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Bradyrhizobium japonicum Does Not Require α-Ketoglutarate Dehydrogenase for Growth on Succinate or Malate

LAURA S. GREEN* AND DAVID W. EMERICH

Department of Biochemistry and Interdisciplinary Plant Group, University of Missouri, Columbia, Missouri 65211

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The *sucA* gene, encoding the E1 component of α-ketoglutarate dehydrogenase, was cloned from *Bradyrhizobium japonicum* USDA110, and its nucleotide sequence was determined. The gene shows a codon usage bias typical of non-*nif* and non-*fix* genes from this bacterium, with 89.1% of the codons being G or C in the third position. A mutant strain of *B. japonicum*, LSG184, was constructed with the *sucA* gene interrupted by a kanamycin resistance marker. LSG184 is devoid of α-ketoglutarate dehydrogenase activity, indicating that there is only one copy of *sucA* in *B. japonicum* and that it is completely inactivated in the mutant. Batch culture experiments on minimal medium revealed that LSG184 grows well on a variety of carbon substrates, including arabinose, malate, succinate, β-hydroxybutyrate, glycerol, formate, and galactose. The *sucA* mutant is not a succinate auxotroph but has a reduced ability to use glutamate as a carbon or nitrogen source and an increased sensitivity to growth inhibition by acetate, relative to the parental strain. Because LSG184 grows well on malate or succinate as its sole carbon source, we conclude that *B. japonicum*, unlike most other bacteria, does not require an intact tricarboxylic acid (TCA) cycle to meet its energy needs when growing on the four-carbon TCA cycle intermediates. Our data support the idea that *B. japonicum* has alternate energy-yielding pathways that could potentially compensate for inhibition of α-ketoglutarate dehydrogenase during symbiotic nitrogen fixation under oxygen-limiting conditions.

Bradyrhizobium japonicum is a gram-negative soil bacterium that forms nitrogen-fixing symbioses with soybean. During the development of the symbiosis, the bacteria induce and invade specialized organs, called nodules, on the root of the host plant (5, 26, 44). The nodules house the bacteria and provide an environment conducive to nitrogen fixation, including a reduced oxygen tension to protect nitrogenase and a steady supply of nutrients to the endosymbiont. Concomitantly with its invasion of the nodule, *B. japonicum* differentiates from a free-living to a symbiotic form (bacteroid), a process involving extensive adjustments to its nitrogen and carbon metabolism (25, 40).

Bacteroid respiration during nitrogen fixation depends entirely on reduced carbon supplied by the host plant. The main carbon compounds used by fully differentiated bacteroids are four-carbon dicarboxylic acids, the metabolism of which has been assumed to occur via the tricarboxylic acid (TCA) cycle (25, 40). However, some studies indicate that the TCA cycle may not be operating to full capacity during bacteroid respiration. For example, the O_2 limitation of bacteroid respiration inside the soybean nodule can lead to increased NADH/NAD ratios and thus to significant inhibition of α-ketoglutarate dehydrogenase (34). Such studies imply that the respiration rate of *B. japonicum* bacteroids may in part be limited by carbon flux through certain steps of the TCA cycle.

To maintain carbon flux under oxygen-limiting conditions, bacteroids could theoretically use alternate metabolic pathways to bypass the inhibited reactions of the TCA cycle. For example, the γ-aminobutyrate (GABA) shunt, whereby α-ketoglutarate is metabolized to glutamate, GABA, and, finally, succinate, can be used by some bacteria to bypass α-ketoglutarate dehydrogenase (Fig. 1) (34). A second pathway, the glyoxylate cycle, can be used to bypass the decarboxylating reactions of the TCA cycle and serves an anapleurotic function necessary for growth of *Escherichia coli* on two-carbon substrates (22). While both the GABA shunt and the glyoxylate cycle have been hypothesized to play a role in *B. japonicum* bacteroid carbon metabolism (23–25, 39, 45), key enzymes, namely, glutamate decarboxylase and isocitrate lyase, may not be present at sufficient activities to support significant carbon flux through these pathways (19, 25, 34, 39).

Our long-term goals are to clarify which metabolic pathways are available for the catabolism of dicarboxylic acid substrates in *B. japonicum* and to understand how their activities are coordinated during this bacterium's differentiation into an effective endosymbiont. To this end, we have cloned and characterized *sucA*, the structural gene for the dehydrogenase (E1) component of *B. japonicum* α-ketoglutarate dehydrogenase, and constructed a strain that is missing this TCA cycle enzyme. This represents the first mutant of *B. japonicum* with a complete block in the TCA cycle and offers a unique opportunity to examine the role of this pathway in *B. japonicum* intermediary metabolism. In this study, we show that the α-ketoglutarate dehydrogenase-deficient mutant grows as well or nearly as well as the wild type on a wide variety of carbon sources but is impaired in its use of glutamate and is inhibited by acetate. In contrast to almost all α-ketoglutarate dehydrogenase mutants described so far, the *B. japonicum* mutant is able to grow well on the TCA cycle intermediates succinate and malate. These results imply the existence of metabolic pathways in free-living *B. japonicum* that can compensate for the loss of α-ketoglutarate dehydrogenase.

MATERIALS AND METHODS

Bacterial strains and media. The strain *B. japonicum* USDA110 was obtained from Art Karr of the University of Missouri, Columbia. This strain was routinely cultured at 28°C, on either rich medium (yeast extract-mannitol [42]) or defined medium (Tully's medium, with 25 mM arabinose as a carbon source [20]). Tully's medium consists of 15 mM $(NH_4)_2SO_4$, 0.7 mM $MgSO_4$, 0.4 mM $CaCl_2$, 20 mM NaCl, 2.5 mM K_2HPO_4, and 40 mM MOPS (3-[*N*-morpholino]propane-sulfonic

* Corresponding author. Mailing address: Department of Biochemistry, 117 Schweitzer Hall, University of Missouri—Columbia, Columbia, MO 65211. Phone: (573) 771-9076. Fax: (573) 882-5635. E-mail: bclgreen@muccmail.missouri.edu.

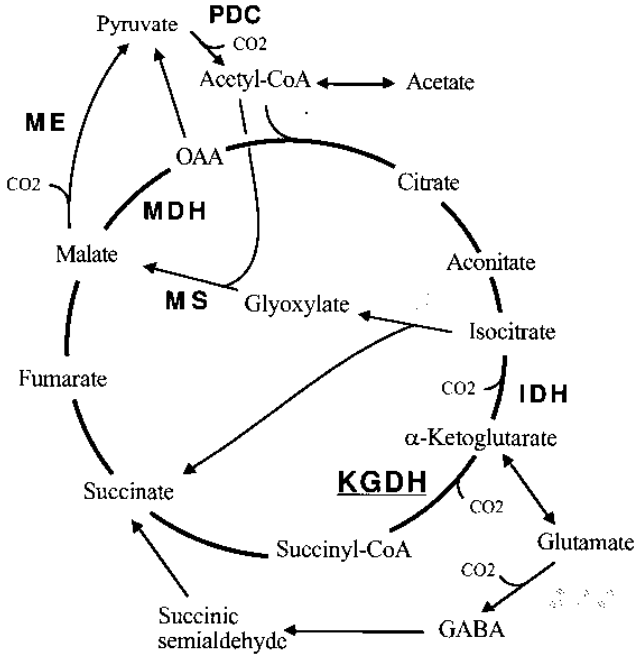

FIG. 1. The TCA cycle and associated pathways. Abbreviations: MDH, malate dehydrogenase; ME, NAD(P) malic enzyme; IDH, isocitrate dehydrogenase; ICL, isocitrate lyase; MS, malate synthase; KGDH, α-ketoglutarate dehydrogenase; GDC, glutamate decarboxylase; OAA, oxaloacetic acid.

acid), pH 6.8, plus micronutrients and a carbon source. Where appropriate, the cultures were supplemented with chloramphenicol (30 μg/ml), kanamycin (100 to 200 μg/ml), and/or tetracycline (36 to 48 μg/ml). For growth under tetracycline selection, $MgSO_4$ was reduced to 0.05 mM in Tully's medium and eliminated entirely from rich medium.

Cloning and sequencing of *sucA*. The *sucA* gene of *B. japonicum* was cloned as part of a cluster of genes encoding TCA cycle enzymes and identified by sequence homology to previously characterized *sucA* genes (42a). Complete sequencing of the gene was accomplished by the dideoxy chain termination method (6, 35), using IBI reagents and protocols. Sequence comparisons were carried out with Geneworks software; the alignments presented here were made with the default parameters for this program.

Transposon mutagenesis of *sucA*. To meet the specific genetic requirements of our mutagenesis strategy, a derivative of pSUP202 (Table 1) (37), pLG51, was created by restricting pSUP202 with *Eco*RI, filling in the cut ends with Klenow fragment, and recircularizing the plasmid; this procedure inactivated the chloramphenicol resistance marker of the parent plasmid. A 6.3-kb *Hin*dIII fragment of the *B. japonicum* genome containing the N-terminal portion of *sucA* was then subcloned from pMDH9.0 (Table 1) into the *Hin*dIII site of pLG51 to form pLG11 and transformed into *E. coli* MC4160 (27). The plasmid was then mutagenized with the transposon Tn*10*-miniKan, delivered via a lambda phage derivative (43). One of the mutagenized plasmids obtained by this method, pLG11::Tn*10*-44, contained an insertion into the N-terminal third of *sucA*. To create a *B. japonicum* strain with an inactivated *sucA*, pLG11::Tn*10*-44 was first transformed into *E. coli* S17.1 and then conjugated into *B. japonicum* USDA110 by the method of Hahn and Hennecke (13). Kanamycin-resistant exconjugates were selected on yeast extract-mannitol plates supplemented with 5 mM succinate, chloramphenicol, and kanamycin, and double recombinants were identified by colony screening (13) using plasmid and kanamycin resistance marker probes. Because of interference from extracellular polysaccharides produced by *B. japonicum* colonies, they could not be screened reliably when grown directly on the hybridization membranes or when transferred to the membranes by conventional means. Therefore, the individual colonies were first grown in a small volume of Tully's medium-arabinose or scraped directly from plates, and then the cells were washed with 0.9% NaCl–0.01% Tween 20 and transferred to the hybridization membrane with a vacuum dot blot apparatus (Bio-Rad).

Southern hybridization was carried out in a sodium dodecyl sulfate-phosphate buffer as previously described (7). Standard procedures were used for plasmid construction, preparation, and analysis.

Enzyme assays. Cultures of *B. japonicum* were grown in Tully's medium-arabinose to mid-log phase. Cells were harvested by centrifugation (6,000 × *g*), washed once with 200 mM NaCl, and resuspended in Breaking Buffer {20 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] [pH 7], 100 mM NaCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 1.5 mM dithiothreitol, 4% [wt/vol] glycerol}. Cells were broken by passing them twice through a French pressure cell at 16,000 lb/in^2. The resulting extracts were clarified by centrifugation (10,000 × *g*) and desalted by dialysis against Breaking Buffer or passage over a Bio-Gel P6 desalting column (Bio-Rad).

α-Ketoglutarate dehydrogenase activity (EC 1.2.4.2) was measured by the method of Reed and Mukherjee (32). Assays were carried out in a reaction buffer that was 50 mM TES (pH 7.5), 1 mM $MgCl_2$, 2 mM NAD^+, 3 mM cysteine, and 0.2 mM cocarboxylase. Cell extract containing 20 to 400 μg of protein was assayed in a total volume of 1 ml, and the reaction was started by the addition of reduced coenzyme A (CoA) (to 0.06 mM) and α-ketoglutarate (to 1 mM). The reaction was monitored by the change in A_{340}. Malate dehydrogenase (EC 1.1.1.37), pyruvate dehydrogenase (EC 1.2.4.1), and isocitrate dehydrogenase (EC 1.1.1.42) activities were assayed as previously described (21). Isocitrate lyase (EC 4.1.3.1) activity was measured by the continuous assay of Dixon and Kornberg (9). Glutamate decarboxylase (EC 4.1.1.15) was assayed by the method of Jin and coworkers (18) in which the GABA formed was determined enzymatically (17). Protein content of the extracts was determined by the method of Bradford (4).

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
S17.1	*thi pro hsd*(r^- m^+)*recA*::RP4-2-Tc^r::mu Km^r::Tn*7* Tp^r Sm^r	37
MC4160	F$^-$ *araD139* Δ(*lacIOPZYA*)U169 *rpsL thiA* Δ*recA*	27
DH5α	Host for plasmid maintenance	14
Bradyrhizobium japonicum		
USDA110	Cm^r	A. Karr
LSG184	110 (*sucA*::Tn*10*-miniKan), Cm^r Km^r	This work
Plasmids and vectors		
pTZ18U	Cloning vector, Ap^r	United States Biochemical
pMDH9.0	pTZ18U containing 9.0-kb *Kpn*I fragment of *B. japonicum* DNA, including part of *sucA*, Ap^r	42a
pSUP202	Ap^r Cm^r Tc^r IncColE1 *mob*	37
pLG51	pSUP202, Cm^r inactivated, Ap^r Tc^r	This work
pLG11	pLG51 containing 6.3-kb *Hin*dIII fragment of pMDH9.0, Ap^r	This work
pLG11-44	pLG11::Tn*10*-miniKan, Ap^r Km^r	This work
pLAFR1	Cosmid vector, Tc^r	12
pLAFR1-*mdh*	pLAFR1 containing 20-kb *Eco*RI partial fragment of *B. japonicum* DNA including *sucA*, Tc^r	This work
λ1105	Suicide vector for delivery of Tn*10*-miniKan	43

Growth measurements. For testing growth on various carbon substrates, *B. japonicum* was first grown to late log phase (A_{630} of 1.0 to 1.5) in Tully's medium-arabinose. Cells were pelleted by centrifugation and washed once with, and then resuspended in, Tully's medium with the carbon source omitted. The cells were then inoculated into the test medium to an A_{630} of 0.05 to 0.1 and returned to culture. Except where noted, carbon substrates were added to a final concentration of 20 mM, with concentrated stock solutions made up in water; neutralized; and filter sterilized before use. Growth was monitored by measuring the A_{630} of 1-ml samples taken at regular intervals. Carbon substrates tested were L-arabinose, succinate, L-malate, glycerol, formate, D-galactose, glyoxylate, L-glutamate, pyruvate, GABA, and D-β-hydroxybutyrate.

Growth on alternate nitrogen substrates was conducted in the same manner except that the cells were washed with and inoculated into Tully's medium with the ammonium sulfate omitted; an alternate nitrogen source was then added to a final concentration of 15 mM. Nitrogen sources tested were $(NH_4)_2SO_4$, K_2NO_3, and L-glutamate.

Nucleotide sequence accession number. The GenBank accession number assigned to the *B. japonicum sucA* nucleotide sequence is U73618.

RESULTS

Characterization of *sucA*. The *sucA* gene of *B. japonicum* was found among a cluster of genes encoding the TCA cycle enzymes malate dehydrogenase and succinyl-CoA synthetase and was identified by sequence homology to previously characterized *sucA* genes (42a). Southern analysis of *B. japonicum* genomic DNA, using the *sucA* gene as a probe and hybridization conditions allowing about 40% mismatch, indicated that there is only one copy of *sucA* in *B. japonicum* (data not shown), the sequence of which was completed for this study. The putative coding region is 2,958 nucleotides long, encoding a protein with a predicted molecular mass of 110,909 Da. The gene displays a codon usage bias similar to that observed for group III (non-*nif* and non-*fix*) *B. japonicum* genes (31); overall, the sequence is 64.5% G+C, with 89.1% of the codons being G or C in the third position. The putative translation product of *B. japonicum sucA* has the indicated overall identities to other *sucA* gene products: *Azotobacter vinelandii* (45% [36]), *E. coli* (44% [8]), and *Bacillus subtilis* (36% [33]). The putative *sucA* gene product of *B. japonicum* contains a well-conserved thiamine pyrophosphate binding domain, found in all known α-ketoglutarate dehydrogenases (30), as well as other highly conserved domains of unknown function (Fig. 2). Single pass sequencing also revealed the N terminus of *sucB*, encoding the E2 component of α-ketoglutarate dehydrogenase, just downstream of *sucA* (data not shown).

Inactivation of *sucA*. The *B. japonicum sucA* gene was inactivated by transposon mutagenesis in *E. coli*, and the interrupted gene (Fig. 3) was used to create a mutant strain of *B. japonicum* by marker exchange. In approximately 5% of the kanamycin-resistant exconjugates obtained, the wild-type genomic copy of *sucA* had been replaced by the insertionally inactivated copy. The genotype of these strains was confirmed by Southern analysis of their genomic DNA (not shown), and since they were all identical, only one, LSG184, was selected for further study. Northern analysis showed that there was no detectable *sucA* or *sucB* transcript in total RNA extracted from LSG184 (data not shown). A positive control transcript from a gene upstream of *sucA*, *mdh*, was present at normal levels in the mutant.

Extracts from the parental strain and LSG184 were assayed for the activities of α-ketoglutarate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, and pyruvate dehydrogenase (Table 2). Wild-type *B. japonicum* had a specific activity for α-ketoglutarate dehydrogenase of 68.5 nmol · min^{-1} · mg of $protein^{-1}$ (Table 2), a value similar to those obtained by other workers (34), and the activity displayed a K_m for α-ketoglutarate of approximately 18 μM (data not shown). A residual level of α-ketoglutarate-dependent dehydrogenase activity

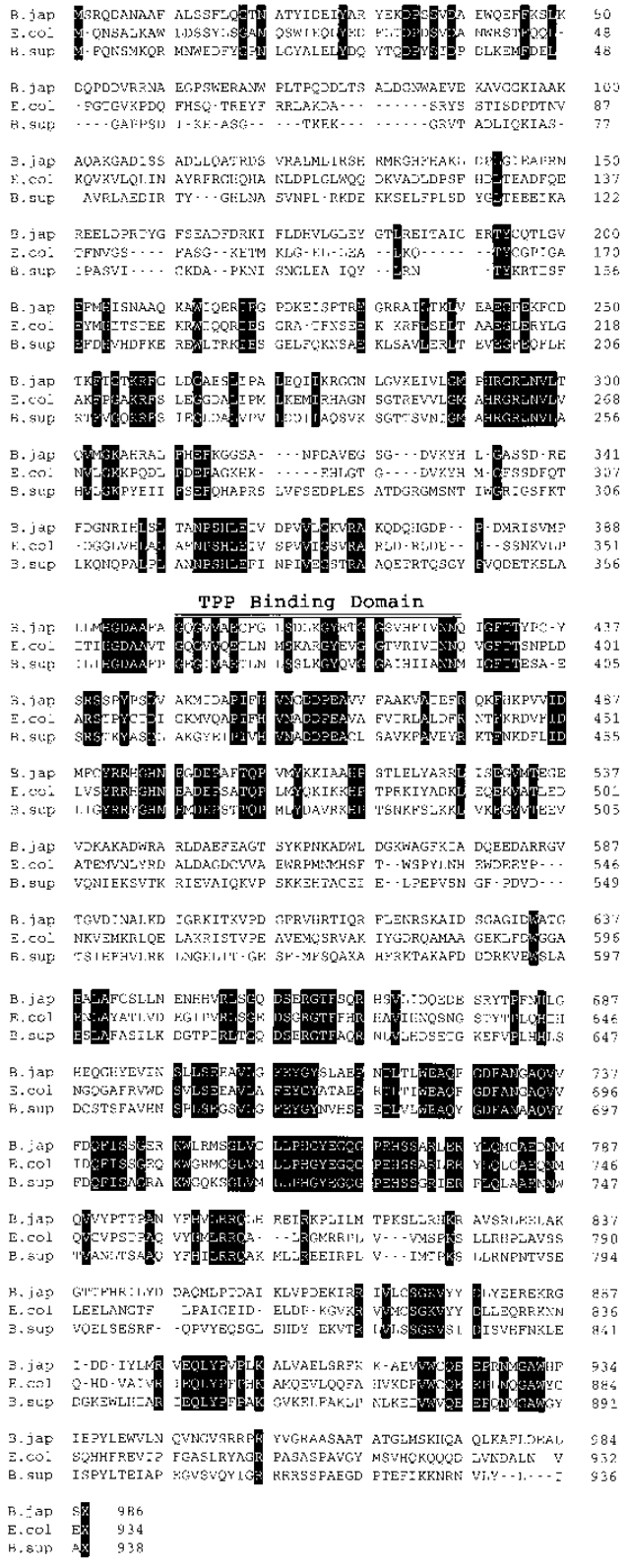

FIG. 2. Comparison of putative translation products of the *sucA* genes of *B. japonicum* (this work), *E. coli* (8), and *B. subtilis* (33). Amino acids that are identical in all three sequences are boxed. The conserved thiamine pyrophosphate (TPP) binding domain is also indicated (30).

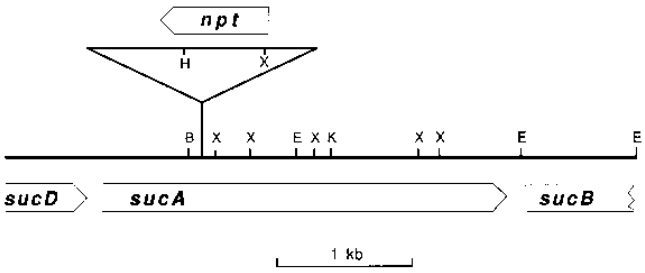

FIG. 3. Physical map of *B. japonicum sucA* interrupted with the transposon Tn*10*-miniKan. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I; K, *Kpn*I.

(amounting to approximately 5% of the total wild-type specific activity) was observed in LSG184. However, this activity was not dependent on CoA and did not display Michaelis-Menten kinetics with respect to the concentration of α-ketoglutarate in the reaction; we conclude that another enzyme or enzymes were responsible for this residual activity. When this CoA-independent activity, which was also observed at a similar level in wild-type extracts, was subtracted out, LSG184 had essentially no α-ketoglutarate dehydrogenase activity (Table 2). In contrast, malate dehydrogenase, isocitrate dehydrogenase, and pyruvate dehydrogenase activities in LSG184 were closer to normal, at 80, 93, and 76%, respectively, of wild-type levels (Table 2).

Growth of the *sucA* mutant on different carbon sources. To see whether the loss of α-ketoglutarate dehydrogenase caused any growth deficiencies in *B. japonicum*, the wild type and LSG184 were tested for their ability to use different carbon sources for growth in batch culture. In most cases, both the wild type and the mutant commenced growth immediately after transfer to the test substrate, without a prolonged lag phase (representative data for the wild type shown in Fig. 4). The exceptions were glutamate and glycerol, on which both strains showed about a 6-h delay before attaining an exponential growth rate. The carbon sources supporting the fastest initial growth of the wild type were the organic acids malate, succinate, and pyruvate (Fig. 5A). However, these growth rates could not be sustained beyond a few generations because, despite the presence of 40 mM MOPS, the pH of the culture rose dramatically. The highest culture densities for the wild type were attained on arabinose, arabinose plus acetate, or arabinose plus pyruvate (Table 3). The latter combination of carbon sources was particularly amenable to the growth of

TABLE 2. Enzyme activities in extracts from various strains of *B. japonicum*

Genotype	Sp act (nmol/min/mg of protein)[a]			
	KGDH	MDH	IDH	PDC
Wild type	68.5 ± 12.2[b]	1,434 ± 264[b]	260[c]	21[c]
LSG184	0.9 ± 0.4[b]	1,154 ± 52[b]	242[c]	16[c]
Wild type/*mdh*[d]	48.6[c]	2,367[c]	ND[e]	ND
LSG184/*mdh*[d]	81.6[c]	2,840[c]	ND	ND

[a] Enzyme abbreviations are as follows: KGDH, α-ketoglutarate dehydrogenase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase.
[b] Values are the means of activities determined for three separate cell extracts ± standard errors.
[c] Values represent the results from a single cell extract.
[d] Strains carrying pLAFR-*mdh*, a complementing cosmid containing the *sucA* and *mdh* region from *B. japonicum.*
[e] ND, not determined.

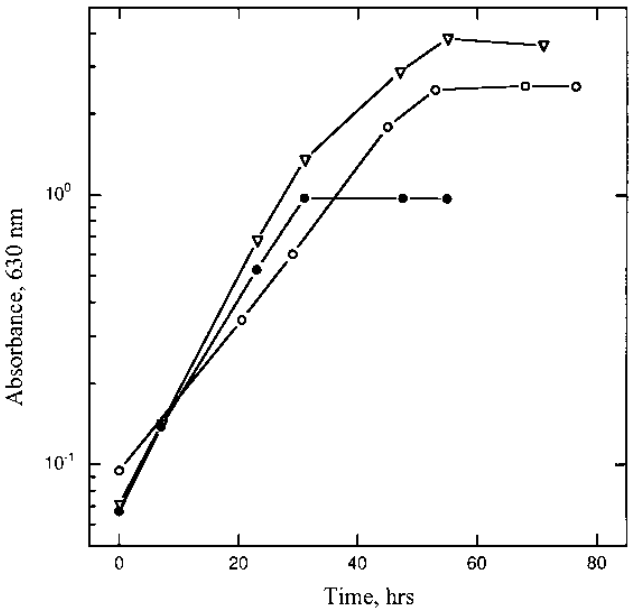

FIG. 4. Growth of *B. japonicum* USDA110 (wild type) on Tully's minimal medium with the following carbon sources: pyruvate (20 mM; closed circles), arabinose (25 mM; open circles), and arabinose plus pyruvate (25 and 20 mM, respectively; open inverted triangles).

wild-type *B. japonicum*, providing for relatively rapid growth (doubling time of 7 h) up to a high cell density (A_{630} of nearly 4.0) in batch culture (Fig. 4 and 5B, "PYR + ARA"). Neither the wild type nor the mutant could grow on GABA or if no carbon was added.

On several of the carbon sources tested, the *sucA* mutant grew as quickly as or only slightly more slowly (up to 12% increase in doubling time) than the wild type (Fig. 5A). These compounds included arabinose, glycerol, formate, galactose, and glyoxylate. Other compounds, including malate, succinate, and β-hydroxybutyrate, supported good growth of the mutant that nevertheless was substantially slower (44 to 59% increase in doubling time) than that of the wild type (Fig. 5A). The mutant was generally able to attain batch culture densities similar to those of the wild type (Table 3) except when acetate was included in the growth medium (see below).

Growth of the *sucA* mutant on acetate and pyruvate. LSG184 grew very slowly compared to the wild type on acetate and pyruvate (Fig. 5B). The mutant also never attained wild-type culture densities when grown on acetate. Arabinose supplementation did not improve the growth rate of the mutant on acetate, indicating that acetate actually inhibited its growth rate under these conditions. At higher concentrations (80 mM), acetate also inhibited the growth rate of wild-type cultures (data not shown). The growth rate of the mutant on acetate was somewhat improved by the addition of succinate (20 mM) (Fig. 5B).

Arabinose supplementation also improved the growth rate of LSG184 growing on pyruvate, but the mutant still grew more slowly under these conditions than on arabinose alone (Fig. 5B). This result indicated that pyruvate, like acetate, inhibited the growth rate of *B. japonicum* lacking α-ketoglutarate dehydrogenase. However, despite having a slower growth rate on pyruvate or pyruvate plus arabinose, the mutant was able to attain culture densities comparable to those of the wild type on these substrates. The addition of succinate to pyruvate-grown cultures led to lysis of both wild-type and LSG184 cells after

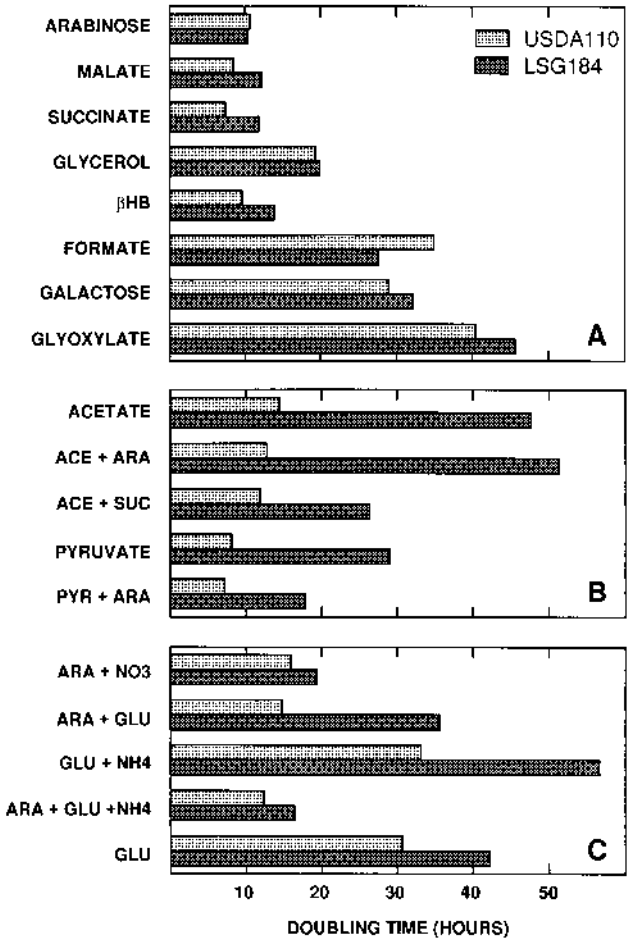

FIG. 5. Doubling times of *B. japonicum* USDA110 (wild type) and LSG184 (*sucA* mutant), grown on Tully's minimal medium with various carbon and/or nitrogen sources. Carbon sources were used at a final concentration of 20 mM except for arabinose, which was used at 25 mM, and nitrogen sources were used at a final concentration of 15 mM. Values are the averages of readings from three cultures; standard errors were all less than 8% of the means. For each culture condition, except for growth on arabinose, differences in doubling times between the wild type and the *sucA* mutant were all significant at at least the 95% confidence level. Abbreviations: βHB, β-hydroxybutyrate; ACE, acetate; ARA, arabinose; SUC, succinate; PYR, pyruvate; NH4, ammonia; NO3, nitrate; GLU, glutamate.

about one generation, accompanied by a large rise in culture pH.

Growth of the *sucA* mutant on alternate nitrogen sources. Because of the position of α-ketoglutarate dehydrogenase at a key branch point between carbon and nitrogen metabolism, we also tested the growth of the *sucA* mutant on different nitrogen sources. The growth rates of the wild type and LSG184 were equal when they were grown on Tully's medium-arabinose with ammonia as the sole nitrogen source (Fig. 5A, "ARABINOSE"). When grown on nitrate in place of the ammonia, the doubling time of both the wild type and LSG184 increased, with the mutant growing somewhat more slowly than the wild type (Fig. 5C, "ARA + NO3"). In contrast, the mutant grew much more slowly than the wild type when it was grown with glutamate as a sole nitrogen source (Fig. 5C, "ARA + GLU").

Glutamate was also a poor carbon source, in terms of the growth rates achieved, for both the wild type and LSG184 (Fig. 5C, "GLU + NH4"), but was not actively inhibitory because supplementation with arabinose restored higher growth rates (Fig. 5C, "ARA + GLU + NH4"). In one growth experiment, we omitted ammonia from the defined medium and forced the cells to use glutamate as their only source of both carbon and nitrogen. The resulting modest growth rates (Fig. 5C, "GLU") confirmed that glutamate is a mediocre carbon source for both the wild type and the *sucA* mutant but also indicated that the slow growth of the LSG184 on glutamate was improved when excess ammonia was omitted.

TABLE 3. Culture densities attained by *B. japonicum* growing on various carbon sources

Carbon source	Final culture density (A_{630})	
	USDA110	LSG184
Arabinose	2.5 ± 0.02[a]	3.0 ± 0.02
Malate	1.5 ± 0.08	1.5 ± 0.02
Succinate	1.5 ± 0.01	1.9 ± 0.02
β-Hydroxybutyrate	1.2 ± 0.02	1.1 ± 0.02
Acetate	0.89 ± 0.003	0.29 ± 0.008
Pyruvate	0.97 ± 0.02	0.80 ± 0.01
Arabinose plus acetate	3.4 ± 0.08	>0.32 ± 0.002[b]
Arabinose plus pyruvate	3.8 ± 0.1	>3.9 ± 0.05[b]

[a] Values are the means of readings for three cultures ± standard errors.
[b] These experiments were ended before the cultures had stopped growing. Values represent the mean culture densities at the end of the experiment.

Complementation of the *sucA* mutant. To determine whether the phenotype of LSG184 resulted entirely from the interruption of *sucA*, we complemented the mutant strain by introducing a wild-type copy of *sucA* carried on the cosmid pLAFR1-*mdh*. Complemented cells regained both their α-ketoglutarate dehydrogenase activity (Table 2) and their ability to grow on 20 mM acetate (data not shown).

Activity of potential bypass pathways in the *sucA* mutant. To test whether LSG184 was inducing the glyoxylate cycle to compensate for the loss of α-ketoglutarate dehydrogenase, we assayed for the presence of isocitrate lyase under various growth conditions. The wild type had no detectable isocitrate lyase activity when grown on arabinose but, when subcultured into Tully's medium-acetate for 24 h, showed specific activities for this enzyme of 45 and 33 nmol · min^{-1} · mg of $protein^{-1}$ in two separate experiments. Extracts from LSG184 had no detectable isocitrate lyase activity whether grown on arabinose or malate. Since the *sucA* mutant grows very poorly on acetate, its ability to induce isocitrate lyase under these conditions was not tested. Consistent with the results of previous workers (34, 39), we could not detect glutamate decarboxylase activity in any of our extracts.

DISCUSSION

In this paper, we report the cloning and sequencing of the *sucA* gene of *B. japonicum*, encoding the dehydrogenase (E1) component of the α-ketoglutarate dehydrogenase complex. The putative translation product of the gene shows high amino acid homology to the predicted products of other bacterial *sucA* genes. The codon usage bias of *B. japonicum sucA* places it in a category distinct from the *nif* and *fix* genes (31), a result consistent with the role of *sucA* in central carbon metabolism. Through transposon mutagenesis and marker exchange, we have constructed a strain of *B. japonicum*, LSG184, in which the *sucA* gene is inactivated. The fact that LSG184 has no residual α-ketoglutarate dehydrogenase activity is consistent with our hybridization experiments (data not shown) indicating that *B. japonicum* has only one copy of the *sucA* gene. This result is in contrast to the phenotype of a *fumC* mutant de-

scribed by Acuna and coworkers (1), which showed that *B. japonicum* has at least two genes coding for the TCA cycle enzyme fumarase.

We were concerned that the interruption of *sucA* might have a polar effect on the transcription of downstream genes and thus that the phenotype of the *sucA* mutant might arise out of more than just the loss of α-ketoglutarate dehydrogenase activity. Indeed, Northern hybridizations of LSG184 RNA show that the insertion of the transposon in *sucA* also eliminates expression of *sucB*; in *B. japonicum* then, as in all other bacteria so far studied, *sucA* and *sucB* appear to be cotranscribed. However, elimination of *sucB* expression is not expected to have any phenotypic effect beyond the loss of α-ketoglutarate dehydrogenase activity already caused by the inactivation of *sucA*.

We do not know what genes lie downstream of *sucB* and might also form part of the *sucA* operon. In *A. vinelandii*, the gene encoding the third component of α-ketoglutarate dehydrogenase, *lpd*, is located just downstream of *sucA* and *sucB* (36). The product of the *lpd* gene also forms part of the pyruvate dehydrogenase complex (PDC). The fact that LSG184 has near-normal levels of PDC activity indicates either that (i) as in many gram-negative bacteria, *lpd* is not located near *sucAB*; (ii) as with *A. vinelandii*, *lpd* is immediately downstream of *sucB* but can be independently transcribed; or (iii) *lpd* is downstream and cotranscribed with *sucAB*, but there is a second copy elsewhere for PDC. The *sucA* mutant was successfully complemented with a 20-kb cosmid clone containing a wild-type copy of *sucAB*, indicating that this strain does not have lesions elsewhere in its genome contributing to its phenotype. However, since the complementing cosmid also carried an extensive stretch of DNA downstream of *sucAB*, the possibility still remains that the insertion in *sucA* has a polar effect on the expression of downstream genes encoding other functions and that these were also complemented by the cosmid pLAFR1-*mdh*.

Growth of the *sucA* mutant on alternate carbon sources. The TCA cycle performs two functions in the cell, energy production and the provision of biosynthetic precursors. The loss of α-ketoglutarate dehydrogenase can affect these functions differentially in bacteria, with the greatest effect generally seen on energy production, especially from carbon substrates requiring catabolism via the TCA cycle. The dual role of α-ketoglutarate dehydrogenase was elegantly demonstrated by the work of Beatty and Gest (2, 3), showing that a mutant of *Rhodopseudomonas capsulata* that was deficient in this enzyme could not grow on succinate, malate, or pyruvate aerobically in the dark. However, when grown photoheterotrophically, with energy derived from light, the mutant was perfectly able to use these substrates for growth. Therefore, the α-ketoglutarate dehydrogenase mutant of *R. capsulata* was blocked for energy production but not for biosynthesis via the TCA cycle.

The inability to use TCA cycle intermediates (or their immediate precursors) as sole carbon sources for growth is a common feature of α-ketoglutarate dehydrogenase mutants. Thus, mutants of *E. coli* (15, 16), *Pseudomonas* strain AM1 (41), *B. subtilis* (11), *Aerobacter aerogenes* (38), *Rhizobium meliloti* (10), and *R. capsulata* (2) were variously unable to grow on acetate, succinate, malate, fumarate, β-hydroxybutyrate, or glutamate, presumably because they weren't able to harvest sufficient energy from these substrates. An exception was an α-ketoglutarate dehydrogenase mutant of *R. meliloti* which, based on colony size on plates, could grow on succinate but not on pyruvate or acetate (10). According to our results, *B. japonicum* is also a striking exception to the rule that α-ketoglutarate dehydrogenase mutants cannot use TCA cycle intermediates for their sole source of carbon and energy. Malate, succinate, and β-hydroxybutyrate all supported good growth rates for LSG184. *B. japonicum*, therefore, does not need a complete TCA cycle to generate sufficient energy from these substrates to support growth.

The ability of the *sucA* mutant of *B. japonicum* to effectively catabolize a variety of carbon compounds, as inferred from its ability to grow on these substrates, implies the existence of some pathway fulfilling the energy production role of the TCA cycle. To test for the presence of the GABA shunt, we grew the wild type and LSG184 on glutamate as a sole carbon source. An inability to grow on glutamate is a common feature of α-ketoglutarate dehydrogenase mutants of bacteria (2, 11, 38). The relatively slow growth of the *sucA* mutant on glutamate, compared to that of the wild type, indicates that the GABA shunt, if present, is not able to compensate completely for the loss of α-ketoglutarate dehydrogenase. Previous workers have been unable to find significant glutamate decarboxylase activity in extracts from wild-type *B. japonicum* (34, 39), nor were we able to detect activity in the *sucA* mutant. However, the fact that the *sucA* mutant grew at all indicates that, unlike *B. subtilis* (11), *A. aerogenes* (38), and *R. capsulata* (2), it has some alternative to the conventional TCA cycle for catabolizing glutamate. Whatever strategy is used by the *B. japonicum* mutant to grow on glutamate seems to be particularly sensitive to the presence of excess ammonia (Fig. 5C, "GLU + NH4").

The presence of a functional glyoxylate cycle in *B. japonicum* has long been debated. Evidence supporting its existence is the well-documented presence of malate synthase (19) and the radiorespirometric data of Stovall and Cole (39) which predicted that up to 50% of the acetyl-CoA in *B. japonicum* bacteroids is metabolized via this enzyme. The fact that USDA110 can grow on acetate also implies the existence of the glyoxylate cycle or another pathway that performs a similar function. However, this enzyme had previously been found only in bacteroids and only at low levels (5 to 7 nmol · min^{-1} · mg of $protein^{-1}$) (24, 45). *B. japonicum* would not grow under the inducing conditions (growth on oleate) used by Johnson and coworkers (19) in their survey of rhizobia for the presence of isocitrate lyase, and they did not assay cells grown on acetate. We found that USDA110 grown on 20 mM acetate as its sole source of carbon had significant isocitrate lyase activity (33 to 45 nmol · min^{-1} · mg of $protein^{-1}$), indicating that, like many bacteria, *B. japonicum* induces the glyoxylate cycle under these conditions. However, we could not detect isocitrate lyase in the *sucA* mutant grown on either arabinose or malate. These results demonstrate that LSG184 does not use the glyoxylate cycle to compensate for the loss of α-ketoglutarate dehydrogenase.

α-Ketoglutarate dehydrogenase mutants of *E. coli* (15) and *A. aerogenes* (38) are succinate auxotrophs under aerobic conditions, indicating that these bacteria are unable to produce enough succinyl-CoA for biosynthetic needs by alternate routes. This is not a general feature of α-ketoglutarate dehydrogenase mutants, as this phenotype was not seen in *B. subtilis* (11), *R. capsulata* (2), *Pseudomonas* strain AM1 (41), or *R. meliloti* (10). Based on our results, *B. japonicum* also falls into this latter group of organisms. The flux of carbon through succinyl-CoA required to support biosynthetic needs is likely to be much lower than that needed for energy production via the TCA cycle (3). Small amounts of succinyl-CoA supplied by running carbon backwards from malate and/or fumarate or as the product of minor pathways or side reactions in the cell are probably sufficient to meet biosynthetic demands in most bacteria.

Acetate sensitivity of the *sucA* mutant. Acetate is oxidized via the TCA cycle in *B. japonicum* (39). It is not surprising, then, that acetate is a poor substrate for growth of the *sucA* mutant of *B. japonicum*. However, the *B. japonicum sucA* mutant failed to grow even when a good carbon source (arabinose) was supplied along with the acetate, indicating that its growth rate was actually inhibited by acetate. A similar, though less dramatic, effect on growth rate was observed when cultures were grown on pyruvate, with the additional difference that the mutant was able to attain wild-type culture densities on pyruvate but not acetate. Surprisingly, β-hydroxybutyrate supported normal growth of the mutant, even though this compound is metabolized via acetate (21).

One way that acetate might cause growth inhibition of a mutant missing α-ketoglutarate dehydrogenase is through unregulated entry of acetate into the TCA cycle and the resulting depletion of four-carbon intermediates (e.g., oxaloacetate). This mechanism is consistent with the fact that acetate was much less inhibitory to cells cultured on succinate. Arabinose may not have been able to support growth of the mutant in the presence of acetate, because it is catalyzed via pyruvate (28) and would be expected, if anything, to increase the internal concentration of acetate. Because LSG184 grew as well as the wild type on formate and β-hydroxybutyrate, the mutant's inability to tolerate acetate is unlikely to be caused by a general sensitivity to weak acids such as that observed in *R. meliloti* (29). Finally, the acetate sensitivity of LSG184 could arise out of a polar effect of the *sucA* insertion on the transcription of a gene downstream of *sucB* which, in turn, has affected some other function in the cell that is necessary for growth in the presence of acetate.

Growth of the *sucA* mutant on alternate nitrogen sources. For a nitrogen source, the *sucA* mutant was able to use ammonia and nitrate nearly as well as the wild type. In contrast, growth of the mutant on glutamate as a sole nitrogen source was much slower than that of normal *B. japonicum*. Removal of the amino group from glutamate for biosynthesis of other nitrogen-containing metabolites will generate α-ketoglutarate, and this byproduct is likely to build up in a mutant that is missing α-ketoglutarate dehydrogenase. Since, in *B. japonicum*, α-ketoglutarate regulates the activity of several enzymes of intermediary metabolism (reviewed in reference 25), a buildup of this metabolite could have complex pleiotropic effects on the growth of *B. japonicum.*

Conclusion. In conclusion, the *sucA* mutant of *B. japonicum* shows a remarkable ability to grow on a variety of carbon substrates, including the TCA cycle intermediates malate and succinate. The main deficiencies displayed by the *sucA* mutant are a reduced ability to use glutamate as a carbon or nitrogen source and an increased sensitivity to growth inhibition by acetate. Our data support the idea that *B. japonicum* has alternate metabolic pathways that could potentially compensate for inhibition of α-ketoglutarate dehydrogenase during symbiotic nitrogen fixation under oxygen-limiting conditions. Indeed, despite abnormalities in early symbiotic development, the *sucA* mutant can form effective bacteroids, capable of fixing nitrogen at normal rates (12a). These results reveal an unexpected metabolic flexibility in *B. japonicum* and, furthermore, that there is still much to learn about the pathways this organism can use for intermediary carbon metabolism.

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