Carbon Metabolism of *Azospirillum brasilense*

Galina V. Vigil

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CARBON METABOLISM OF \textit{AZOSPIRILLUM BRASILENSE}

BY

GALINA V. VIGIL

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science
Major in Microbiology

South Dakota State University
1982
CARBON METABOLISM OF AZOSPIRILLUM BRASILENSE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Carl A. Westby
Thesis Advisor

Robert M. Peng
Head, Microbiology
ACKNOWLEDGEMENTS

I am indebted to Dr. C. Westby for his help in the preparation of this manuscript and for his encouragement in moments of crises. I am also thankful for his friendliness and understanding.

Special thanks to Wilbur for his help and caring.

I am grateful to Kay Lynn for her everlasting smile and the ability of taking "typing abuse".

Thanks to everyone in the Department who had faith in me.

A million thanks to my husband, Rudy, without whom all this would not be possible.
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Azospirillum brasilense is a soil bacterium that fixes nitrogen as a free-living organism or in association with the roots of economically important forage grasses and cereal crops (14). It is widely distributed in tropical and subtropical regions, and occurs to a lesser extent in temperate zones (17,58).

The bacterium was first isolated in 1921 by Beijerinck, who called it *Azotobacter spirillum* and initially claimed it fixed nitrogen (4). Unable to confirm this property he withdrew the claim and renamed the organism *Spirillum lipoferum* on the basis of its vibroid form and preference for organic acid salts as the carbon and energy source (4). Not until 1963 was the organism reisolated by Becking who conclusively demonstrated dinitrogen fixation in pure culture (3).

Interest in this bacterium was stimulated anew after Dobereiner and Day in 1974 isolated it from the roots of some tropical grasses and detected nitrogenase activity in root pieces obtained from the washed roots of *Digitaria decumbens* (14).

The taxonomic status of *Spirillum lipoferum* was recently reexamined by Krieg et al (33) and Tarrand et al (55). Because of important differences among various isolates of *S. lipoferum* the group was subdivided into two new taxons, *A. brasilense* and *A. lipoferum*. The two new species were included in the Approved Lists of Bacterial Names by the International Journal of Systematic Bacteriology in 1980 (50). The new genus is distinguished from *Spirillum* by cell size, relation to O₂ and the G+C ratio. *Spirillum* cells are larger, typically
microaerophilic and have a G+C ratio of 38%. Azospirillum cells, on the other hand, are smaller, can grow under aerobic conditions and have a G+C ratio of 70% (33). Azotobacter is distinguished from Azospirillum by its large ovoid cells, its ability to form thick-walled cysts and the respiratory protection for its nitrogenase (55).

Members of the genus Azospirillum are Gram negative curved rods exhibiting pleomorphism on different media and possessing prominent poly-β-hydroxybutyrate granules (55). They have mainly a respiratory type of metabolism and fix nitrogen only under microaerophilic conditions (55). The mode of motility, a spinning of the cell on its longitudinal axis accompanied by fast movement in various directions is very characteristic of the two recognized species (14,27). The two species differ in their nucleotide sequence as shown by DNA-homology studies (55). A. lipoferum which closely resembles Beijerinck's S. lipoferum can use glucose as the sole carbon source for growth and nitrogen fixation, produces an acidic reaction in peptone-based glucose media, requires biotin, and forms S-shaped or helical cells in semisolid nitrogen-free media (33,55). A. brasilense lacks all of these characteristics except that in older cultures S-shaped forms are also observed (33,55).

The main point of interest in most studies of Azospirillum has been its nitrogen-fixing efficiency. A. brasilense is clearly capable of efficient nitrogen fixation in pure culture as demonstrated by the acetylene-reduction assay (11,14,52). Dobereiner (11) reported up to 50 mg N₂ fixed per g of malate, and Stephan (52) observed 93mg N₂ fixed
per g of lactate. However, the extent to which nitrogen fixation by this bacterium benefits the plant has not been determined (59). It has not yet been possible to demonstrate a statistically significant increase in plant yield with field inoculations of *Azospirillum* (8,59). More studies are necessary to elucidate the relationship between this bacterium and grass roots.

Grasses may derive nitrogen from the atmosphere through the participation of free-living or associated microorganisms by three mechanisms: 1) decaying plant carbonaceous material may support growth on N₂ by free-living bacteria which may in the long run contribute fixed nitrogen to the soil; 2) nitrogen fixation may occur through the release of carbon compounds from living plants into the soil which also would result in a long-term nitrogen enrichment of the soil as above; 3) associative nitrogen fixation occurring in, on, or close to the root may be linked to plant carbon metabolism (e.g. carbon-nitrogen exchange between plant and bacteria). The *Azospirillum*-grass association has been classified in the third category but there is no definite evidence in support of this classification (59).

To understand the nature of bacteria-plant associations both plant and bacterial metabolism studies are important. Research into the biochemistry of photosynthesis has revealed that besides the reductive pentose phosphate cycle (C-3), many grasses also possess the C-4 dicarboxylic acid cycle (6). Grasses possessing this pathway utilize their available nitrogen more efficiently in producing dry matter and fixing atmospheric CO₂ (6). Dobereiner et al (15) suggested that this pathway is important in grasses for their ability to stimulate nitrogen
fixation in rhizosphere bacteria. The general importance of the C-4 dicarboxylic acid pathway in tropical grasses had previously been widely accepted (12,14,39).

Azospirillum spp. are thought to be mainly responsible for the nitrogen fixation that occurs in grasses (39). The preference of these bacteria for malate and other organic acids as the carbon source of choice has been demonstrated in several studies (8,11,43). Malate is the primary product of photosynthesis in some of the C-4 grasses and it has been hypothesized that malate accumulation in the plant leaf leads to its transport into the roots, favoring nitrogen fixation (59).

Child et al (10) demonstrated the induction of nitrogenase by S. lipoferum (presently A. brasilense) and Rhizobium spp. grown in direct association with plant cell tissue culture. They showed that the plant tissue culture supplied TCA cycle intermediates needed to induce nitrogenase in both organisms. The stimulation of nitrogenase activity in S. lipoferum by organic acids has been reported by Okon et al (41). The positive chemotactic response of this bacterium to arabinose, galactose, fructose, malate, pyruvate, succinate, glutamate, leucine, and alanine was recently demonstrated (42). The above compounds are found in plant root exudates (48,60). Among other substances occurring in plant root exudates is lactate which is one of the best growth substrates for A. brasilense (61). Chemotaxis may play a role in the association of this bacterium with the roots of appropriate grasses (42). Kyung et al (35) demonstrated a great increase in root exudation by sorghum seedlings inoculated with A. brasilense and Azotobacter vinelandii. This indicates a close association of these nitrogen fixers
with certain plant roots.

Before the nature of the plant-bacterial association can be understood well the nature of the bacterium itself has to be better understood. Little attention has been paid to metabolic pathways in *A. brasilense* not concerned with nitrogen fixation. Okon et al (43) measured oxygen uptake by cell suspensions and crude cell-free extracts of *S. lipoferum* following the addition of Krebs cycle intermediates, sugar phosphates, and sugars. Their results suggested that the bacterium possesses an operative Krebs cycle, but that glycolytic and pentose phosphate pathways are only weakly functional (43). An oxidative pathway by which L-arabinose is converted to 2-ketoglutarate has been demonstrated in crude cell-free extracts of *A. brasilense* by Novick and Tyler (40).

In the present study I examined carbon metabolic pathways in *A. brasilense* (ATCC 29145) by measuring certain enzyme activities in crude cell-free extracts obtained from cells grown singly on gluconate, glycerol, lactate, and malate as growth substrates. Two mutants CW-1 and CW-2, which grow very poorly on either malate, lactate, or glycerol, were also studied to determine the phenotypic site of mutation, to study regulation of carbon metabolism, and to ascertain the role of carbon metabolism enzymes in *A. brasilense*. 
MATERIALS AND METHODS

Bacterial Strains

*Azospirillum brasilense* Sp 7 (ATCC 29145), the wild type, was obtained from ATCC, Rockville, MD. Mutants CW-1 and CW-2 were obtained from Cutshall and Westby, South Dakota State University. The mutants were isolated on nutrient agar plates following 45 min mutagenesis of Sp 7 with diethyl sulfate at 1.5% and 3.0%, for CW-1 and CW-2, respectively (D. S. Cutshall and C. A. Westby, unpublished data). The mutants unlike the wild type grow very poorly on malate-, lactate-, or glycerol-minimal agar (24-72 h of incubation at 30°C).

All cultures were maintained by weekly transfers on BHI slants. *A. brasilense* Sp 7 cultures were stored at room temperature and the mutants after 17-20 h incubation at 30°C were kept at 4°C to prevent reversion to the wild type.

Media and Cultural Conditions

The synthetic medium used in this study contained the basal salts of Day and Dobereiner (11) plus 2.3g/l NH₄Cl and 5g/l of either potassium D-gluconate, potassium DL-malate, sodium DL-lactate or glycerol. The carbon sources in concentrated aqueous solutions were filter sterilized separately and after the addition of each aseptically to salts (heat sterilized) there was a final pH adjustment to 6.8 with concentrated sterile KOH. These media were used for growing both the inoculum and batch cultures of Sp 7. The mutants were grown on basal salts plus NH₄Cl with gluconate as the carbon source.

Batch cultures were grown in 2-liter Erlenmeyer (wide-mouth)
flasks with 250 ml of medium per flask. In a typical run 4-6 flasks inoculated 1:15 (vol/vol) with log phase cells were incubated at 30°C(±1)C on a reciprocal shaker (64 strokes/min). Growth was followed by measuring the absorbance change with time at 520nm on a Bausch and Lomb Spectronic-20 spectrophotometer, and generation times were calculated from the growth curves (Fig. 1). Cultures were grown to an absorbance of 0.6-1.3, which corresponds approximately to 3-8 x 10^8 colony-forming units/ml (Fig. 2).

**Harvesting and Washing of the Cells**

Cultures were checked for purity by morphological observation under the phase contrast microscope and by streaking them on BHI agar for a colony morphology check. Typical colonies are round to irregular, translucent, mucoid, becoming dry and developing a light pink pigment within a week at 30°C (44). Microscopic observations reveal highly motile, curved, rod-shaped and plump cells, occurring in pairs or singly, and containing refractile granules. Mutants were checked for reversion by streaking on basal salts NH₄Cl agar with malate and checking for growth after 24-96 h.

Cells were harvested by centrifugation at 10,000 x g for 15 min at 4°C; washed twice with chilled physiological saline (250 ml total per culture flask) and resuspended in 1-3 ml of 0.02M Tris-hydrochloride buffer, pH 7.5.

**Crude Extract Preparation**

Washed cells were ruptured by four 30-sec sonic bursts at 0-4°C. The Branson model S-110 sonifier (small probe) was
adjusted to 4 amperes of power output. Debris and residual cells were removed from the sonicate by centrifugation at 39,900 x g for 30 min at 0-4°C. The supernatant was crude cell-free extract. Crude extracts were stored at -60°C (1-3 days) when not assayed immediately.

The protein concentration in the extracts was determined by the Bio-Rad procedure (Bio-Rad Technical Bulletin 1069), with bovine serum albumin as a standard (0.20-1.0 mg/ml). The extracts were diluted with chilled deionized water to obtain protein concentrations in assay mixtures ranging between 0.20-0.70 mg/ml.

**Enzymes**

**General**

All enzyme assays were carried out at room temperature in a Beckman model DU single beam spectrophotometer with a Gilford model-2000 attachment. The final volume of all reaction mixtures was 3.0 ml. An essential substrate defined by the assay procedure was omitted from each blank system and 15-sec readings were taken of both the test and blank reaction mixtures to determine the initial rates of the reactions. When necessary extracts were diluted sufficiently with ice-cold 0.02M Tris-hydrochloride buffer, pH 7.5, to obtain an absorbance change in the reaction mixture not exceeding 0.100 per min at the appropriate wavelength.

One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 micromole of product per min under defined conditions. The following millimolar extinction coefficients were used to calculate enzyme activities: NAD(P) and
NAD(P)H at 340nm, 6.22 (26); NADH at 366nm, 3.4 (1); phosphoenolpyruvate (PEP) at 230nm, 3.0 (26), and 1.75 at 240nm (25); and the formazan product of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction at 550nm, 8.1 (26). Specific activities are expressed as milliunits per mg protein, and represent averages of activities obtained from at least two extracts prepared from separate cell batches. All assays for each extract were performed in triplicate.

**Enzyme Assays**

The following enzymes were assayed according to the referenced procedures: gluconate 2-dehydrogenase (EC 1.1.99.3) (26); phosphogluconate dehydrogenase (EC 1.1.1.44) (57); glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (57); NAD-dependent glyceraldehyde-phosphate (GAP) dehydrogenase (EC 1.2.1.12) and NADP-dependent GAP dehydrogenase (EC 1.2.1.13) (Sigma Technical Bulletin); phosphoglycerate kinase (EC 2.7.2.3) (9); phosphoglycerate mutase (EC 2.7.5.3) (24); enolase (EC 4.2.1.11) (64); fructose-bisphosphate aldolase (EC 4.1.2.13) (26); fructose-bisphosphatase (EC 3.1.3.11) (22); 6-phosphofructokinase (EC 2.7.1.11) (26); glucose-phosphate isomerase (EC 5.3.1.9) (22); glycerokinase (EC 2.7.1.30) (7); lactate dehydrogenase (EC 1.1.1.27), and malate dehydrogenase (EC 1.1.1.37) (Sigma Technical Bulletin No. 340-UV). The combined activity of phospho-2-keto-3-deoxygluconate (PKDG) aldolase (EC 4.1.2.14) and phosphogluconate dehydratase (EC 4.2.1.12) was assayed according to procedures described by Kovachevich and Wood (31,32).

The following enzymes were assayed using a modification of the referenced procedures: gluconokinase (EC 2.7.1.12) (57) and pyruvate
kinase (EC 2.7.1.40) (Worthington Enzyme Manual, 1972). For glucono-kinase the reaction mixture contained 7.5mM ATP instead of 2.5mM and 0.5U phosphogluconate dehydrogenase (EC 1.1.1.44, yeast, type V) instead of 0.1U. For pyruvate kinase the reaction mixture contained 28.3mM imidazole buffer (pH 7.6) instead of 43.3mM, 7.1mM PEP instead of 0.71mM and 3.4U DL-lactic dehydrogenase (EC 1.1.1.27, rabbit muscle, type XI) instead of 0.3-0.5U.

Yeast phosphogluconate dehydrogenase, type V; yeast glucose-6-phosphate dehydrogenase, type XV; and rabbit 6-phosphofructokinase, type III were used as positive controls in the appropriate enzyme assays.

Chemicals and Enzymes

All essential chemicals and enzymes used in the assays were obtained from Sigma Chemical Co., St. Louis, Missouri. They were stored according to Sigma recommendations. BHI agar was from Difco Laboratories, and other chemicals not used in the assays were of reagent grade. A sodium glyceraldehyde-phosphate liquid concentrate used in the GAP dehydrogenase assays was prepared from the diethylacetel barium salt according to a Sigma Technical Bulletin.
RESULTS AND DISCUSSION

Growth Characteristics

The generation times of wild type *A. brasilense* grown on glycerol, lactate, malate, or gluconate as the single carbon source are shown in Table 1. The generation times of the two EMP-mutants grown on gluconate alone are also shown. All cultures were batch grown, aerobically at 30°C, in liquid synthetic medium containing the basal salts of Day and Dobereiner (11), NH₄Cl and the carbon source. Generation times were determined graphically as the time of absorbance.

Table 1. Generation times of *Azospirillum brasilense* Sp 7 (wild type) and two EMP-mutants, CW-1 and CW-2.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Sp 7 (wild type)</th>
<th>CW-1</th>
<th>CW-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>11.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Malate</td>
<td>3.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gluconate</td>
<td>5.2</td>
<td>7.3</td>
<td>7.4</td>
</tr>
</tbody>
</table>

*a Each growth substrate was tested singly at 5g/l in a synthetic medium containing the basal salts of Day and Dobereiner and 2.3 g/l NH₄Cl. Cultures were incubated aerobically at 30°C(+1°C).

*ND is not determined. CW-1 and CW-2 grew very poorly even after 48-72 h incubation at 30°C on 5g/l glycerol-, lactate-, or malate- in the above medium solidified with 15g/l agar (personal communication, D.S. Cutshall and C. A. Westby).
doubling in the exponential growth phase (Fig. 1) (18). Cultures to be used for enzyme studies were harvested at an absorbance of 0.6-1.3 at 520nm which corresponds to 3-4 generations of growth and to approximately 3-8 x 10^8 colony-forming units (CFU) per ml (Fig. 2). Low numbers (approximately 3 x 10^8 CFU/ml) were typical of glycerol-grown wild type cells and the gluconate-grown mutants (Fig. 2, A, E and F). This may be somewhat misleading, however, because these cultures uniquely tended to have large numbers of cell clumps thereby yielding CFUs/ml that were not a close approximation of cells/ml. The CFUs/ml more closely approximated the cells/ml in other cultures.

Observations under the phase contrast microscope typically revealed the presence of large ovoid bodies along with normal single cells and cell clumps in wild type cultures grown on glycerol and in mutant cultures grown on gluconate. Similar structures may have been observed by Eskew et al (20) in cultures of *S. lipoferum* grown on N-free malate basal salts medium. They suggested that these "cystlike" forms are several cells within a single body. The cell clumps that I observed may be related to the large ovoid bodies.

**Metabolic Pathways**

The activities of a number of enzymes involved in the metabolism of gluconate, glycerol, lactate, and malate were measured in crude cell-free extracts prepared from *A. brasilense* cells grown singly on these substrates. The mutants CW-1 and CW-2 were tested for the presence of the same enzymes in extracts obtained from cells grown on gluconate as the sole carbon source.
Fig. 2. Absorbance versus colony-forming units per ml of *A. brasi* Sp 7 and EMP-mutants, CW-1 and CW-2. The conditions are same as those of Fig. 1. The growth substrates are (A) g (B) lactate, (C) malate, and (D) gluconate, for Sp 7; (E) gluconate, for CW-1, and (F) gluconate, for CW-2.
The purpose of the experiments was to determine which carbon metabolism pathways occur in *A. brasilense*, to identify the phenotypic site of mutation in CW-1 and CW-2 and to study the regulation of carbon metabolism in this bacterium.

The enzymes found in this study to be present in *A. brasilense* are arbitrarily labeled as constitutive or inducible (or derepressible). All enzymes whose specific activity in one or more of the extracts was at least 2.5 times that of the basal level (lowest concentration found) are accordingly defined as being inducible (or derepressible), and the enzymes whose specific activity was less than 2.5 times that of the basal level are similarly defined as constitutive (Table 2). It is assumed in the different assay systems that feedback inhibition (37) did not occur and therefore does not account for specific activity differences detected among various of the extracts.

My results indicate that of the carbon metabolism enzymes I tested for in *A. brasilense* only NAD-dependent GAP dehydrogenase (#7)*, phosphoglycerate mutase (#10), and fructose bisphosphatase (#14) are constitutive (Table 2). The other carbon metabolism enzymes found to be present are all inducible (or derepressible) (Table 2).

**Gluconate Metabolism Enzymes**

My results indicate that Sp 7 and the two mutants contain gluconokinase (#1), phosphogluconate dehydratase (#3) and PKDG aldolase (#4) (Table 3). This indicates the presence in *A. brasilense* of a

*Numbers following each enzyme name correspond to those given in figure 3 and tables 2-6.*
Table 2. Inducible (or derepressible) and constitutive enzymes of carbon metabolism in *A. brasiliense*.

<table>
<thead>
<tr>
<th>Enzyme&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inducible&lt;sup&gt;b&lt;/sup&gt; (or derepressible)</th>
<th>Constitutive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconokinase (#1)</td>
<td>Glyceraldehyde-phosphate dehydrogenase (NAD+) (#7)</td>
<td></td>
</tr>
<tr>
<td><strong>&lt;sup&gt;c&lt;/sup&gt;Phosphogluconate dehydratase-PKDG aldolase (#3-4)</strong></td>
<td>Phosphoglycerate mutase (#10)</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-phosphate dehydrogenase (NADP+) (#8)</td>
<td>Fructose bisphosphatase (#14)</td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase (#9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enolase (#11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase (#12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase (#13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosephosphate isomerase (#16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerokinase (#19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase (#20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase (#21)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Enzymes not included in the table are gluconate 2-dehydrogenase (#2), glucose-6-phosphate dehydrogenase (#6), phosphogluconate dehydrogenase (#5), and 6-phosphofructokinase (#15). These were either absent or were present in very small amounts in crude extracts of *A. brasiliense* grown in the tested substrates. Glucokinase (#17) and glucose dehydrogenase (18) were not assayed.

<sup>b</sup>Enzymes whose specific activity in one or more of the test crude extracts was found to be 2.5 times or higher than that of the basal level are considered inducible (or derepressible).

<sup>c</sup>This coupled enzyme system appears to be inducible, however, the inducibility of each separate enzyme was not determined.

<sup>d</sup>This enzyme demonstrates low specific activity only in glycerol grown cells.
functional Entner-Doudoroff (ED) pathway for gluconate catabolism. All strains lack phosphogluconate dehydrogenase (#5) and glucose-6-phosphate dehydrogenase (#6). Yeast phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase were tested as positive controls under the same conditions employed for crude extracts of *A. brasiliense* and showed good activity. The absence of phosphogluconate dehydrogenase (#5) in *A. brasiliense* is particularly noteworthy because it indicates a non-functional hexose monophosphate (HMP) pathway (30).

Gluconokinase (#1) was induced only in gluconate-grown cells while the coupled ED enzyme system was present at different levels in all the extracts tested. The range of these levels, 41.0-121 mU/mg of protein, means that under the guidelines used to define induction that the ED enzyme system (or one of the enzymes) is inducible in this bacterium (Table 3). Induction of the ED pathway enzymes by microorganisms grown in the presence of glucose or gluconate has been reported in a variety of bacteria including *E. coli* (19), *Pseudomonas* spp. (26,46,62), *Azotobacter* spp. (53,54), and *Rhizobium* spp. (30,47). Quay et al (46) have evidence which suggests that gluconate or some derivative is the inducer of gluconokinase (#1) and phosphogluconate dehydratase (#3) in *Pseudomonas* spp. and also possibly in other organisms. Other workers (19,32) have shown that PKDG aldolase (#4) can be induced without the simultaneous induction of phosphogluconate dehydratase (#3). Fradkin and Fraenel (21) suggest that PKDG aldolase (#4) is induced by PKDG itself. They postulate that low non-induced
Table 3. Gluconate pathway enzymes in *A. brasilense* Sp7 and two EMP-mutants, CW-1 and CW-2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth(^a) substrate</th>
<th>Gluconokinase (1)</th>
<th>Gluconate 2-dehydrogenase (2)</th>
<th>Phospho-(^c) gluconate dehydratase PKDG (3–4)</th>
<th>Phospho-gluconate dehydrogenase (5)</th>
<th>Glucose 6-phosphate dehydrogenase (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7 (wild type)</td>
<td>Glycerol</td>
<td>0</td>
<td>0.8</td>
<td>69.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactate</td>
<td>0</td>
<td>1.0</td>
<td>121</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malate</td>
<td>0</td>
<td>2.1</td>
<td>81.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gluconate</td>
<td>19.1</td>
<td>1.2</td>
<td>92.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CW-1</td>
<td>Gluconate</td>
<td>20.4</td>
<td>0.7</td>
<td>41.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CW-2</td>
<td>Gluconate</td>
<td>23.0</td>
<td>0.5</td>
<td>56.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Growth conditions were as described in Table 1.

\(^b\)Enzyme activities in crude cell extracts were measured as described in Methods. One enzyme unit (U) is the amount of enzyme which catalyzes the formation of 1 micromole of product per min under the conditions of the assay. Glucose dehydrogenase (18) was not assayed.

\(^c\)Combined activity of these two enzymes was measured.
levels of PKDG aldolase (#4) are sufficient for catalyzing the formation of a small amount of PKDG from pyruvate and glyceraldehyde-3-phosphate (Fig. 3). The PKDG thus synthesized would provide according to their theory enough substrate for inducing higher levels of the aldolase. This postulate would explain the presumed induction of PKDG aldolase (the dehydratase may be induced instead because the coupled assay was used) in glycerol-, lactate-, and malate-grown cells of *A. brasilense* (Fig. 3). However, it does not account for induction of phosphogluconate dehydratase (#3) in glycerol, lactate or malate-grown cells since glucose-6-phosphate dehydrogenase (#6) is, as previously mentioned, absent and this would prevent the gluconeogenic formation of 6-phosphogluconate, the most likely inducer, from the growth substrates (Fig. 3). The formation of 6-phosphogluconate from PKDG in a reverse reaction can be disregarded because the reaction is irreversible (2). It is possible that phosphogluconate dehydratase (#3) is constitutive in *A. brasilense*, however, in other bacteria so far tested it is inducible (26,46).

The ED pathway has been shown to be the major route of gluconate dissimilation in a number of bacteria (19,26,46) including nitrogen-fixers such as *Azotobacter* spp. (53,54) and *Rhizobium* (30,47). A concurrent pathway of gluconate oxidation, the keto-gluconate (KG) pathway was hypothesized by Keele et al (30) to be important in *Rhizobium japonicum*. In this pathway gluconate is oxidized to 2-ketogluconate which is subsequently oxidized to 2,5-diketogluconate. The latter is, in turn, decarboxylated to a five-carbon compound which
Figure 3. Carbon metabolism of A. brasilense. Enzymes are numbered as follows: 1. gluconokinase; 2. gluconate 2-dehydrogenase; 3. phosphogluconate dehydratase; 4. phospho-2-keto-3-deoxygluconate aldolase; phosphogluconate dehydrogenase; 6. glucose-6-phosphate dehydrogenase; 7. glyceraldehyde-phosphate dehydrogenase (NAD+); 8. glyceraldehyde phosphate dehydrogenase (NADP+); 9. phosphoglycerate kinase; 10. phosphoglycerate mutase; 11. enolase; 12. pyruvate kinase; 13. fructose bisphosphate aldolase; 14. fructose bisphosphatase; 15. 6-phosphofructokinase; 16. glucosephosphate isomerase; 17. glucokinase; 18. glucose dehydrogenase; 19. glycerokinase; 20. lactate dehydrogenase; 21. malate dehydrogenase. Dashed arrows correspond to reactions that are missing in this bacterium or are detectable at only very low levels in all extracts. Unnumbered solid arrows correspond to reactions not tested in the present study. The asterisk(*) at phosphoglycerate kinase indicates the proposed mutation site in CW-1 and CW-2.
is then converted by unknown reactions to 2-ketoglutarate, the Krebs cycle intermediate (Fig. 3). Oxidation of gluconate to the above intermediates was first demonstrated in 1953 in acetic acid bacteria (29) and more recently it was again seen in cowpea rhizobia (Stowers, personal communication). In the present study A. brasilense was not analyzed for the KG pathway, however, very low levels of gluconate 2-dehydrogenase (#2), the first enzyme of this pathway, were consistently and reproducibly found in all extracts (Table 3). The very low levels that were detected were observed in soluble fractions, particulate fractions were not analyzed. Unfortunately, the bulk of the enzyme may have been present in just those particulate fractions and consequently missed in my study. The enzyme is membrane-bound in many other bacteria (23).

The enzymes leading to the formation of 6-phosphogluconate from 2-ketogluconate via 6-phospho-2-ketogluconate (Fig. 3) were not tested in this study, however, this route is found in Pseudomonas spp. (23,62).

Glucose dehydrogenase (#18) was not assayed for in this study, it was assumed to be absent in A. brasilense because the organism does not use glucose for growth (55). An alternate possibility is that the organism has an intracellular glucose dehydrogenase for endogenous glucose but no transport mechanism for moving exogenous glucose through the cell membrane. If this is the case then A. brasilense would be unlike the pseudomonads which have a membrane-bound glucose dehydrogenase (23).
Embden-Meyerhof-Parnas (EMP) Triosephosphate Enzymes

Okon et al (43) suggest from respiratory studies with whole cells and extracts that the glycolytic pathway is only weakly functional in *S. lipoferum*. My results indicate that the triosephosphate enzymes of this pathway are decidedly present in this bacterium (Table 4). Levels of the inducible enzymes (#8, 9, 11 and 12) in induced cells are comparable with those found in other organisms (26, 57). Phosphoglycerate kinase (#9) is induced in high levels in *A. brasilense* cells grown on glycerol and gluconate (Table 4) which indicates that the triosephosphate part of the EMP pathway is catabolically functional in this organism (Fig. 3). The pathway is also most likely used anabolically in lactate and malate grown cells where levels of the kinase are lower (Table 4) but still detectable. NAD-dependent GAP dehydrogenase (#7) is present constitutively in *A. brasilense* (Table 4) in much higher levels than reported in other bacteria (26, 57). This enzyme is one of the key triosephosphate enzymes and its high activity in *A. brasilense* grown on both gluconeogenic and glycolytic substrates is an indication of the importance of this pathway in the carbon metabolism of this bacterium. The NADP-dependent GAP dehydrogenase (#8) will be discussed in a latter section.

EMP Hexosephosphate Enzymes

Among the EMP hexosephosphate enzymes (Table 5), only 6-phosphofructokinase (#15) was not detectable in any of the extracts. This means a catabolic EMP hexosephosphate route is absent (26, 57) in *A. brasilense* (Fig. 3). Rabbit muscle 6-phosphofructokinase was tested
Table 4. EMP tiosephosphate enzymes in A. brasilense Sp7 and two EMP-mutants, CW-1 and CW-2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth substrate</th>
<th>Glyceraldehydephosphate dehydrogenase (NAD+) (7)</th>
<th>Glyceraldehydephosphate dehydrogenase (NADP+) (8)</th>
<th>Phosphoglycerate kinase (9)</th>
<th>Phosphoglycerate mutase (10)</th>
<th>Enolase (11)</th>
<th>Pyruvate kinase (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7 (wild type)</td>
<td>Glycerol</td>
<td>2,223</td>
<td>3.7</td>
<td>107</td>
<td>16.6</td>
<td>38.8</td>
<td>52.8</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>1,573</td>
<td>23.5</td>
<td>61.4</td>
<td>36.3</td>
<td>383</td>
<td>72.0</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>1,262</td>
<td>2.9</td>
<td>66.7</td>
<td>17.4</td>
<td>445</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>2,514</td>
<td>24.0</td>
<td>172</td>
<td>26.5</td>
<td>379</td>
<td>136</td>
</tr>
<tr>
<td>CW-1</td>
<td>Gluconate</td>
<td>1,197</td>
<td>2.7</td>
<td>12.9</td>
<td>16.0</td>
<td>403</td>
<td>14.9</td>
</tr>
<tr>
<td>CW-2</td>
<td>Gluconate</td>
<td>1,146</td>
<td>3.0</td>
<td>19.9</td>
<td>15.2</td>
<td>285</td>
<td>34.0</td>
</tr>
</tbody>
</table>

aGrowth conditions were as described in Table 1.
bMeasurements were as stated in Table 2.
at the same time as a positive control in my studies and showed normal activity, indicating the assay procedure was acceptable. *P. aeruginosa* (26), *Azotobacter* spp. (53), and *Rhizobium* spp. (47, Stowers, personal communication) have also been shown, using essentially the same assay, to lack this enzyme. The presence of fructose bisphosphate aldolase (#13), fructose bisphosphatase (#14) and glucosephosphate isomerase (#16) in all of the *A. brasilense* extracts tested indicates an operational anabolic hexosephosphate EMP route. Fructose bisphosphatase (#14) does not catalyze a reverse reaction ruling out catabolic usage of this pathway (37). The anabolic EMP hexosephosphate route is presumably used by *A. brasilense* for gluconeogenesis when either gluconate or glycerol are the growth substrates (Fig. 3). It is also apparently used with the triosephosphate enzymes for gluconeogenesis when malate or lactate are the growth substrates (Fig. 3).

As mentioned in the beginning of this discussion, enzymes of the HMP pathway were not detected in any of the extracts of *A. brasilense*. Other enzymes for the formation of pentose-phosphate, such as transaldolase and transketolase, were not examined in this study but they are found in other nitrogen-fixing bacteria which also lack the HMP pathway (53, Stowers, personal communication). The anabolic EMP and catabolic ED pathways presumably provide starting substrates (GAP and fructose-6-phosphate) for pentose-phosphate synthesis (Fig. 3).

Some bacteria that lack a catabolic EMP pathway are able to use glucose via glucose dehydrogenase or glucokinase (26,53). The presence of glucose dehydrogenase (#18) in *A. brasilense* is ruled out for reasons
Table 5. EMP hexosephosphate enzymes in *A. brasilense* Sp7 and two EMP-mutants, CW-1 and CW-2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth substrate</th>
<th>Specific activity (mU/mg of protein)</th>
<th>Fructose-bisphosphate aldolase (13)</th>
<th>Fructose-bisphosphatase (14)</th>
<th>6-Phosphofructokinase (15)</th>
<th>Glucose-phosphate isomerase (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7 (wild type)</td>
<td>Glycerol</td>
<td>15.5</td>
<td>28.0</td>
<td>0</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>22.0</td>
<td>29.0</td>
<td>0</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>13.5</td>
<td>20.0</td>
<td>0</td>
<td>77.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>36.0</td>
<td>41.0</td>
<td>0</td>
<td>61.0</td>
<td></td>
</tr>
<tr>
<td>CW-1</td>
<td>Gluconate</td>
<td>12.8</td>
<td>33.0</td>
<td>0</td>
<td>52.5</td>
<td></td>
</tr>
<tr>
<td>CW-2</td>
<td>Gluconate</td>
<td>14.9</td>
<td>35.0</td>
<td>0</td>
<td>73.3</td>
<td></td>
</tr>
</tbody>
</table>

*aGrowth conditions were as described in Table 1.*

*bMeasurements were as stated in Table 2. Glucokinase (17) was not assayed.*
discussed earlier. Glucokinase (#17) was not tested in this study but because the organism does not grow on glucose (55) the enzyme is presumed to be absent. Even if this was not true and the lack of growth on glucose was due to an inadequate glucose uptake system, endogenous glucose could not be catabolized via glucokinase (#17) because the necessary follow-up enzymes, 6-phosphofructokinase (#15) and glucose-6-phosphate dehydrogenase (#6) are lacking in *A. brasilense*. This is evidenced by the results of Tables 3 and 5.

**Utilization of Lactate, Malate, and Glycerol**

Among the four substrates tested in this study, malate and lactate were utilized the most vigorously by *A. brasilense* (Table 1). This is an indication of a functional Krebs cycle (Fig. 3). Lactate dehydrogenase (#20) is induced (or derepressed) in lactate- and malate-grown cells (Table 6). The true inducer of lactate dehydrogenase in bacteria is not known (65), however, synthesis of this enzyme appears to be under catabolite repression control (65). If such a control exists in *A. brasilense* it is tempting to suggest that the catabolite that regulates synthesis of the enzyme is pyruvate or is related to pyruvate. Presumed higher pyruvate levels resulting from gluconate degradation via the ED pathway (each PKDG molecule is converted to two molecules of pyruvate) would then account for the lactate dehydrogenase repression seen in gluconate-grown cells. On the other hand, presumably lower pyruvate levels in malate- or lactate-grown cells (only one molecule formed per molecule of growth substrate) would account for derepression of the enzyme in cells grown on these substrates. It is not clear,
Table 6. Glycerol, lactate and malate utilization enzymes in *A. brasilense* Sp7 and two EMP-mutants, CW-1 and CW-2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth substrate</th>
<th>Specific activity&lt;sup&gt;b&lt;/sup&gt; (mU/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glycerokinase (19)</td>
</tr>
<tr>
<td>Sp7 (wild type)</td>
<td>Glycerol</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>1.1</td>
</tr>
<tr>
<td>CW-1</td>
<td>Gluconate</td>
<td>1.2</td>
</tr>
<tr>
<td>CW-2</td>
<td>Gluconate</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Growth conditions were as described in Table 1.

<sup>b</sup>Measurements were as stated in Table 2.
however, why the enzyme is repressed in glycerol-grown cells if this hypothesis is true.

Malate dehydrogenase (#21) levels were quite high in cells grown on all the substrates (Table 6) with the highest levels observed in wild type gluconate-grown cells. Malate dehydrogenase is known to be inhibited by oxaloacetate and activated by malate (34), however, very little is known about its repression or induction in bacteria. Higher levels of malate dehydrogenase in gluconate-grown cells of _A. brasilense_ may be related to higher levels of pyruvate. Pyruvate is used for acetyl-CoA production and subsequent condensation with oxaloacetate to form citrate. The latter reaction is the rate-limiting step in the Krebs cycle (63), and higher pyruvate concentrations would be expected to drive it forward giving impetus also to the later malate dehydrogenase reaction. This proposed sequence should minimize accumulation of oxaloacetate which could very well derepress malate dehydrogenase. By contrast, lower endogenous pyruvate levels in glycerol-, lactate, and malate-grown wild type cells should not drive this sequence as rapidly resulting in more oxaloacetate accumulation and possible repression of malate dehydrogenase.

Glycerokinase (#19) has been reported to be induced by glycerol in a number of bacteria (38) but growth on this substrate was noted in those studies to be very slow. I observed the same things in _A. brasilense_ (Table 1 and 6). The most likely explanation for these observations relates to the known tendency of bacteria to convert dihydroxyacetone phosphate (DHAP) to methylglyoxal, a bactericidin (38).
Limited substrate induction of glycerokinase (#19) and glycerol-3-phosphate dehydrogenase (DHAP enzyme) which has been documented in a number of bacteria (38) would permit catabolism of glycerol but limit DHAP and subsequently methylglyoxal synthesis, minimizing toxicity.

Markedly lower levels of enolase (#11) were observed in extracts of glycerol-grown cells as compared with levels of this enzyme in cells grown on other substrates (Table 4). It is not clear why this occurs.

**GAP Dehydrogenase and its Role**

NADP-dependent GAP dehydrogenase (#8) was detectable in all of the *A. brasilense* extracts (Table 4), however, NAD-dependent GAP dehydrogenase (#7) was present in much higher levels. The difference was over 100 fold for gluconate-grown cells and over 400 fold for malate-grown cells. This is contrary to the general trend in other bacteria (26, 57). In previous studies in *Pseudomonas* spp. (26, 57) and *Azotobacter* spp. (53) it was shown that the NADP-dependent enzyme catalyzes the reaction at higher rates than the NAD-dependent enzyme. It was also noted in that pseudomonad study that levels of the NADP-dependent enzymes were much higher in glucose-grown cells than in succinate-grown cells (57). A parallel is apparent in *A. brasilense* (Table 4) where levels of this enzyme in gluconate-grown cells are higher than in malate-grown cells. Ruiz-Amil et al (49) suggest that both GAP dehydrogenases are induced by GAP. This control mechanism would explain high levels of the NADP-dependent enzyme observed in gluconate-grown cells of *A. brasilense* since GAP, the inducer, is only three steps removed from gluconate. Lower levels seen in malate-grown
cells can be similarly explained because GAP is five or six steps removed from that substrate. Low levels of the NADP-dependent enzyme in glycerol-grown cells most likely results from limited DHAP synthesis which, as mentioned before, occurs in glycerol-grown bacteria. Since DHAP is the direct precursor of GAP, a limitation of DHAP should also limit GAP. High levels of the NADP-dependent enzyme in lactate-grown cells cannot, however, be explained by the above mechanism.

From the results of my study it is difficult to assess the overall importance of the NADP-dependent enzyme. Quantitatively it appears, however, to be less important than the NAD-dependent enzyme in the catabolism (assay is run in this direction) and possibly also in the anabolism of *A. brasilense*. Results to be discussed below with CW-1 and CW-2 give some indication that the NADP-dependent enzyme may be regulated by repression and that its role is mainly catabolic in *A. brasilense*.

**Mutation Site and its Effect on the Metabolism of CW-1 and CW-2**

All of the enzymes detected in extracts of *A. brasilense* Sp 7 (wild type) were also found in the mutant extracts (Tables 3, 4, 5 and 6). Several enzymes, however, were present at reduced levels, and phosphoglycerate kinase (#9) and pyruvate kinase (#12) in particular were present in considerably lower amounts in the mutants than in the wild type (Table 4). Both the wild type and the mutants were grown on gluconate and the specific activity of phosphoglycerate kinase (#9) was 13 and 8 times lower in CW-1 and CW-2, respectively, than in Sp 7. The specific activity of pyruvate kinase (#12) was 9 and 4 times lower
in CW-1 and CW-2, respectively, than in Sp 7. Mutant cell levels of these two enzymes did not even come close to levels detected in wild type cells grown on any of the other substrates (Table 4).

A double mutation cannot be entirely ruled out without genetic analysis as the cause for the deficiencies in CW-1 and CW-2. Nevertheless, the likelihood of this is very small and the few mutants deficient in the triosephosphate enzymes that have been isolated from other microorganisms have all been shown to be single point mutants (1,28,36). The most likely site of the mutation in CW-1 and CW-2 is on either the phosphoglycerate kinase (#9) or the pyruvate kinase (#12) genes. A mutation in the former, however, would be more consistent with the mutants overall phenotypic properties. For example, both mutants as opposed to Sp 7 do not grow or grow very poorly on lactate and malate and mutants deficient in pyruvate kinase (#12) would not be expected to behave this way because they, like most microbes (23), should still be able to convert oxaloacetate directly to phosphoenolpyruvate (Fig. 3). Mutants blocked in phosphoglycerate kinase (#9), on the other hand, would be expected, as CW-1 and CW-2 do, to behave in exactly this fashion. With regard to the predicted growth behavior of pyruvate kinase (#12) mutants, it has in fact been seen in *Saccharomyces cerevisiae*, where Spargue (51) observed that such mutants grow on lactate as well as the wild type. Growth on lactate is presumably possible via the Krebs cycle and gluconeogenesis without participation of pyruvate kinase.

An alternate possibility with regard to the site of mutation in
CW-1 and CW-2 is that it is located on the pyruvate kinase (#12) gene. The growth characteristics of the mutants on lactate and malate could be explained by a secondary physiological effect of the mutation (not associated with a second mutation) on phosphoglycerate kinase (#9) and other carbon enzymes (see below). Regardless of whether the mutation is on the phosphoglycerate kinase (#9) or pyruvate kinase (#12) gene, it appears to be leaky since none of the deficiencies observed are absolute.

If it is assumed that the mutation is on the phosphoglycerate kinase (#9) gene then an explanation must be given for CW-1 and CW-2 deficiencies in pyruvate kinase (#12) (Table 4), the ED enzymes (#3 and 4) (Table 3), NADP-dependent GAP dehydrogenase (#8) (Table 4), fructose-bisphosphate aldolase (#13) (Table 5), and malate dehydrogenase (#21) (Table 6) in gluconate-grown cells. A possible explanation is that a deficiency in the kinase (#9) in gluconate-grown cells results in accumulation of 3-phosphoglyceroyl-phosphate, which represses the NADP-dependent GAP dehydrogenase (#8), the ED enzymes (#3 and 4) and fructose-bisphosphate aldolase (#13) (Fig. 3). Repression of pyruvate kinase (#12) may be part of a separate mechanism for the maintenance of adequate PEP levels as an important endogenous energy reserve. PEP is used for translocation of sugars and other substrates (PEP: phosphotransferase system) and in various biosynthetic pathways of bacteria (23). Repression of pyruvate kinase (#12) would prevent the excessive drainage of high energy phosphate in PEP which would already be in short supply because of the deficiency in phosphoglycerate
kinase (#9). Lower levels of malate dehydrogenase (#21) in the mutants could be related to lower endogenous levels of pyruvate possibly through the mechanism discussed earlier. Repression of pyruvate kinase (#12) in the mutants should result in lower pyruvate levels. Higher lactate dehydrogenase (#20) levels in the mutants suggest its derepression by low pyruvate levels resulting from the repression of pyruvate kinase (#12).

Usage of the Triosephosphat e Pathway in Carbon Metabolism

From this study it is clear that the triosephosphate pathway plays an important role in *A. brasilense* for the dissimilation and assimilation of various carbon compounds. This pathway for example could theoretically be used by *A. brasilense* for dissimilation of fructose via a PEP: phosphotransferase system, 1-phosphofructokinase, and fructose-bisphosphate aldolase as has been shown in other bacteria (45). In this pathway fructose is phosphorylated to fructose-1,6-bisphosphate by the first two enzymes and the bisphosphate product is then converted to two triosephosphate intermediates by the last enzyme. The triosephosphate pathway in combination with the Krebs cycle and an L-arabinose to 2-ketoglutarate branch described recently by Novick (40) may be used by *A. brasilense* for the gluconeogenesis of L-arabinose. Galactose, which is also used by this bacterium (55) may enter the triosephosphate pathway via the D-tagatose-6-phosphate branch (56). In this branch as has been shown in other bacteria (56), galactose is converted in a series of reactions to DHAP and GAP.
SUMMARY AND CONCLUSIONS

Crude cell-free extracts of *A. brasilense* Sp 7 (wild type) and two EMP-mutants (CW-1 and CW-2) were tested for the presence of carbon metabolism enzymes. Gluconate, glycerol, lactate, and malate were singly used in a basal salts (including NH$_4$Cl) liquid medium for growing the wild type, and gluconate was used in the same medium for growing the mutants. All cultures were grown aerobically at 30°(+1)C.

The conclusions of this study are as follows:

1. The range of generation times of *A. brasilense* Sp 7 grown on gluconate, glycerol, lactate or malate in the above synthetic medium is between 2.9 and 11.2 h. *A. brasilense* grows fastest when lactate is the sole carbon source (2.9 h) and slowest when it is glycerol (11.2 h).

2. *A. brasilense* Sp 7 lacks a catabolic but has an anabolic EMP hexosephosphate pathway and possesses amphibolic triosephosphate enzymes.

3. The hexose monophosphate pathway is not present in this bacterium.

4. An inducible gluconokinase (#1) and ED enzyme system (#3 and 4) are used by this organism for gluconate dissimilation to pyruvate and GAP. The latter is metabolized via the triosephosphate pathway and pyruvate is presumably metabolized by the Krebs cycle. The very low levels of gluconate 2-dehydrogenase (#2) in the soluble fractions which were examined in this study do not necessarily indicate the enzymes' absence in *A. brasilense* because it is membrane-bound in other bacteria.

5. The presence of lactate and malate dehydrogenases (#20 and
21) in *A. brasilense* and the short generation times on these substrates suggest an active Krebs cycle. It is not entirely clear how the two enzymes are regulated in *A. brasilense*, however, it is suggested that lactate dehydrogenase (#20) is subject to catabolite repression and pyruvate or a related compound is the co-repressor. It is also possible that malate dehydrogenase (#21) is a derepressible enzyme in *A. brasilense* and that oxaloacetate is the co-repressor.

6. Glycerokinase (#19) is presumably induced by glycerol, but growth on this substrate is very slow. The slow growth on glycerol is typical of bacteria and is related to limited induction of glycerol kinase and glycerol-3-phosphate dehydrogenase. Glycerol is apparently dissimilated via the triosephosphate and the Krebs cycle enzymes.

7. Glucokinase (#17) and glucose dehydrogenase (#18) are presumed to be absent in *A. brasilense* Sp 7 because this strain does not grow on glucose as the sole energy and carbon source.

8. Most of the enzymes examined in this study are inducible (or derepressible), only NAD-dependent GAP dehydrogenase (#7), phosphoglycerate mutase (#10) and fructose bisphosphatase (#14) are constitutive. The constitutive nature of the two former emphasizes their importance both in the gluconeogenesis of lactate and malate and in the dissimilation of glycerol and gluconate.

9. The role of NADP-dependent GAP dehydrogenase (#8) in the metabolism of *A. brasilense* is uncertain. The possibility exists that this enzyme is regulated by repression and that its role is mainly catabolic.
10. CW-1 and CW-2 appear to be leaky mutants, since none of the deficiencies observed are absolute. The mutation site in CW-1 and CW-2 has been limited to either the phosphoglycerate kinase (#9) or the pyruvate kinase (#12) gene. Genetic analysis, however, needs to be run before a definite conclusion can be made here.

11. The overall phenotypic properties of the mutants are most consistent with a single-site mutation effecting phosphoglycerate kinase (#9). The deficiency in this enzyme explains the mutants' very poor growth on malate and lactate due to impaired gluconeogenesis and slower growth on gluconate presumably because of lower internal pyruvate levels.

12. The deficiency in phosphoglycerate kinase (#9) in the mutants is apparently related to reduced levels of pyruvate kinase (#12), the ED enzymes (#3 and 4), NADP-dependent GAP dehydrogenase (#8), fructosebisphosphate aldolase (#13), and malate dehydrogenase (#21). Higher lactate dehydrogenase (#20) levels in the mutants suggest its derepression by low pyruvate levels resulting from the phosphoglycerate kinase (#9) deficiency.

The triosephosphate pathway is an important over-riding part of carbon metabolism in *A. brasilense*. 
LITERATURE CITED


