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Lesions in Citrate Synthase That Affect Aerobic Nitrogen Fixation by *Azotobacter chroococcum*

JUAN L. RAMOS† AND ROBERT L. ROBSON*

AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, United Kingdom

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A class of *Azotobacter chroococcum* mutants induced by Tn*1* that were defective in normal aerobic nitrogen fixation when grown on sugars (Fos^-) were corrected by provision of α-ketoglutarate or glutamate. In a representative mutant, Fos252, rates of evolution of $^{14}CO_2$ from [^{14}C]acetate or [^{14}C]glucose were 5% of the parental values, although uptake and incorporation were normal for both substrates. The results suggest that a lesion affects the entry of substrates into the tricarboxylic acid cycle. The activity of citrate synthase in Fos252 in vitro was 5% that of the parents. The citrate synthase (*gltA*) gene from *Escherichia coli* was cloned into broad-host-range vectors and mobilized into Fos252. The plasmids restored parental citrate synthase activities to Fos252 and complemented the inability to fix N_2 in air. The data indicate that a mutation causing an intrinsic limitation in respiratory capacity abolishes normal aerobic N_2 fixation, which is consistent with the hypothesis of respiratory protection for nitrogenase in *Azotobacter* species.

Nitrogenase, the enzyme which catalyzes the reduction of N_2 to NH_4^+, is an extremely oxygen-labile enzyme. However, some obligately aerobic bacteria have the capacity to fix N_2. In the obligately aerobic heterotrophic genus *Azotobacter*, the problem posed by O_2 for N_2 fixation has been countered by at least two protective mechanisms. In respiratory protection, low levels of intracellular O_2 are maintained by the specialized respiratory system found in these organisms (8, 9, 18). Conformational protection involves the formation of an inactive, yet O_2-tolerant, complex between the nitrogenase components (MoFe and Fe proteins) and a third protein which may be unique to azotobacters (13, 21). The complex is probably formed in vivo when respiratory protection is overburdened.

The study of O_2-sensitive mutants of *Azotobacter* species should provide much information about mechanisms of O_2-protection for N_2 fixation. In an earlier study, we described the isolation of a broad group of mutants of *Azotobacter chroococcum* which are defective in aerobic N_2 fixation. They share the common property that the phenotype is expressed when sugars are supplied as the sole carbon source. Generally, the provision of a utilizable carboxylic acid corrected the mutant phenotype. We called these mutants Fos^- (J. L. Ramos and R. L. Robson, J. Gen. Microbiol., in press). In this study, we describe the characterization of a group of such mutants that were corrected with α-ketoglutarate or glutamate. We show that one such mutant is deficient in citrate synthase activity, which leads to a reduced flux through the tricarboxylic acid (TCA) cycle, which is responsible for the failure to fix N_2 in air.

MATERIALS AND METHODS

Organisms and growth conditions. Strains and plasmids used in this work are listed in Table 1. *Azotobacter* strains were routinely cultured aerobically at 30°C on enriched Burk-type medium (RM), or when defined growth conditions were required, a modified Burk medium (18) that contained 0.1 mM Ca^{2+} (AM) was used. RM and AM usually contained sucrose (55 mM) as the sole carbon source and have been described previously (23), but when glucose was supplied in place of sucrose, it was added at 36 mM. Acetate, α-ketoglutarate, or glutamate was supplied at 10 mM. When a combined nitrogen source was required, $(NH_4)_2SO_4$ (5 mM) was added. *Escherichia coli* strains were routinely grown on LB medium at 30°C. Minimal growth medium for strain W620 was that of Vogel and Bonner (27) supplemented with the following (in grams per liter): glucose (1), thiamine (0.005), uracil (0.035), and glutamate (0.34). Antibiotics were added, when appropriate, at the following concentrations for azotobacters (in micrograms per milliliter): nalidixic acid (Nal; 10), streptomycin (Sm; 20), kanamycin (Km; 1), carbenicillin (Cb; 50), tetracycline (Tc; 2). For *E. coli* the concentrations (in micrograms per milliliter) are as follows: Sm (50), Cb (50), Km (20), Tc (10).

Enzyme assays. Nitrogenase activity in vivo was assayed by the C_2H_2 reduction technique. Assays were carried out at 30°C for 1 h in 35-ml conical flasks sealed with rubber stoppers containing 5 ml of cell suspensions (0.1 to 1 mg of protein ml^{-1}) under 10% (vol/vol) C_2H_2 in air. Citrate synthase activity was assayed in cell extracts by the method of Srere (26). One unit (U) of enzyme activity catalyzed the formation of 1 μmol of coenzyme A min^{-1}.

Radioisotope experiments. *Azotobacter* cell suspensions (3 ml; 0.4 to 0.6 mg of cell protein per ml), in a flask with a 25-ml nominal volume provided with a side arm, and sealed with a rubber stopper, were incubated at 30°C with 2 to 5 μCi of [1-^{14}C]glucose (specific activity, 4.1 mCi $mmol^{-1}$; 1.52 GBg $mmol^{-1}$) or [1-^{14}C]acetate (specific activity, 5 mCi $mmol^{-1}$; 1.86 GBg $mmol^{-1}$) for 10 to 15 min. Rates of uptake and assimilation of the labeled substrate were linear for at least 30 min in the conditions described above. Duplicate 1-ml portions were centrifuged at 12,000 rpm for 1 min, washed once with 1.5 ml of 5 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 0.5 $MgCl_2$ (23), suspended in 100 μl of water, and transferred into scintillation vials. $^{14}CO_2$ produced by *Azotobacter* suspensions was collected in 200 μl of 1 M methylbenzethonium hydroxide in methanol which was in the side arm of the flask. The radioactivity in the cells and the CO_2 evolved was measured with a Beckman scintil-

* Corresponding author.
† Present address: Departement de Biochimie Médicale, Centre Médical Universitaire, 1211 Geneva 4 Switzerland.

TABLE 1. Strains and plasmids

Strain and plasmid	Description or genotype	Source or reference
Strains		
E. coli		
W620	*thi-1 pyrD36 gltA6 galK30 rpsL129*	5
HB101	F⁻ *hsdD20* (r_B^-, m_B^-) *recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-6 mtl-1 supE44* λ⁻	6
A. chroococcum		
MCD1	Str^r Nal^r	23
Fos252	Str^r Nal^r Ap^r (Tn*1* derivative of MCD1 which requires α-ketoglutarate for growth on sugars)	Ramos and Robson, in press
Plasmids		
pDB2	Ap^r *gltA* ColE1 replicon	5
pRK2103	Km^r Tra^r	10
pKT230	Km^r Sm^r *mob*$^+$ IncQ	2
pRK2501	Km^r Tc^r *mob*$^+$ IncP	14
pJRL1	Tc^r *gltA mob*$^+$ IncP	This study
pJLR2 and pJLR3	Sm^r Ap^r *gltA mob*$^+$ IncQ	This study
pJLR4 and pJLR5	Km^r Ap^r *gltA mob*$^+$ IncQ	This study

lation counter in 10 ml of Cocktail T Scintran (British Drug House, Poole, United Kingdom) scintillation fluid with a counting efficiency of 50%.

Methods with nucleic acids. Genomic DNA from *A. chroococcum* MCD1 and *E. coli* for Southern blot analysis were prepared from exponentially growing cells as described previously (23). Southern blot analysis and nick translation of DNA were also performed as described previously (13a, 25). Plasmid DNA was prepared by the method of Birnboim and Doly (4). Sizes of DNA fragments were determined by electrophoresis in 0.8% (wt/vol) agarose gels (type II; Sigma Chemical Co., Poole, United Kingdom) in 40 mM Tris acetate buffer containing 1 mM EDTA (16) at 1.5 V cm^{-1} for 14 to 17 h. Fragments obtained by digesting phage lambda DNA with *Hin*dIII or *Hin*dIII-*Eco*RI were used as standards. Ligation of DNA fragments was performed as described by Maniatis et al. (16).

Construction of wide-host-range plasmids carrying *E. coli* citrate synthase. The source of the citrate synthase gene was plasmid pDB2 (5). pDB2 is a pBR322 derivative which carries a 3.24-kilobase (kb) *Hin*dIII-*Eco*RI fragment of *E. coli* DNA containing the citrate synthase gene (*gltA*) (Fig. 1). Since ColE1 derivatives are not stably maintained in *A. chroococcum* we constructed a series of plasmids which carry *E. coli* citrate synthase in wide-host-range mobilizable vectors pRK2501 (14) and pKT230 (2).

pJLR1 is a derivative of pRK2501 which carries the 2.7-kb *Hin*dIII-*Bgl*II fragment of pDB2 inserted into the large *Hin*dIII-*Bgl*II fragment of pRK2501 (Fig. 1). pJLR4 and pJLR5 are cointegrates of pDB2 and pKT230 joined at their unique *Eco*RI sites. pJLR5 is shown in Fig. 1. pJLR2 and pJLR3 are cointegrates of pDB2 and pKT230 joined at their unique *Hin*dIII sites. All pJLR plasmids complemented *E. coli* W620 for growth on minimal medium without glutamate. Citrate synthase activities in strain W620 carrying pJLR1 (3 to 4 U per mg of protein), pJLR2, pJLR3, or pJLR5 (5 to 7 U per mg of protein) compared favorably with those of W620 (pDB2) (7 U per mg of protein) (5). However, W620(pJLR4) showed lower citrate synthase levels (1 U per mg of protein). Repression of citrate synthase activity in *E. coli* by glucose was retained in all pJLR plasmids, as was the case for pDB2 (5).

Introduction of pJLR plasmids into *A. chroococcum*. The pJLR plasmids were introduced into *A. chroococcum* strains by conjugation from the *E. coli* donor in the presence of helper strain *E. coli* HB101(pRK2013). Matings were performed on RM agar with glucose (1% [wt/vol]) at 30°C for 24 h. The frequency of transfer was 10^{-5} to 10^{-6} per recipient for pJLR1 and 10^{-3} to 10^{-4} per recipient for pJRL2 through pJRL5.

Other methods. Measurement of respiration rates of whole organisms was determined in a Clark-type O_2 electrode (Rank Brothers, Bottisham, Cambridge, United Kingdom) as described previously (Ramos and Robson, in press). Growth of organisms under poorly aerated conditions (60

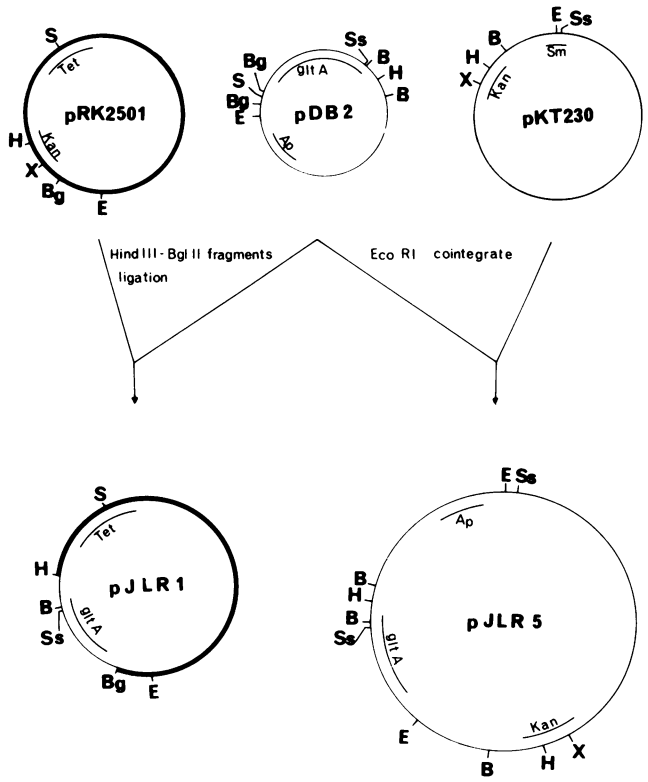

FIG. 1. Construction of broad-host-range plasmids containing *gltA* (citrate synthase) from *E. coli*. pJLR1 (12.7 kb) and pJLR5 (19.5 kb) were constructed from pDB2 (7.6 kb) and pRK2501 (11 kb) or pKT230 (11.9 kb), respectively. Restriction enzyme sites are denoted as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Ss, *Sst*I; X, *Xho*I.

TABLE 2. Influence of pJLR1 and pJLR5 on growth and respiration in *A. chroococcum* MCD1 and Fos252

Strain	Carbon source utilized for growth:									
	Sucrose						Sucrose–α-ketoglutarate			
	Doubling time (h)[a]	Citrate synthase (U mg of protein $^{-1}$)[b]	Respiration characteristics			CO_2 evolution[f]	Doubling time (h)[a]	Respiration characteristics[g]		CO_2 evolution[f]
			V_{max}[c]	$K_s(O_2)$[d]	$K_s(O_2)$[e]			V_{max}	$K_s(O_2)$	
MCD1	3.5	1.00	135	20	7	1.4	2.4	125	5	1.3
MCD1(pJLR1)	3.5	1.30	128	20	7	1.3	ND[h]	138	5	ND
MCD1(pJLR5)	3.8	3.00	142	20	7	1.4	2.4	142	5	1.2
Fos252	10.5	0.05	25	25	25	0.1	3.0	90	7	0.03
Fos252(pJLR1)	4.0	0.85	109	22	9	1.0	ND	130	5	ND
Fos252(pJLR5)	4.1	1.60	112	22	10	1.1	2.6	128	6	1.0

[a] Cells were grown on AM medium with or without 10 mM α-ketoglutarate at 60 rpm in a Gallenkamp orbital incubator at 30°C.
[b] Cells were grown on RM medium.
[c] Respiration rate with sucrose or sucrose plus acetate. Values are given as micromoles of O_2 per milligram of protein per hour.
[d] K_s for O_2 (in micromolar) with sucrose.
[e] K_s for O_2 (in micromolar) with sucrose-acetate.
[f] CO_2 evolved from acetate. Values are given as micromoles of CO_2 protein per hour.
[g] Respiration rates with sucrose–α-ketoglutarate. Values as micromoles of O_2 per milligram of protein per hour for V_{max} and in micromolar for K_s (O_2).
[h] ND, Not determined; values represent average of at least three independent determinations.

rpm in a Gallenkamp orbital incubator) or highly aerated conditions (160 to 200 rpm in the same incubator) was followed turbidimetrically with a Klett-Summerson photoelectric colorimeter with a no. 64 filter (Klett Manufacturing Co., Inc.). Pulse-labeling of proteins in vivo was carried out with $^{35}SO_4^{2-}$ as described by Robson (22). *E. coli* cells were transformed with plasmid DNA essentially as described by Cohen et al. (7). Cell extracts were prepared by disrupting cells by passing them through a French pressure cell at 18,000 lb/in^2. Cell debris was removed by centrifugation at 5,000 × *g* for 15 min. Estimations of cellular protein were done essentially as described by Gornall et al. (12).

Chemicals. 5,5′-Dithiobis(2-nitrobenzoic acid) and methylbenzethonium hydroxide were purchased from Sigma Chemical Co. Cocktail T Scintran was supplied by British Drug House. [1-^{14}C]glucose and [1-^{14}C]acetate were purchased from Amersham International Inc., Amersham, United Kingdom.

RESULTS

Physiological characteristics of Fos252 and MCD1. The isolation of a class of Tn*1*-induced mutants of *A. chroococcum* MCD1 which grew poorly with N_2 on sucrose or glucose, unless α-ketoglutarate was provided, has been described previously (Ramos and Robson, in press). Since glutamate substituted for α-ketoglutarate, these mutants resembled glutamate auxotrophs of *E. coli* that lack citrate synthase (1, 11). We studied this possibility in detail with a representative strain, Fos252, which had only 5 to 10% of the citrate synthase activity as that measured in the parent strain (Table 2).

Lesions affecting the entry of metabolites into the TCA cycle should lead to a decreased oxidation of acetate. We observed that Fos252, unlike the parent strain, did not use acetate for growth with either N_2 or NH_4^+ as nitrogen sources.

When [^{14}C]acetate was added to glucose-grown strains MCD1 and Fos252, both took up acetate at a similar rate (10 to 12 μmol per mg of protein h^{-1} for MCD1 versus 7 to 9 μmol per mg of protein h^{-1} for Fos252). However, the mutant oxidized [^{14}C]acetate to $^{14}CO_2$ at only 5 to 10% of the rate of MCD1 (Table 2). This limitation in the oxidation of [^{14}C]acetate to $^{14}CO_2$ was also observed in the mutant, even under conditions in which it exhibited good growth, e.g., when sugars plus α-ketoglutarate were provided (acetate uptake is constitutive). Similarly, the evolution of $^{14}CO_2$ from [^{14}C]glucose was only 3 to 5% that of the parent, despite little difference in glucose uptake.

Respiration rates of Fos252 with glucose (25 ± 5 μmol of O_2 per mg of protein h^{-1}) were lower than those of the parent strain (135 ± 15 μmol of O_2 per mg of protein h^{-1}) with the same substrate. However, both MCD1 and Fos252 showed similar respiration rates with glucose when α-ketoglutarate was provided (90 ± 10 μmol of O_2 per mg of protein h^{-1} for Fos252 as compared with 125 ± 15 μmol of O_2 per mg of protein h^{-1} for MCD1; Table 2).

These results suggest that the mutation in Fos252 does not substantially lower the rate of entry of glucose or acetate but does affect the rate of oxidation. Since α-ketoglutarate can be respired at high rates, the lesion may be located early in the TCA cycle, which is consistent with the low levels of citrate synthase activity.

The lowered capacity of the TCA cycle affects growth, more markedly in N_2 than in NH_4^+. When sugars were used as the carbon source and NH_4^+ was used as the nitrogen source, Fos252 grew more slowly than did the parent strain under both poorly and highly aerated conditions. It exhibited doubling times of 1.7 to 2 times longer than that of the parent. However, when N_2 was the nitrogen source, the doubling times were three- to fourfold longer than those in the parent strain under poorly aerated conditions (Table 2). Nitrogenase activity of Fos252 (16 to 30 μmol of C_2H_2 per mg of protein h^{-1}) in these cultures was lower than that of MCD1 (60 to 80 μmol of C_2H_4 per mg of protein h^{-1}).

Diazotrophic growth of Fos252 with sugars was prevented in highly aerated cultures. The addition of α-ketoglutarate to these cultures restored growth in N_2. Thus, mutations in the TCA cycle lead to increased O_2 sensitivity of N_2 fixation in *A. chroococcum*. An increase in the O_2 concentration from 1 to 20 ± 2 μM in AM medium led to an 80 to 100% inhibition of nitrogenase activity in the mutant but not the parent (Table 3). This inhibition was not observed when α-ketoglutarate was supplied. Values for nitrogenase activity were 55 and 74 μmol of C_2H_4 per mg of protein h^{-1} at 1 and 20 μM O_2, respectively. No repression of nitrogenase synthesis in Fos252 was observed at 20 μM O_2 in the medium, as determined by $^{35}SO_4^{2-}$ pulse-labeling studies (data not shown). Hence, lesions which lead to a decreased flux

TABLE 3. Influence of the *gltA* gene on nitrogenase activities of MCD1 and Fos252 at different O_2 concentrations[a]

Strain	Nitrogenase activity[b] at the following concn of O_2:	
	1 μM	20 μM
MCD1	56 ± 10	88 ± 22
MCD1(pJLR5)	60 ± 14	80 ± 15
Fos252	23 ± 4	4 ± 4
Fos252(pJLR5)	48 ± 9	64 ± 9

[a] Cultures were maintained in AM medium at 30°C with dissolved oxygen at the given values. Acetylene reduction was followed for 5 min.

[b] Nitrogenase activity is expressed as micromoles of C_2H_4 per milligram of protein per hour. The values given are the average and standard deviation of three experiments.

through the TCA cycle in turn render the mutant susceptible to growth inhibition by O_2 when fixing N_2.

Complementation of Fos252. To confirm that citrate synthase was affected in Fos252, we attempted to clone the *A. chroococcum* wild-type gene for use in complementation studies. We first tried to isolate, from a gene bank of *A. chroococcum*, a clone which might complement *gltA* mutants of *E. coli*. This approach was not successful, although an alternative method was suggested by our finding that a fragment of DNA carrying *E. coli* citrate synthase hybridized to genomic DNA of *A. chroococcum* (Fig. 2). This observation led us to attempt to use the *E. coli* gene in our experiments. Several wide-host-range plasmids carrying *E. coli* citrate synthase were constructed, starting with pDB2 (for details, see Fig. 1 and Materials and Methods). Two of the resultant plasmids, pJLR1 and pJLR5, were introduced into MCD1 and Fos252. Table 1 shows that in MCD1, both plasmids enhanced citrate synthase activity. The increase was greater with pJLR5. This could be a gene dosage effect if the copy number of pJLR5 were greater than that of pJLR1. No significant effects on growth rates, respiration characteristics, or nitrogenase activity were observed (Tables 2 and 3). Both plasmids complemented Fos252 as shown by the following: (i) citrate synthase activity was elevated to the same level as in the parent (Table 2); (ii) growth on acetate was restored and the rate of $^{14}CO_2$ evolution from [^{14}C]acetate was similar to that of the parent strain (Table 2); (iii) respiration rates with sugars were similar to those of MCD1 (Table 2); (iv) growth rates with sugars with N_2 or NH_4^+ under poorly or highly aerated conditions were comparable to those of the parent strain.

Growth in N_2 was aerotolerant in the complemented mutant. This was demonstrated by the following experiments. Cultures of MCD1, MCD1(pJLR5), Fos252, and Fos252(pJLR5) were grown to similar cell densities. Then, the aeration rate of the culture was increased for a given period of time and then restored to the initial rate. Growth was measured at timed intervals during the experiment. Figure 3 shows that the increased agitation caused growth of Fos252 to cease, but not in the case of Fos252(pJLR5) or MCD1 with and without pJRL5. Reimposition of low aeration allowed growth of Fos252 after a short lag time. An increase in the O_2 concentration in the medium from 1 to 20 ± 2 μM in cultures of Fos252(pJLR5) did not result in the switch off of nitrogenase activity in contrast to that seen in Fos252 cultures (Table 3). Inhibition of growth of Fos252

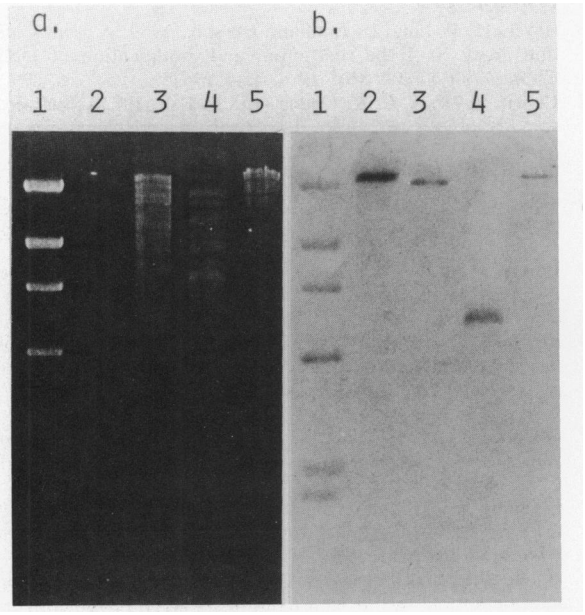

FIG. 2. Hybridization of a DNA fragment containing *E. coli gltA* gene to genomic DNA from *A. chroococcum*. (a) Genomic DNA digested with restriction enzymes, fractionated on an agarose gel, and visualized with ethidium bromide. Lane 2, *E. coli* (JC5466) DNA digested with *Eco*RI; lanes 3, 4, and 5, *A. chroococcum* (MCD1) DNA digested with *Eco*RI, *Bst*EII, and *Hin*dIII, respectively. (b) Autoradiogram of Southern blot of gel as described in (a) after hybridization to the *E. coli gltA* gene contained on the 2.7-kb *Hin*dIII-*Bgl*II fragment from pDB2. Lane 1 contains ^{32}P end-labeled DNA fragments from a *Hin*dIII digest of phage λ DNA.

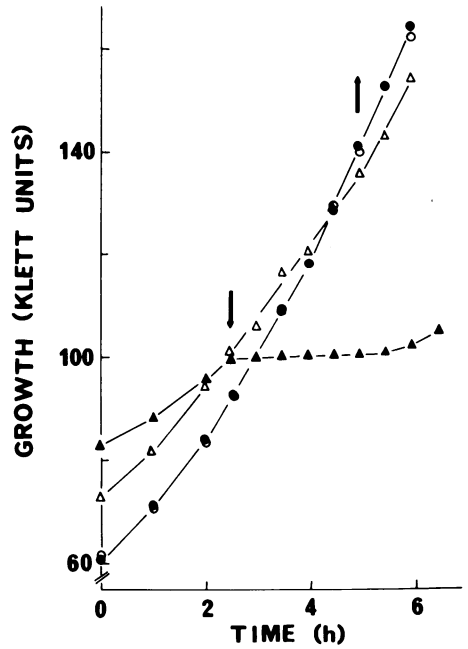

FIG. 3. Influence of the *E. coli gltA* gene in pJLR5 on O_2 tolerance of Fos252 when fixing N_2. Cultures were grown in AM medium in Erlenmeyer flasks fitted with side arms. Cultures were incubated at 30°C under air with shaking at 60 rpm. The first arrow indicates an increase in the shaking rate to 160 rpm. The second arrow indicates the time of restoration of the shaking rate to 60 rpm. Growth was determined turbidimetrically in a Klett-Summerson colorimeter. Cultures were as follows: ▲, Fos252; △, Fos252(pJLR5); ●, MCD1; ○, MCD1(pJLR5).

was not observed when NH_4^+ or α-ketoglutarate was supplied in similar experiments.

We have observed previously (Ramos and Robson, in press) that the addition of acetate to sucrose-grown and sucrose-respiring MCD1 cells decreased the apparent K_s value for O_2 from 20 to 7 μM O_2. The addition of acetate to Fos252 grown on sucrose did not change the apparent K_s value for O_2 nor allow aerotolerant diazotrophic growth. However, the presence of pJLR1 and pJLR5 in Fos252 restored the effect of acetate on the apparent K_s value for O_2 (Table 2). The acetate-induced decrease in the apparent K_s value for O_2 seems to require a fully operative citrate synthase gene product.

DISCUSSION

The mutation in Fos252, which leads to a requirement for α-ketoglutarate or glutamate for good growth, could affect either citrate synthase as in the *gltA* (citrate synthase) mutants of *E. coli* (1, 11) or isocitrate dehydrogenase (15) or both enzymes (3, 15). Complementation of Fos252 by the *gltA* gene from *E. coli* conclusively demonstrates the first alternative to be correct, as was first indicated by the low activities of this enzyme in the mutant. Intergeneric expression of the *E. coli* citrate synthase gene has not been demonstrated before, and the plasmids constructed in this work should be useful for a wide range of gram-negative bacteria.

The location of the Tn*1*-induced mutation in Fos252 is of interest because it does not abolish enzymatic activity completely. As a mutation which completely abolished citrate synthase activity would be expected to be lethal in an obligate aerobe, it is likely that Tn*1* is inserted in such a way as either to lower the expression of the gene or to inactivate only a distal portion of the protein which is relatively unimportant for catalysis. The mutation produces 20-fold lower citrate synthase activity in crude extracts of the mutant, and the rate of oxidation of both glucose and acetate through the TCA cycle is reduced by a comparable amount in vivo. However, that the rate of uptake and incorporation of both substrates was close to that in parents implies that there is no feedback inhibition of uptake and incorporation of these substrates resulting from an accumulation of acetyl coenzyme A or precursor metabolites. The assimilated portion of substrates not oxidized are probably channeled into the formation of poly-β-hydroxybutyrate. This suggests that strains which overproduce this polymer first might be selected by screening for a requirement for glutamate or α-ketoglutarate for good growth.

The lesion led to restricted growth of Fos252, and this was more evident when the nitrogen source was N_2 rather than NH_4^+. This might be explained by an inadequate supply of ATP or reducing power for nitrogenase, or exacerbated O_2 sensitivity. Our data show that the mutant is particularly O_2-sensitive for N_2 fixation, as compared with the parent strain. The mutant was unable to grow in N_2 on solid media, unless it was provided with a microaerobic environment, and it grew in liquid culture only when high inoculum levels or low aeration regimes were used (Ramos and Robson, in press). These data provide direct evidence that an intrinsic limitation in respiratory capacity abolishes normal aerobic nitrogen fixation in *A. chroococcum* and is entirely consistent with the proposal of respiratory protection of nitrogen fixation in the genus (8, 9).

Recent studies of O_2-protective mechanisms for N_2 fixation in *Azotobacter vinelandii* conclude that conformational protection or compartmentalization of nitrogenase play an important role, at least when aeration is extreme (19, 20). It is difficult to envisage how a passive mechanism, as proposed for conformational protection (24), acts in any other way than a fail-safe system. Compartmentalization per se is unlikely to be effective, unless it optimizes the active removal of O_2 by respiration. If conformational protection or compartmentalization are crucial to ongoing N_2 fixation in *Azotobacter* species, then lesions affecting these systems may be found among O_2-sensitive mutants.

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