

Glucose Transport System in a Facultative Iron-Oxidizing Bacterium, *Thiobacillus ferrooxidans*

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Properties of a heat-labile glucose transport system in *Thiobacillus ferrooxidans* strain AP-44 were investigated with iron-grown cells. [^{14}C]glucose was incorporated into cell fractions, and the cells metabolized [^{14}C]glucose to $^{14}\text{CO}_2$. Amytal, rotenone, cyanide, azide, 2,4-dinitrophenol, and dicyclohexylcarbodiimide strongly inhibited [^{14}C]glucose uptake activity, suggesting the presence of an energy-dependent glucose transport system in *T. ferrooxidans*. Heavy metals, such as mercury, silver, uranium, and molybdate, markedly inhibited the transport activity at 1 mM. When grown on mixotrophic medium, the bacteria preferentially utilized ferrous iron as an energy source. When iron was exhausted, the cells used glucose if the concentration of ferrous sulfate in the medium was higher than 3% (wt/vol). However, when ferrous sulfate was lower than 1%, both of the energy sources were consumed simultaneously.

Thiobacillus ferrooxidans has been classified as a strict autotroph (15). However, Shafia et al. (5, 6) and Tabita and Lundgren (11) have shown that when autotrophically grown cells are transferred to a medium containing iron plus glucose, cells preferentially utilize ferrous iron, and when the iron is exhausted, they use glucose. These glucose-adapted cells can grow on glucose as the sole energy source upon transfer into a glucose-salts medium. Some conditions for adaptation and the enzymes involved in glucose metabolism have been studied by several workers (5, 6, 11-13).

Recently, Harrison et al. (2) isolated a pair of stable phenotypes from several presumably pure cultures of *T. ferrooxidans*. One phenotype was a strict autotroph utilizing sulfur or ferrous iron as the energy source and was unable to utilize glucose; the other phenotype was an acidophilic obligate heterotroph capable of utilizing glucose but not sulfur or ferrous iron. From the results of studies of DNA homology, it was concluded that the acidophilic heterotroph was of a different genotype from that of *T. ferrooxidans* or *T. acidophilus* (1), and the authors warned that the cultures of *T. ferrooxidans* reported to be capable of utilizing organic compounds should be carefully examined for contamination.

We have also isolated two kinds of iron-oxidizing bacteria from our culture of iron-grown *T. ferrooxidans* strain SPP (8). One (strain AP-19) was a strict autotroph utilizing ferrous iron or sulfur as the energy source and

was unable to utilize glucose or organic substances; the other (strain AP-44) was a facultative iron-oxidizing bacterium which obtains its energy from ferrous iron or elemental sulfur in addition to organic substances, such as glucose, galactose, gluconic acid, citric acid, peptone, and yeast extract. The most distinct physiological difference between these strains was their ability to take up [^{14}C]glucose into the cells.

The problem of whether there is a facultative strain of iron-oxidizing *T. ferrooxidans* which can use ferrous iron, sulfur compounds, and organic substances as the energy sources has not yet been established. In this paper, the [^{14}C]glucose uptake system of iron-grown AP-44 was studied to clarify whether the system operated in glucose metabolism of this bacterium.

MATERIALS AND METHODS

Microorganism. The iron-oxidizing bacterium, *T. ferrooxidans* strain AP-44 (8) was used throughout this study.

Media and conditions of cultivation. The organism was grown on three media: (i) 9K medium (7), which contained ferrous iron as the sole energy source; (ii) iron-glucose medium, which contained glucose added to the 9K medium and was used for mixotrophic growth; (iii) glucose-salts medium, which contained 0.5% glucose and salts of 9K medium (excluding ferrous sulfate) and was used for heterotrophic growth. In the growth experiments, 100 ml of the iron-glucose medium described above was inoculated with 2 ml of an autotrophically grown culture and shaken at 28°C. The method used for large-scale production of

autotrophically grown cells, which was used for the measurement of [^{14}C]glucose uptake, was that described earlier (10).

Growth rate. All cultures were filtered through Toyo filter paper no. 5C and diluted to the required level with 0.1 N sulfuric acid. The growth rate was determined by direct cell counts of the filtrate with a Thomas counting chamber.

Ferrous iron and glucose determination. The determination of ferrous iron or glucose concentration in the medium was as described previously (8).

[^{14}C]glucose uptake activity. The radioactivity of [$U\text{-}^{14}\text{C}$]glucose taken up by iron-grown strain AP-44 or glucose-salts-grown strain AP-44 was measured by the method previously described (8). The composition of the reaction mixture was as follows: 2 ml of 0.1 M β -alanine sulfuric acid buffer, pH 3.0; log-phase cells which were washed three times with 0.01 M phosphate buffer, pH 7.5 (20 mg of protein); carrier glucose, 2 μmol ; [$U\text{-}^{14}\text{C}$]glucose, 1.2 μCi . Total volume was 4.5 ml. Each of the metabolic inhibitors tested was added to the reaction mixture.

The reaction mixture, except glucose, was incubated at 30°C for 10 min before addition of glucose. The reaction was stopped by adding 0.5 ml of 20 mM mercuric chloride. The reaction mixture was immediately centrifuged at $14,000 \times g$ for 10 min, and the cells were washed twice with 10 ml of distilled water. The radioactivity of the washed cells was determined by using an Aloka LSC-635 liquid scintillation system. The counting vial contained 2 ml of the washed cell suspension (4 mg of protein) plus 4 ml of counting mixture consisting of PCS (Amersham Corp.) (total volume, 6 ml). Counting efficiency was measured by the external standard method. The amount of glucose taken up was expressed in terms of micromoles per milligram of protein.

Distribution of [^{14}C]glucose in the cells. Iron-grown cells were treated with [$U\text{-}^{14}\text{C}$]glucose for 1 h in the following reaction mixture: 54 ml of 0.1 M β -alanine sulfuric acid buffer, pH 3.0; iron-grown cells (725 mg of protein); carrier glucose, 20 μmol ; [$U\text{-}^{14}\text{C}$]glucose, 12 μCi . Total volume was 100 ml. The reaction was stopped by adding 10 ml of 20 mM mercuric chloride, and the cells were washed immediately four times with 90 ml of distilled water. The [$U\text{-}^{14}\text{C}$]glucose-treated cells were disrupted by sonic oscillation (20 kHz) for 30 min. The broken cells were centrifuged at $10,000 \times g$ for 20 min. The crude extract was further centrifuged at $105,000 \times g$ for 60 min to separate the particulate fraction (plasma membrane fraction) and supernatant fraction (cytosol fraction). The radioactivity of each fraction was determined by using the liquid scintillation system and the method as described above.

$^{14}\text{CO}_2$ evolution. $^{14}\text{CO}_2$ evolved from the [$U\text{-}^{14}\text{C}$]glucose-treated cells was trapped into a pair of tubes containing 10 ml of monoethanolamine, using the method and the apparatus previously described (8). The composition of the reaction mixture was as follows (experiment A): 3.0 ml of 0.1 M β -alanine sulfuric acid buffer, pH 3.0; iron-grown cells (20 mg of protein); carrier glucose, 2 μmol ; [$U\text{-}^{14}\text{C}$]glucose, 1.2 μCi . Total volume was 5.0 ml. The same concentration of a 10-min boiled fraction was also used to determine the amount of $^{14}\text{CO}_2$ evolution (experiment B). The reaction was stopped after 30 min by adding 0.5 ml of 20 mM mercuric chloride. The radioactivity trapped in

monoethanolamine was determined by using the method described above.

Protein determination. The protein content was determined by the biuret method (3), using crystalline bovine serum albumin as the reference protein.

RESULTS

Distribution of the radioactivity in iron-grown cells after the incorporation of [^{14}C]glucose. The time course of [^{14}C]glucose uptake into iron-grown cells is shown in Fig. 1. The level of radioactivity increased with time, and the profile of the curve was similar to that obtained with glucose-salts-grown cells (8). The [^{14}C]glucose uptake activity was nearly completely destroyed by heating the cells in a water bath at 70°C for 10 min.

To distinguish whether [^{14}C]glucose is taken up into the cells or merely attached tightly on the surface of the outer membrane, a study of the distribution of the radioactivity in the iron-grown cells was carried. As shown in Table 1, a large amount (52.3%) of the radioactivity taken up into the cells was in the cytosol fraction, suggesting that [^{14}C]glucose is taken up into the iron-grown cells.

$^{14}\text{CO}_2$ evolution of iron-grown strain AP-44. The amount of $^{14}\text{CO}_2$ evolved from the [^{14}C]glucose-treated iron-grown cells was determined by trapping $^{14}\text{CO}_2$ into monoethanolamine. As shown in Table 2, significant $^{14}\text{CO}_2$ evolution occurred with intact cells, in contrast to the controls with boiled cells. Similar results were also obtained for glucose-salts-grown strain AP-44 (8). The results suggest that [^{14}C]glucose is

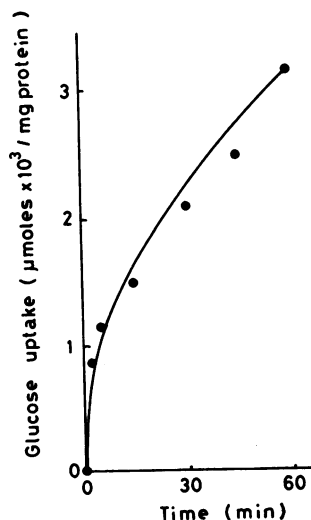


FIG. 1. Time course of [$U\text{-}^{14}\text{C}$]glucose uptake into iron-grown cells. The method for analysis and the composition of reaction mixture were described in the text.

TABLE 1. Distribution of the radioactivity in the cells of iron-grown *T. ferrooxidans* strain AP-44 after the incorporation of [U - ^{14}C]glucose

Fraction ^a	Protein (mg)	Total radioactivity (cpm)	Distribution (%)
Cell-free crude extract	482	1,037,294	79.6
Cell debris	260	266,200	20.4
Cytosol fraction	162	682,789	52.3
Plasma membrane fraction	238	390,443	29.9

^a Each of the fractions was prepared from [U - ^{14}C]glucose-treated cells by the method described in the text. The washed cells before fractionation contained 1,201,018 cpm total radioactivity.

not only taken up into the cells, but is also metabolized to carbon dioxide and water.

Effect of various inhibitors on [^{14}C]glucose uptake activity. Table 3 shows that [^{14}C]glucose uptake activity was strongly inhibited by several respiratory inhibitors or uncoupling reagents, such as amytal, rotenone, cyanide, azide, 2,4-dinitrophenol, and dicyclohexylcarbodiimide, in both iron-grown cells and glucose-salts-grown cells. Heavy metals, such as mercury, silver, uranium, and molybdate, also markedly inhibited the [^{14}C]glucose transport activity in both iron-grown cells and glucose-salts-grown cells.

Figure 2 shows the effects of ferrous and ferric iron on the [^{14}C]glucose uptake activity. The activity of iron-grown cells was markedly inhibited by ferrous iron but not by ferric iron at similar concentrations. Similar results were obtained for glucose-salts-grown cells.

We usually observed that when the bacterium was grown mixotrophically (iron-glucose medium), cells preferentially utilized ferrous iron as an energy source if the concentration of ferrous

TABLE 2. $^{14}CO_2$ evolution from [^{14}C]glucose-incorporated iron-grown cells

Fraction	Radioactivity (cpm)	
	Expt A ^a	Expt B ^b
Cells	30,735	620
Respiratory $^{14}CO_2$ ^c		
Flask 1	670	3
Flask 2	4	4

^a Results obtained with intact iron-grown cells.

^b Results obtained with 10-min boiled iron-grown cells.

^c $^{14}CO_2$ evolved from the [U - ^{14}C]glucose-treated cells was trapped into a pair of flasks containing 10 ml of monoethanolamine; flasks 1 and 2 were connected to the reaction vessel successively. The method for analysis and the composition of reaction mixture were described in the text.

TABLE 3. Effect of respiratory inhibitors and uncoupling reagents on [^{14}C]glucose uptake activity

Compound ^a	Final concn (M)	[^{14}C]glucose uptake activity ($10^3 \mu\text{mol/mg}$ of protein)	
		Iron-grown cells	Glucose-grown cells
None		1.17	1.92
Amytal	5×10^{-5}	0.00	0.00
Rotenone	5×10^{-5}	0.00	0.00
Quinacrine	10^{-3}	0.91	1.71
Progesteron	10^{-3}	0.94	1.79
Cyanide	10^{-4}	0.64	
	10^{-3}	0.07	0.28
Azide	10^{-4}	0.82	
	10^{-3}	0.00	0.65
2,4-DNP	10^{-3}	0.14	0.18
DCCD	5×10^{-5}	0.00	0.00

^a DCCD, Dicyclohexylcarbodiimide; 2,4-DNP, 2,4-dinitrophenol.

sulfate in the medium was higher than 10 mM, and when the ferrous iron was exhausted, they utilized glucose (Fig. 3). The same phenomenon was also observed by Tabita and Lundgren (11). If the concentration of ferrous iron in the medium was below 3.5 mM, cells utilized both ferrous iron and glucose at the same time (Fig. 4). The reason why the bacterium was not able to utilize glucose at the early stage of growth in high-iron mixotrophic medium is well explained by the properties of the [^{14}C]glucose transport

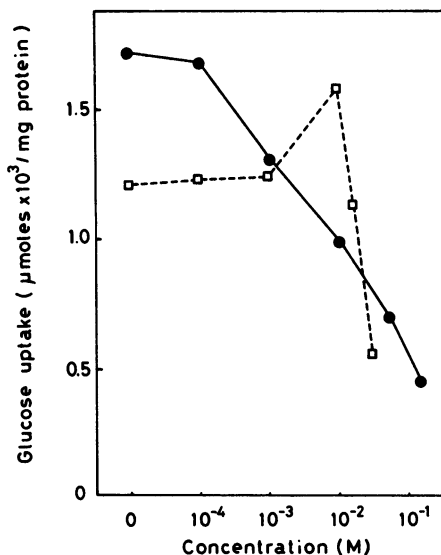


FIG. 2. Effect of ferrous iron and ferric iron on [U - ^{14}C]glucose uptake activity of iron-grown cells. The method for analysis and the composition of reaction mixture were described in the text. Symbols: ●, ferrous iron; □, ferric iron.

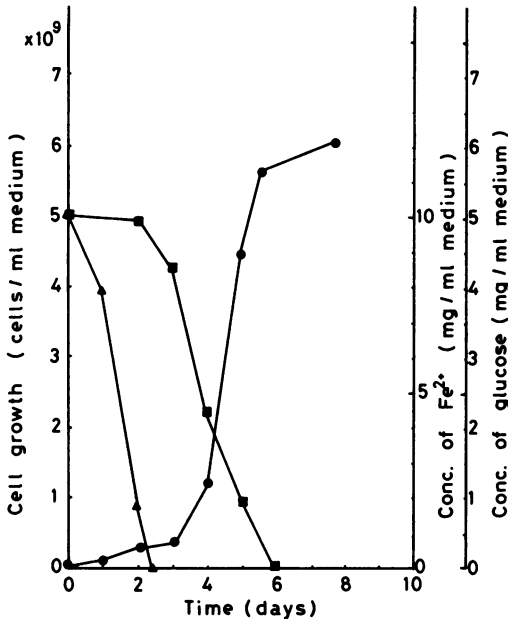


FIG. 3. Growth of *T. ferrooxidans* strain AP-44 on 5% ferrous sulfate-0.5% glucose-salts medium. The composition of the medium and the method for cultivation were described in the text. Symbols: ●, cell growth; ▲, concentration of ferrous iron; ■, concentration of glucose.

system. Since at the initial stage of growth shown in Fig. 3, 18 mM of ferrous iron in the medium strongly inhibited glucose uptake into the cells, the organism utilized ferrous iron first. However, when ferrous iron was oxidized to ferric iron, the condition of the medium became preferable for the organism to utilize glucose, and in this way, ferrous iron concentration controls the utilization of glucose.

DISCUSSION

The following properties are required to show that strain AP-44 is a facultative iron-oxidizing bacterium: (i) glucose in the medium must be taken up into the cells; (ii) glucose taken up must be metabolized to carbon dioxide and water, and energy must be generated from the reaction; (iii) the metabolism of glucose must be satisfied with iron-grown cells.

There have been few reports about glucose uptake in iron-grown *T. ferrooxidans*. Tuovinen and Kelly (14) have shown that suspensions of *T. ferrooxidans* incorporated a small amount of ¹⁴C-labeled glucose when incubated in its presence at pH 2.0 in Warburg flasks. The amount of [¹⁴C]glucose incorporated was dependent on the presence of ferrous iron, and the activity was increased about 2.5-fold by adding 60 μmol of ferrous iron. In contrast, we found that the

activity in iron-grown strain AP-44 was independent of the presence of ferrous iron; rather, it was markedly inhibited by high concentrations of the ions. Tuovinen and Kelly (14) did not discuss whether the incorporated glucose was utilized as the energy source or as the carbon source.

Matin et al. (4) reported that 2-deoxy-D-glucose uptake activity by washed cell suspensions of *T. novellus*, which is a facultative sulfur-oxidizing bacterium, was inhibited by azide, cyanide, and 2,4-dinitrophenol. We also observed heat-labile and energy-dependent [¹⁴C]glucose uptake in iron-grown *T. ferrooxidans* strain AP-44 and propose that the system plays an important role on the glucose metabolism of this bacterium for the following reasons: (i) strain AP-19, which does not have any [¹⁴C]glucose uptake activity, could not utilize glucose for energy and carbon source (8), but the cell-free crude extract of iron-grown AP-19 had a level of glucose-metabolizing activity similar to that of iron-grown strain AP-44; (ii) 52.3% of the total radioactivity taken up into the cells of strain AP-44 as [¹⁴C]glucose was found to be distributed in the cytosol fraction; (iii) ¹⁴CO₂ evolution was observed from [¹⁴C]glucose with iron-grown cells, and the reaction was very sensitive to cyanide or azide, suggesting an

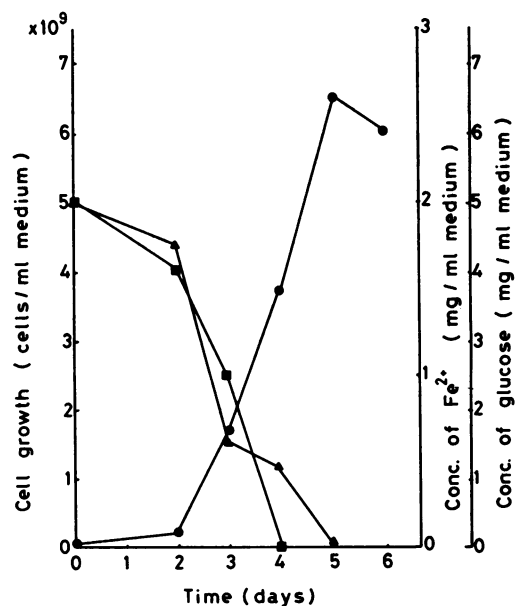


FIG. 4. Growth of *T. ferrooxidans* strain AP-44 on 1% ferrous sulfate-0.5% glucose-salts medium. The composition of the medium and the method for cultivation were described in the text. Symbols: ●, cell growth; ▲, concentration of ferrous iron; ■, concentration of glucose.

involvement of a terminal electron transport system in the reaction; (iv) the properties of [^{14}C]glucose uptake in iron-grown cells were similar to those of glucose-salts-grown cells; (v) the pH optimum of [^{14}C]glucose uptake activity (pH 3.0 in both iron-grown strain AP-44 and glucose-salts-grown AP-44) closely corresponded with that for growth on glucose-salts medium (pH 2.5 to 3.0); (vi) the reason why the bacterium was not able to utilize glucose at the early stage of growth in the high-iron mixotrophic medium is well explained by the properties of the [^{14}C]glucose transport system.

The physiological and enzymatic data obtained in our laboratory (8–10) also suggest the existence of a facultative iron-oxidizing bacterium. We observed activities of enzymes or an enzyme system involved in glucose metabolism, such as glucose-oxidizing activity, glucose dehydrogenase, and NADH oxidase (NADH:acceptor oxidoreductase), in glucose-salts-grown cells and also in iron-grown cells.

From DNA homology, Harrison et al. (2) warned that cultures of *T. ferrooxidans* reported to be capable of utilizing organic compounds should be carefully examined for contamination. They proposed that it is possible that the contaminants, such as acidophilic heterotrophic *thiobacilli* or acidophilic obligate heterotrophs, utilize trace amounts of organic substances from the atmosphere of the incubation chamber or as impurities on the inorganic ingredients in the medium.

If the contaminant cells utilize the organic

substances described above, the amount of cells obtained must be negligibly small as compared with the autotrophically grown *T. ferrooxidans* on 9K medium. We observed that iron-grown *T. ferrooxidans* remained viable for a long time without autolysis. Thus, only small numbers of contaminant cells could be grown by utilizing autolyzed organic substances for the energy and carbon source after the stationary phase of the bacterium. A negligibly small amount of contaminant cells would be present at the early stage of cultivation because iron-grown cells were always harvested at the late logarithmic stage of growth after 5 or 6 days of cultivation.

The most reliable way to check homogeneity of the facultative iron-oxidizing bacterium strain AP-44 may be to obtain a single colony on a glucose agar plate and to prove whether the isolated organism can grow on autotrophic media or heterotrophic media.

We tried to make a single colony on the glucose agar plate. The most desirable glucose agar plate for the purpose was found to be 0.5% ferrous sulfate–0.5% glucose–1.0% agar–salts (pH 3.5). Isolated, tiny brown opaque colonies were obtained on the plate. After 1 or 2 days of cultivation on the plate, the agar turned to yellow, suggesting the formation of ferric hydroxide. The isolation on the plate described above was repeated five times, and each of the finally isolated colonies was maintained on an agar slant of the same composition.

Figure 5 shows cells from a single colony grown on autotrophic media or glucose-yeast

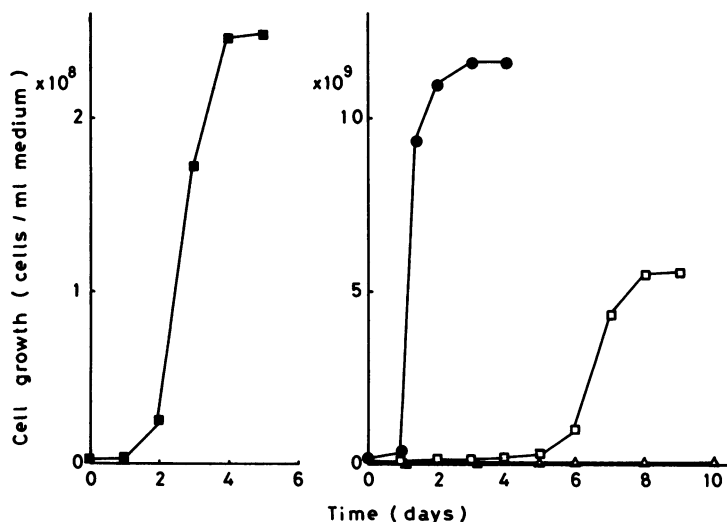


FIG. 5. Growth of a single colony obtained on a ferrous-glucose-salts-agar plate from strain AP-44 on autotrophic, mixotrophic, and heterotrophic media. The methods for isolation and maintenance were described in the text. Symbols: ■, 5% ferrous sulfate-salts medium (9K medium); □, 5% ferrous sulfate–0.5% glucose-salts medium; △, 0.5% glucose-salts medium; ●, 0.5% glucose–0.1% yeast extract-salts medium.

extract media. The organism could not grow on glucose-salts medium, suggesting the requirement of some growth factor(s). In contrast, it could grow on ferrous sulfate-glucose salts medium without growth factor(s).

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