marmur (5) was used to calculate sample G+C ratios, using a G+C ratio of 51 ± 0.25 mol% for *Escherichia coli* reference DNA. To determine DNA homology, we denatured DNA solutions by heating them at 90°C for 10 min followed by rapid cooling on ice. A known quantity of DNA was absorbed onto a nitrocellulose disk and baked for 2 h at 80°C. A prehybridization mixture was prepared by dissolving 5 mg of sonicated salmon sperm DNA in 10 ml of 6× SSC and adding 20 μl of Denhardt solution. Prehybridization was carried out at 65°C for 2 h. The hybridization mixture was prepared by adding 0.5 μg of alkali-denatured nick-translated reference DNA (specific activity, 5×10^6 to 8×10^6 cpm/μg) and 25 mg of sonicated salmon sperm DNA to 3 ml of 20× SSC–100 μl Denhardt solution, and the volume was made up to 10 ml with water. After incubating at 65°C overnight, the samples were washed three times with 0.3× SSC–0.2% sodium dodecyl sulfate at 65°C for 15 min, and the remaining radioactivity was determined.

The mol% G+C ratios of the *T. ferrooxidans* FD1 strains grown in 9K iron medium and in FeG iron-glucose medium were identical (60.5 mol% G+C) and the same as the mol% G+C of the obligately autotrophic ATCC 19859 strain (Table 1). The graph of the hyperchromic shift at an optical density at 260 nm of DNA isolated from the *T. ferrooxidans* FD1 strain grown in FeG iron-glucose medium as a function of temperature was not biphasic. The utilization of glucose was confirmed (see Fig. 1) in the *T. ferrooxidans* FD1 cultures used for mol% G+C and DNA homology studies. The mol% G+C of *Acidiphilium* Het FD2 strain (67.9 mol% G+C) differed from the *Thiobacillus* strains (Table 1). A high degree of DNA homology was observed between the iron- and iron-glucose-grown FD1 strains, indicating that they were the same organism and related to the ATCC 19859 strain (Table 1). Although the *T. ferrooxidans* FD1 strain only showed 39% homology with the ATCC 19859 strain, it was within the range of the seven DNA homology groups reported by Harrison (3). Zero DNA homology was ob-

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TABLE 1. DNA base composition and DNA homology between *T. ferrooxidans* ATCC 19859 strain grown in 9K iron medium, *T. ferrooxidans* FD1 strain grown in 9K medium and FeG glucose-iron medium, and *Acidiphilium* Het FD2 grown in GYE glucose medium

Bacterial strain	Energy source	mol% G+C	% Homology to FD1	% Homology to Het FD2
ATCC 19859	Iron	60.5	39	2
FD1	Iron	60.5	100	3
FD1	Iron and glucose	60.5	85	5
Het FD2	Glucose	67.9	0	100

served between the *T. ferrooxidans* FD1 strain after growth in iron-glucose medium and the *Acidiphilium* strain. This total lack of DNA homology between the *Acidiphilium* strain and the *T. ferrooxidans* FD1 cultures which utilized 2.5 of glucose per liter in the iron-glucose medium (see Fig. 1) indicated that the mixotrophic *T. ferrooxidans* FD1 cultures were not contaminated with the heterotrophic *Acidiphilium* strain.

The growth of the *T. ferrooxidans* FD1 strain in iron medium and iron medium supplemented with glucose was compared (Fig. 1). The rate of iron oxidation was faster in 9K medium containing 9 g of ferrous iron per liter than in FeG medium which contained 4 g of ferrous iron per liter. The glucose present in FeG medium could have been responsible for the slower rate of iron oxidation. The amount of glucose present in the FeG medium decreased from 5 g/liter to 2.5 g/liter as the iron was oxidized. This decrease was not due to acid hydrolysis of glucose as no glucose was degraded in an uninoculated control. The yield of *T. ferrooxidans* cells was 0.08 and 0.06 g (dry weight) per liter on FeG medium and 9K medium, respectively. Based on the quantity of iron oxidized, the yield of *T. ferrooxidans* cells was ca. threefold greater in the glucose-containing medium. This increase in yield together with the disappearance of glucose from FeG medium indicated that the FD1 strain was able to utilize glucose.

The growth of *T. ferrooxidans* FD1 in a mixotrophic medium with different concentrations of ferrous sulfate and a constant amount of glucose was studied to determine whether ferrous iron was utilized preferentially or whether ferrous iron and glucose were utilized simultaneously (Fig. 2). At a concentration of 5 g of ferrous iron per liter, the FD1 strain utilized both iron and glucose concurrently, although iron was exhausted first. At 7 g of ferrous iron per liter, there was a slight lag phase during which ferrous iron alone was utilized. When the concentration of iron dropped to ca. 5 g/liter, both iron and glucose were used simultaneously. At 9 g of ferrous iron per liter, there was complete inhibition of iron oxidation and glucose utilization, suggesting that at this iron concentration the glucose was inhibitory. The addition of 0.5 g of glucose per liter to the medium with 9 g of ferrous iron per liter also resulted in the organism being unable to use either substrate. However, at lower iron concentrations, both iron and glucose were used simultaneously, which indicated that the glucose concentration per se was not toxic but rather that a lower iron/glucose ratio is important for growth. The effect of the ferrous iron concentration on the

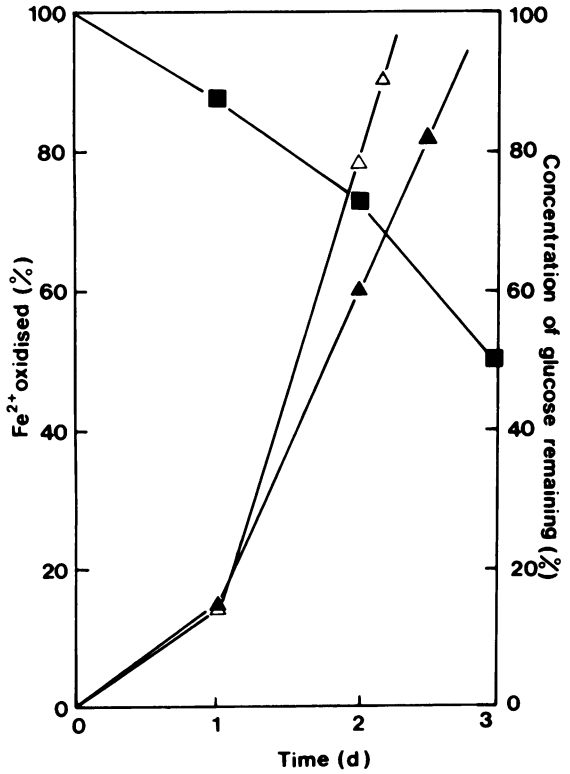

FIG. 1. Comparison between ferrous iron oxidation of *T. ferrooxidans* FD1 in iron medium (△) and in iron-glucose medium (▲). ■, Glucose utilization in iron-glucose medium; d, day.

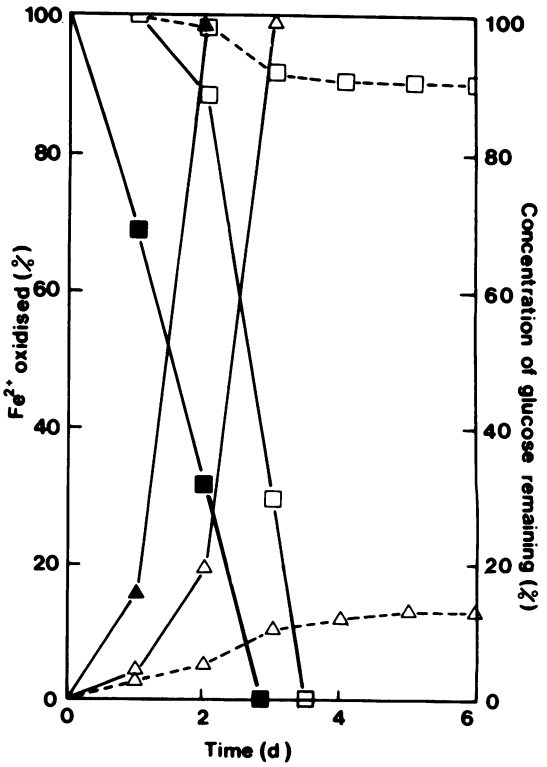

FIG. 2. Utilization of glucose by *T. ferrooxidans* FD1 in iron medium supplemented with glucose, at different ferrous iron concentrations. Initial glucose concentrations was 5 g/liter. Initial ferrous iron concentrations were 4 g/liter (▲), 7 g/liter (—△—), and 9 g/liter (--△--). Glucose utilization is shown by the squares; iron oxidation is shown by the triangles. d, Day.

ability of the *T. ferrooxidans* FD1 strain to utilize glucose is interesting. Most work on *T. ferrooxidans* growth is carried out in 9K medium with a ferrous iron content of 9 g/liter, which would prevent the detection of other mixotrophic *T. ferrooxidans* strains with the same characteristics as the FD1 strain.

Our results indicate that certain *T. ferrooxidans* strains are able to grow mixotrophically and utilize glucose in the presence of iron. This is not due to contamination by obligately heterotrophic *Acidiphilium* strains which often occur in close association with *T. ferrooxidans* strains (1, 4) since the FD1 strain had an identical DNA G+C content and high DNA homology when it was grown in iron and iron-glucose media. Furthermore, this bacterium did not grow on organic medium in the absence of iron.

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