JOURNAL OF BACTERIOLOGY, Oct. 1971, p. 328-333
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Vol. 108, No. 1
Printed in U.S.A.

Utilization of Glucose and the Effect of Organic Compounds on the Chemolithotroph *Thiobacillus ferrooxidans*

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Received for publication 18 June 1971

The utilization of glucose by the chemolithotroph *Thiobacillus ferrooxidans* results in a repression of the ability to oxidize iron, the substrate for autotrophic growth. An assay with resting cells was used to measure iron oxidation rates. Concomitant with the decreased iron oxidation rates, the enzyme responsible for carbon dioxide fixation, ribulose diphosphate (RuDP) carboxylase, was also repressed. Maximum iron oxidation rates precede peak RuDP carboxylase levels, consistent with the role of these processes in autotrophic metabolism in nonrepressed cells. The degree of iron oxidation repression depends on the organic substrate supplied, as does the level of RuDP carboxylase. The uptake of glucose parallels an increase in synthesis of glucose-6-phosphate dehydrogenase and the accumulation in cells of poly-β-hydroxybutyrate. The organism is also capable of growing on glucose and other organic supplements in the absence of its inorganic energy source; growth rates depend on the organic substrate supplied.

The acidophilic iron-oxidizing bacteria include those organisms previously referred to as *Ferrobacillus ferrooxidans* but now recognized as *Thiobacillus ferrooxidans* (22). In a predominantly inorganic environment, the acidophilic iron-oxidizing bacteria obtain energy for the reduction of carbon dioxide from the oxidation of reduced iron and sulfur compounds. However, these organisms can metabolize and grow on glucose as the sole energy source (14; Remsen and Lundgren, Bacteriol. Proc., p. 33, 1963). Lundgren et al. (9) have shown that when autotrophically grown cells are transferred to a medium containing iron plus glucose, cells preferentially utilize ferrous iron and when iron is exhausted, the cells use glucose. These cells can grow on glucose as the sole energy source upon transfer into a glucose-salts medium. Some strains of iron-oxidizing bacteria are unable to adapt to glucose, and it has been suggested that this trait may be considered as a basis for species differentiation (14).

Glucose has been shown to inhibit the oxidation of iron and elemental sulfur in manometric experiments with resting cells prepared from cultures grown on either inorganic substrate (16). Concomitant with the inhibition of substrate oxidation, autotrophic fixation of carbon dioxide is drastically reduced in the presence of high concentrations of glucose (16). Silver (15) has recently found that glucose inhibited CO_2 fixation from 40 to 66% when sulfide, thiosulfate, tetrathionate, dithionate, and sulfite served as the source of reduced sulfur. Other studies (J. H. Tuttle and P. R. Dugan, Bacteriol. Proc., p. 64, 1969) have shown that several organic acids inhibit both iron and sulfur oxidation. In this study, we have examined the effects of glucose on the growth of *T. ferrooxidans* in an attempt to understand metabolic changes induced by the oxidation and subsequent metabolism of this sugar.

MATERIALS AND METHODS

Organism and media. *T. ferrooxidans*, a subculture of the original Leathen isolate, strain TM (6), has been used in these studies. Cultures were grown on three media: (i) the 9K medium, previously described (17), used for iron-grown cells; (ii) the 9KG medium containing 0.5% glucose in the 9K medium; (iii) the glucose-salts medium containing 0.5% glucose and the salts of the 9K medium (no iron). Filter-sterilized glucose was used, and the final *p*H of all media was 2.7. When other sugar compounds replaced glucose, they were filter sterilized and tested at the same 0.5% concentration; glutamate (0.5%) was also tested, and it was autoclaved separately and added aseptically to the 9K salts solution.

Cultural procedures. Iron-grown cells were harvested after 60 hr of incubation at 30 C (late lag phase) as

previously described (17). Iron-glucose-grown cells were cultured by using a 10-ml inoculum of iron-grown cells (5×10^9 cells) in 100 ml of the 9KG medium contained in a 250-ml Erlenmeyer flask. The flask was shaken on a reciprocal shaker at 30 C for 60 hr at which time all the ferrous iron was oxidized. After a second transfer into fresh 9KG medium, followed by incubation as above, *T. ferrooxidans* was readily grown on glucose as the sole energy source. This was done by transferring 10 ml of the 9KG culture into 90 ml of the glucose-salts medium and incubating on a shaker at 30 C. Cells have been maintained on the glucose-salts medium for over 1 yr by using bimonthly transfers to fresh medium. Large masses of glucose-grown cells were used for preparing crude enzyme extracts; cells were grown by using a 10% inoculum from a fresh started culture and inoculating into 500 ml of the glucose-salts medium contained in 2,800-ml Fernbach flasks. Flasks were shaken at 30 C on a three-tiered rotary shaker (New Brunswick Scientific Co.) set at 150 cycles/min.

The 9K-fructose-, 9K-sucrose-, and 9K-glutamate-grown cells were cultured in a similar manner with the exception that the appropriate sugar or amino acid substrate was substituted for glucose.

Contamination and purity of cultures was rigorously monitored by streaking iron-glucose- and glucose-grown cultures on a number of complex media adjusted to different *p*H values (2 to 7). The low *p*H (2.7) is optimum for heterotrophic growth of thiobacillus as it is for autotrophic growth. The test procedures were similar to those used by Smith and Hoare (18) to check for purity of cultures of *Nitrobacter agilis* grown on acetate. Plates were incubated at various temperatures for at least 10 days, and in no instance was contamination (growth) observed. Microscopic examination of the cultures was also used to monitor purity.

Growth rates. Growth rates for cultures grown on sugars or other organic substrates as energy sources were determined by using 250-ml Nephalo flasks fitted with side arms (Bellco Glass Inc., Vineland, N.J.). The flasks contained 20 ml of medium and were shaken at 150 cycles/min at 30 C; turbidity was followed spectrophotometrically at 550 nm in a Spectronic 20 colorimeter (Bausch & Lomb, Inc.), and absorbance was plotted as a function of culture time. The generation time was calculated from the exponential portion of the growth curve and is defined as the time required for absorbance at 550 nm to double. The specific growth constant, k, was determined from the expression $k = \ln 2/(t_2 - t_1)$ where $(t_2 - t_1)$ equals the time interval required for cell doubling (7).

Preparation of cell-free extracts. Cells grown in the media containing ferrous iron plus an organic substrate were harvested at 4 C in a Sorvall RC2-B centrifuge and suspended in a buffer (*p*H 7.9) containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 12 mM β-mercaptoethanol, 50 mM $NaHCO_3$, and 15 mM ethylenediaminetetraacetic acid. Ferric iron precipitate was then removed by centrifuging the suspension at $150 \times g$ for 20 min at 4 C. The cells, contained in the supernatant fluid, were collected by centrifugation at $37,000 \times g$ for 15 min. The cell pellet was suspended in the same buffer, and the washing procedure was repeated three times. Washed cells were stored overnight at 4 C in the buffer and broken in a water-cooled (10 kc/sec) Raytheon sonic oscillator. Sonic treatment for 20 min was needed for breakage of a 20% (w/v) suspension of cells. Residual whole cells and debris were removed by centrifugation at $15,000 \times g$, and the resulting supernatant fluid was used as the crude cell-free extract.

Cell extracts were also prepared from cells harvested at different times during growth. Two milliliters of a 20% cell suspension (w/v) was treated by using a sonifier (Branson Instruments, Inc., Plainview, N.Y.) equipped with a mini-probe. The cell suspension was chilled in an ice bath, and intermittent bursts of energy amounting to a total of 5 min were used to break the cells.

Assays and analytical methods. Ribulose 1,5-diphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] was determined as described by Stukus and DeCicco (19) in a two-step coupled assay essentially. A unit of activity is defined as the number of micromoles of 3-phosphoglyceric acid formed in 1 min as measured by observing the oxidation of reduced nicotinamide adenine dinucleotide at 340 nm. Activity is expressed as units per milligram of protein calculated from the linear portion of a curve plotting enzyme activity versus protein concentration. A Coleman no. 124 recording spectrophotometer, equipped with wavelength expansion from 0 to 0.1 absorbance, was used for the assay.

Glucose-6-phosphate dehydrogenase [D-glucose-6-phosphate:nicotinamide adenine dinucleotide phosphate (NADP) oxidoreductase, EC 1.1.1.49] was determined after the reduction of NADP at 340 nm in the following assay system: 15 mM Tris-hydrochloride (*p*H 7.9), 0.83 mM glucose-6-phosphate, 6.67 mM $MgCl_2$, 0.33 mM $NADP^+$, extract and water to 3.0 ml. The concentrations of substrates were all at least 10 times their K_m. One enzyme unit is defined as the amount of enzyme required to reduce 1 μmole of $NADP^+$ per min, calculated from the extinction coefficient of reduced NADP at 340 nm. Specific activity is expressed as units per milligram of protein.

Protein was estimated from the absorbance at 280 and 260 nm by the method of Warburg and Christian as described by Layne (5).

Ferric iron in the medium was determined colorimetrically by the α,α'-dipyridyl reaction (20). Determinations were made on culture supernatant fluids after removing the cells by centrifugation. All dilutions of the supernatant fluid, prior to assay, were made in acidified distilled water (*p*H 2.5) to minimize autooxidation of residual ferrous ions. Glucose was assayed on the same supernatant fluids by using the Glucostat enzyme reagent (Worthington Biochemical Corp., Freehold, N.J.). Dilutions were made in 10 mM potassium phosphate (*p*H 7.0), and the iron-phosphate precipitate which formed was removed by centrifugation. The supernatant fluid (at *p*H 7.0) was used for the glucose assays.

Ferrous iron oxidation by intact resting cells was determined by the procedure of Schnaitman, Korczynski, and Lundgren (13). In this assay, the intensity of the $FeCl_3$ complex (410 nm) formed is used as a measure of iron oxidation.

All reagents were prepared in glass-distilled water. α,α'-Dipyridyl was purchased from Fisher Chemical Co., Fairlawn. N.J. Ribulose 1,5-diphosphate (barium salt), NADP, glucose-6-phosphate, and other special reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Utilization of glucose by iron-grown cells. Iron-grown cells required at least two transfers in 9KG medium before glucose was utilized as a sole source of energy. Figure 1A and B shows the relationship of iron and glucose utilization by iron-grown cells. Heavy inocula [5 g (wet weight)] were used in these experiments, ensuring that all the ferrous iron was rapidly oxidized and leaving glucose as the sole energy source. Ferrous iron was oxidized to ferric iron by 2 to 3 hr, whereas the glucose level remained constant for over 62 hr. When an inoculum of these cells was transferred to fresh 9KG medium, cells quickly oxidized the iron and, after a lag, oxidized the glucose (Fig. 1B). This observation had been noted previously (9) and was reproducible in this study. The increase in the level of glucose-6-phosphate dehydrogenase, a key enzyme for the dissimilation of glucose, is also shown (Fig. 1B). The utilization of glucose resulted in the accumulation of poly-β-hydroxybutyric acid (PHB) as previously reported (23).

Heterotrophic growth of T. ferrooxidans. *T. ferrooxidans* was cultured on glucose as the sole energy source. Figure 2 shows a typical growth curve relating increase in optical density and glucose utilization with time. Glucose disappeared from the medium by 50 hr, and the cell doubling time was 4.5 hr. Glucose-grown cells, examined under the phase microscope, were found in pairs and contained large amounts of the refractile storage product PHB. Glucose could be replaced in the defined media by several sugars or organic substrates (14). Growth rates of the organism grown on five different types of substrates are compared in Table 1. Fructose appeared to be the preferred substrate with a generation time of 3.4 hr; all other substrates tested gave generation times greater than glucose-grown cells. Cells grown heterotrophically on any of the aforementioned substrates, when transferred back to the 9K medium, oxidized iron slowly. However, if these cells were then transferred again to fresh 9K medium, normal iron oxidation rates were obtained. The reason for the delay in regaining normal iron oxidation rates was not investigated, but it was noted that cells kept on glucose for many transfers generally lost their autotrophic growth capacity.

No absolute requirement for vitamins or cofactors was noted for cells grown in the organic media. However, low concentrations of yeast extract when added to the glucose-salts media markedly stimulated growth. For example, the addition of 0.01% yeast extract gave a specific growth rate constant of 0.263 as opposed to 0.154 in the absence of yeast extract. This corresponds to doubling times of 2.5 and 4.5 hr, re-

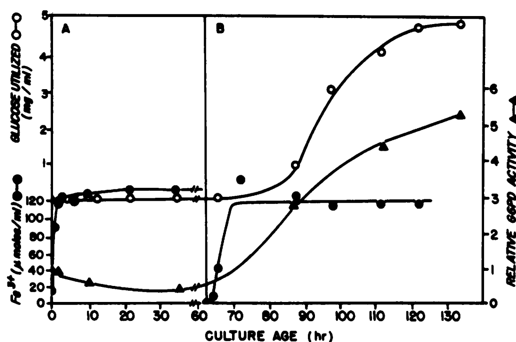

FIG. 1. *Utilization of glucose by iron-grown Thiobacillus ferrooxidans. Iron-grown cells [5 g (wet weight)], suspended in the salts of the 9K medium, were added to 500 ml of the 9KG medium (pH 2.7) contained in a 2-liter Erlenmeyer flask. The flask was incubated at 30 C and shaken at 150 cycles/min. At zero time and intervals thereafter, samples were removed for ferric iron, glucose, and enzyme assays. After 62.5 hr of incubation, 50 ml of medium containing about 0.5 g of cells was placed in a flask containing fresh medium, and the shaking was continued. Samples were removed aseptically and analyzed as before (Fig. 1B). Glucose-6-phosphate dehydrogenase (G6PD) activity (units per milligram of protein) is expressed relative to the resting cell inoculum.*

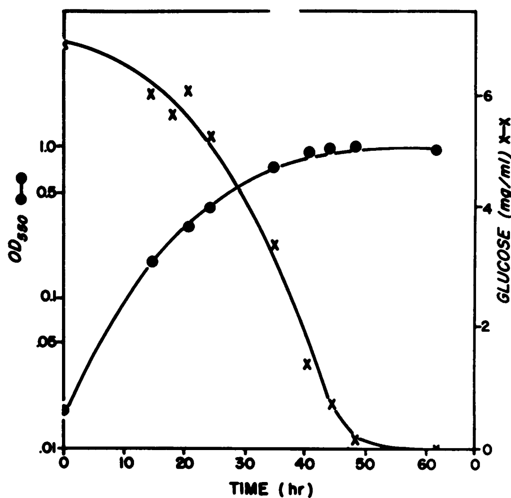

FIG. 2. *Growth of Thiobacillus ferrooxidans in the glucose-salts medium. One milliliter of an actively growing glucose-grown culture served as the inoculum.*

TABLE 1. *Growth of Thiobacillus ferrooxidans on organic substrates*

Growth substrate (0.05%)	Generation time (hr)	Growth rate constant k (hr^{-1})
Glucose	4.5	0.154
Sucrose	6.6	0.105
Fructose	3.4	0.204
Glutamate	7.4	0.094
Gluconate	7.0	0.099
Iron	8.0	0.087
Sulfur[a]	22.3	0.031

[a] Data of Silver (15).

spectively. At high yeast extract concentrations (above 0.25%), a 50-hr growth lag was noted. Glycerol, acetate, pyruvate, and succinate (at a concentration of 0.5%) did not support growth. No other organic substrates have been tested.

Iron oxidation by resting cells. *T. ferrooxidans* was cultured on ferrous iron plus an organic supplement, and the effect of the substrate on the iron-oxidizing capacity was tested by using washed resting cells. Resting cells were prepared from cultures harvested after the primary energy source, iron, was exhausted. At the time of cell harvest (60 to 66 hr), the organic energy source was still being actively metabolized (9), with about 40% of the initial glucose level remaining at the time of harvest. Results of these experiments are shown in Table 2. The rate of iron oxidation by cells previously grown in the various supplement media varied with the organic substrate present. Cells grown in the 9KG medium lost almost 80% of the iron-oxidizing ability (Table 2), whereas iron-fructose-grown cells oxidized iron at a rate nearly comparable to cells grown solely on ferrous iron. These results demonstrate that once organic carbon is oxidized by the cells, the autotrophic energy-generating system is affected; however, the degree depends upon what metabolic reactions are involved.

RuDP carboxylase. The levels of ribulose diphosphate (RuDP) carboxylase in cell-free extracts prepared from cells grown in the various supplemented media are shown in Table 3. Extracts from iron-fructose- and iron-glutamate-grown cells possessed about 70% of the carboxylase activity of autotrophically grown cells. However, both sucrose and glucose almost completely repressed the synthesis of this enzyme. This repression is comparable to that noted in another facultative *Thiobacillus*, namely *T. intermedius* (8). The effect of fructose is similar to that observed in *Hydrogenomonas facilis* (10).

The relationship between iron oxidation, as expressed in resting cells previously grown in a

TABLE 2. *Effect of organic supplement(s) on iron oxidation by resting cells of Thiobacillus ferrooxidans previously grown in the iron-supplemented medium*[a]

Growth substrate(s)	Micromoles of Fe^{3+} formed per min per mg of cell (dry weight)[b]	Percentage of control
9K	0.625	100.0
9K-fructose	0.477	76.3
9K-sucrose	0.224	35.8
9K-glutamate	0.188	30.1
9K-glucose	0.139	22.2

[a] In each case, cells were harvested after 60 hr of growth, washed in β-alanine-sulfate buffer (*p*H 3.6), and suspended in this buffer.

[b] Iron oxidation was determined by using the kinetic assay as described by Schnaitman et al. (13).

TABLE 3. *Ribulose 1,5-diphosphate carboxylase levels in extracts of Thiobacillus ferrooxidans grown in the iron-supplemented media*[a]

Growth substrate[b]	10^{-3} Units/mg	Autotrophic level (%)
Iron	9.88	100.0
Iron plus glucose (60 hr)	1.55	15.7
Iron plus glucose	0.54	5.5
Iron plus sucrose	0.51	5.2
Iron plus fructose	6.62	67.0
Iron plus glutamate	7.17	72.6

[a] All cells were harvested after 66 hr of growth except for one instance (60 hr).

[b] Organic supplements were added to a final concentration of 0.5%.

9KG medium, and carboxylase activity in extracts of these cells is illustrated in Fig. 3. The greatest amount of carboxylase activity (3.5 hr) was noted just after the completion of iron oxidation; thereafter enzyme activity diminished to the level of the cell inoculum (t_0). Washed resting cells, prepared from cells previously grown in the 9KG medium for 1 to 6 hr and then assayed for iron oxidation and enzyme activity, exhibited the highest iron oxidation rate at 1 hr. At this time, the Fe^{3+} ion was actively accumulating in the growth medium. Maximum iron oxidation rates preceded peak RuDP carboxylase activity. These results indicate that the iron oxidation and CO_2 fixation processes, so necessary for autotrophic existence, are related and that the former must precede the maximum expression of carboxylase. The presence of glucose in the medium did not affect the RuDP carboxylase activity or iron oxidation rates, for at the early

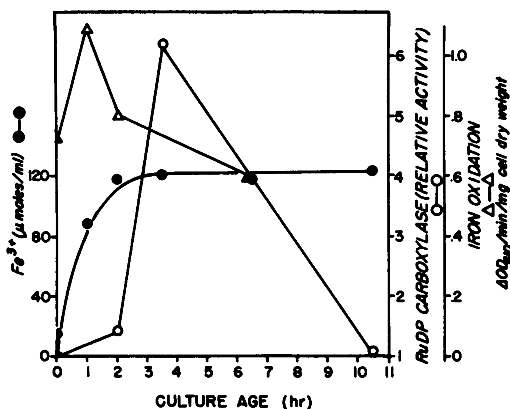

FIG. 3. *Relationship of the rate of iron oxidation by resting cells to ribulose diphosphate (RuDP) carboxylase activity. Cells were grown in 500 ml of the 9KG medium (pH 2.5) by using 5 g of iron-grown cells as an inoculum.* Fe^{3+} *was determined in culture supernatant fluids at the times indicated. For iron oxidation and RuDP carboxylase assays, 9KG-grown cells were harvested and washed as previously described. Iron oxidation by resting cells was assayed spectrophotometrically at 410 nm (3). RuDP carboxylase (units per milligram of protein) is expressed relative to the level of the enzyme found in the cell inoculum (zero time).*

hours of growth only ferrous iron was oxidized. However, once glucose was oxidized, the apparent repression as noted in Table 3 occurred.

DISCUSSION

The results of this study document the ability of *T. ferrooxidans* to grow on glucose as the sole energy source. Shafia and Wilkinson (14) had confirmed an earlier observation from this laboratory of such growth. To grow heterotrophically, the organism must first be cultured in a combination medium containing iron and glucose. Cells prefer iron to glucose when the autotrophic mechanism of the cells is active; Rittenberg (12) suggested that such behavior may indicate that the inorganic substrate provides energy for the initial transport of glucose across the cell membrane. Thus, the observed lag period before glucose is oxidized may represent a period when the cell is synthesizing "transport specific" enzymes such as Enzyme II of the phosphotransferase system of Kundig, Ghosh, and Roseman (4). No experimental evidence is available to support the transferase suggestion. Alternatively (or concurrently) the cell may be synthesizing necessary inducible enzymes for glucose dissimilation, such as glucose-6-phosphate dehydrogenase and related enzymes (Fig. 1). The enzymatic significance of glucose adaptation is the subject of another report (21).

Once adapted to glucose from the 9KG medium, *T. ferrooxidans* was able to grow on glucose as its sole energy source. This situation is similar to that for *T. novellus* and various *Hydrogenomonas* species which grow on simple organic compounds. Yeast extract, when added to the glucose-salts medium, markedly enhanced the growth rate of *T. ferrooxidans*, suggesting a cofactor requirement for maximum growth, such as para-aminobenzoic acid as proposed by Shafia and Wilkinson (14). No studies have been made to establish this.

In contrast to the studies of Shafia and Wilkinson (14) with strain BCU-4, we found that the adaptation to glucose has a profound effect on the constitutive iron oxidation ability of the cells. This effect was also found when other organic compounds were added to the iron-containing media. The degree of inhibition was dependent on the particular organic energy source. There is support for the belief that repression of the autotrophic mechanism is likely, for in these experiments assays were done with washed resting cells in the absence of any organic supplement. London and Rittenberg (8) reported repression of thiosulfate oxidation in *T. intermedius*, as well as a decreased level of RuDP carboxylase in cell-free extracts prepared from cells propagated in a medium containing thiosulfate and an organic supplement. Another *Thiobacillus*, namely *T. novellus*, when grown heterotrophically also has reduced RuDP carboxylase levels (1). A partial repression by acetate for this enzyme has also been noted in *N. agilis* (18), and McFadden and Tu (10) noted a repression of carboxylase activity in *Hydrogenomonas facilis*.

In *T. ferrooxidans*, a reduction of RuDP carboxylase was found in cell-free extracts prepared from iron-glucose-, iron-sucrose-, iron-fructose-, and iron-glutamate-grown cells. There is a relationship between inorganic iron oxidation and RuDP carboxylase activity. Maximum iron oxidation, during growth and by resting cells, precedes maximum RuDP carboxylase activity. This is to be expected since the oxidation of ferrous iron provides energy (adenosine triphosphate) which is then utilized in the reduction of CO_2 via the Calvin cycle. Evidence for the Calvin cycle in *T. ferrooxidans* has been demonstrated (3, 11); RuDP carboxylase is the key enzyme of this pathway (2).

ACKNOWLEDGMENTS

We acknowledge the help of Carl Bodo with the iron oxidation experiments. This work was supported by grant 14010 DAY from the Federal Water Quality Administration, U.S. Department of the Interior.

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