Isolation and Properties of Mutants of *Azotobacter chroococcum* Defective in Aerobic Nitrogen Fixation

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Mutants of *Azotobacter chroococcum* which showed unusually O₂-sensitive N₂ fixation when grown on sugars were isolated following mutagenesis with nitrosoguanidine or the transposon Tn1. These mutants we called Fos⁻ (inability to fix N₂ on sugars). Aerotolerant growth of Fos⁻ mutants was, in general, restored by carboxylic acids, Ca²⁺ or a combined-N source. Two main groups of mutants were distinguished: (1) regulatory mutants which failed to synthesize nitrogenase under aerobic conditions and which were complemented by the *Klebsiella pneumoniae* *nifA* gene product; (2) mutants affected at different steps in intermediary metabolism. The latter strains exhibited low respiration rates or high apparent K_s(O₂) values (when grown on sucrose) compared with the parent strain. They seemed to be defective in respiratory protection of nitrogenase. The restoration of aerotolerant N₂-dependent growth of Fos⁻ mutants by carboxylic acids was correlated with their ability to induce a decrease in the apparent K_s(O₂) value; however, in both the mutant and the parent strains maximum respiration rates did not change significantly. The Ca²⁺ requirement for diazotrophic growth in *A. chroococcum* seems to be related to the capability of these organisms to fix N₂ in air. Ca²⁺ might be required for high fluxes in the tricarboxylic acid cycle and might act at the level of phosphoenolpyruvate carboxylase, which is involved in the replenishment of the tricarboxylic acid cycle and/or at the level of tricarboxylic acid cycle enzymes *per se*. It is suggested that respiratory protection requires the maintenance of high respiratory rates even at low O₂ tension.

INTRODUCTION

Biological N₂ fixation is an O₂-sensitive process in which nitrogenase, the enzyme responsible for reduction of dinitrogen, is irreversibly damaged by exposure to air. Members of the aerobic genus *Azotobacter* can grow in air with N₂ as a N source as a result of at least two mechanisms which overcome the problem of O₂ in the environment. Nitrogenase in these organisms can exist in an inactive yet O₂-tolerant form known as the conformationally protected state (Dalton & Postgate, 1969*a*). Protection arises from the formation of a complex between nitrogenase component proteins, the MoFe- and the Fe-proteins, and a third protein which may be unique to *Azotobacter* (Haaker & Veeger, 1977; Robson, 1979*a*). Phillips & Johnson (1961) suggested that when these organisms are fixing N₂, nitrogenase is protected by the scavenging of O₂ by respiration, a process which was later confirmed and termed 'respiratory protection' (Dalton & Postgate, 1969*a*; Drozd & Postgate, 1970). When respiratory protection is inadequate, conformational protection stabilizes existing nitrogenase whilst synthesis of nitrogenase *de novo* is repressed (Robson, 1979*b*; Postgate *et al.*, 1981).

Azotobacter can exhibit remarkably high rates of respiration (Burk, 1930), which are achieved through a reduction in energy conservation (Dalton & Postgate, 1969*b*). Reduction in energy conservation is believed to be brought about by the routing of electrons through

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Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

alternative limbs of a branched electron transport chain which are not associated with proton translocating sites (Jones *et al.*, 1973a), and by a decrease in the efficiency of energy coupling at those loops of the electron transport chain which are associated with an energy conservation site (Downs & Jones, 1975; Jones *et al.*, 1973b).

In order to learn more about the mechanism of protection of N₂ fixation against O₂-damage in *Azotobacter* we have isolated mutants of *Azotobacter chroococcum* MCD-1 which are unable to fix N₂ aerobically. The physiological properties of some of these mutants are described.

METHODS

Organisms and growth conditions. *Azotobacter chroococcum* MCD-1 (streptomycin (Sm) and nalidixic acid (Nal) resistant) is a non-aggregating, non-gummy derivative of NCIMB 8003 cured of three out of five indigenous plasmids (Robson *et al.*, 1984). *Escherichia coli* HB101(pGS18) was used for TnI mutagenesis; pGS18 is the transposon TnI suicide vector. It comprises the transfer (Tra) genes from the IncN plasmid pCUI cloned into pACYC184. TnI is inserted at an apparently neutral site. The plasmid encodes carbenicillin (Cb) and chloramphenicol (Cm) resistance and is incapable of replication in *Azotobacter* (Selvaraj & Iyer, 1983). *E. coli* HB101(pCK1, pRK2013) is described elsewhere (Kennedy & Robson, 1983). *Azotobacter* strains were routinely cultured aerobically at 30 °C on an enriched Burk's type medium (RM) or, when defined growth conditions were required, a modified Burk's medium (AM) which contained 0.1 mM-Ca²⁺. RM and AM contain sucrose (55 mM) as the sole C source and are described elsewhere (Robson *et al.*, 1984). When carboxylic acids (usually 10 mM) were required they were supplied from neutralized filter-sterilized 1 M stock solutions. When a source of combined N was required, urea (10 mM), (NH₄)₂SO₄ (2.5 to 5 mM), or CH₃COONH₄ (5 to 10 mM) were used interchangeably. *E. coli* was grown routinely at 30 °C on Luria-Bertani medium. Solidified media contained agar at 1.5% (w/v) unless otherwise stated.

Mutagenesis. For *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis, exponentially growing organisms were harvested (at a density of approx. 10⁸ cells ml⁻¹), resuspended in PEM buffer (Robson *et al.*, 1984) and starved for 5 h by incubation aerobically at 30 °C. The population was then exposed to MNNG (5 to 10 µg ml⁻¹) for 10 min to give 10 to 50% survivors. Organisms were outgrown for 24 to 48 h in AM plus 5 mM-(NH₄)₂SO₄ and the appropriate carboxylic acid, and were then N-starved for 6 to 8 h prior to enrichment in which carbenicillin (10 µg ml⁻¹) and cycloserine (10 µg ml⁻¹) were used to select against unwanted phenotypes under the appropriate conditions. For TnI mutagenesis, *A. chroococcum* MCD-1 was mated with *E. coli* HB101(pGS18) for 24 h at 30 °C on RM agar containing glucose (1%, w/v). Cb^r derivatives of *A. chroococcum* MCD-1 were selected on RM agar containing Sm (20 µg ml⁻¹), Nal (10 µg ml⁻¹) and Cb (50 µg ml⁻¹) at a frequency of 10⁻⁴ recipients. Ninety-nine per cent of the Cb^r colonies were Cm^s, indicating the failure of pGS18 to replicate in *A. chroococcum* MCD-1. This was confirmed by examination of the plasmid profiles of the strains. Three per cent of these isolates were auxotrophic or Nif⁻, indicating transposition of TnI from pGS18. However, 1% of the Cb^r transconjugants were also Cm^r (at 30 µg ml⁻¹) but did not contain pGS18; they may have arisen by replicon fusion between pGS18 and either the chromosome or the indigenous plasmids in *A. chroococcum* MCD-1 (Robson *et al.*, 1984).

Measurement of respiration rates. Respiration was measured in a Clark-type O₂ electrode (Rank Bros, Bottisham, Cambridge, UK) at 30 °C. For measuring respiration of intact organisms, cells were grown in 5 ml AM medium with or without carboxylic acids in 25 ml nominal volume flasks at 80 r.p.m. in a Gallenkamp orbital incubator at 30 °C. Cells (0.2 to 1 ml) were transferred to the chamber of the O₂ electrode, which contained AM medium with the carboxylic acid utilized for growth, to reach a final volume of 3 ml. Respiratory membrane preparations were made by disrupting organisms by passage through a French pressure cell at 18000 lbf in⁻² (124 MPa). They were collected by centrifugation at 140000 g for 2 h after first removing intact organisms and cell debris by centrifugation at 15000 g for 15 min. The membrane-enriched pellet was resuspended in 50 mM-HEPES buffer (pH 7.0) containing 5 mM-MgCl₂. Uptake of O₂ was followed in this buffer with substrates added at saturating concentrations. Values for apparent K_s(O₂) of whole cells or membranes were estimated from the trace obtained with the O₂ electrode. At any given O₂ concentration the rate of O₂ uptake was calculated from the tangent to the trace. The apparent K_s(O₂) is defined as the O₂ concentration at which the respiration rate was half maximal. This definition was used because plots of the reciprocal of the O₂ concentration versus the reciprocal of the respiration rate did not fit statistically a straight line.

Other methods. Growth of organisms was followed turbidimetrically using a Klett-Summerson photoelectric colorimeter with a no. 64 filter (Klett MFG, New York, USA); 100 Klett units was equivalent to 0.7 mg protein ml⁻¹ or 1.4 mg dry weight ml⁻¹. For growth curve experiments, exponentially growing cells (50 to 100 Klett units) were diluted tenfold in 10 ml culture media, transferred to conical flasks (100 ml nominal volume) provided with a side-arm and shaken at 140 r.p.m. in a Gallenkamp orbital incubator at 30 °C. For cellular protein estimation, cells were harvested, resuspended in 3 ml 1 M-NaOH and incubated for 16 to 24 h at 4 °C, after which protein determinations were done essentially as described by Gornall *et al.* (1949), using crystalline bovine serum albumin (Sigma) as a standard.

Table 1. *A. chroococcum* Fos⁻ mutants and their isolation

Mutant no.	Mutagen	Selection
1-188	MNNG	Aerobic growth in N ₂ with sucrose plus acetate, not sucrose alone
201-249	TnI	
249-260	TnI	
189-200	TnI	Failure to grow in N ₂ with pyruvate
250-258	TnI	Aerobic growth in N ₂ with sucrose plus 2-oxoglutarate, not sucrose alone
261-264	TnI	Failure to grow in N ₂ on malate
265-278	TnI	Citrate resistance (5 mM)

RESULTS

Isolation of mutants unusually O₂-sensitive for N₂ fixation: the Fos⁻ phenotype

Approximately 50% of mutants of *A. chroococcum* MCD-1 which were unable to grow diazotrophically on AM behaved in an unstable manner. When patched or replica-plated they were able to grow with N₂ in air, which suggested that inoculation at relatively high cell density afforded mutual protection. That this phenotype was due to unusual O₂ sensitivity when fixing N₂ in air was demonstrated in two ways. Organisms were inoculated to a density of approximately 10² colony forming units (c.f.u.) ml⁻¹ into molten AM containing agar at 0.5% (w/v), which was then distributed into test tubes. The tubes were incubated for 3 d at 30 °C. With the parent strain, colonies were observed at the agar-air interface, but with the mutants, colonies were observed in a zone starting approximately 2 to 3 mm below the agar surface. When NH₄⁺ was supplied, growth was observed at the interface in all strains. In a second approach, organisms were spread on AM agar plates at a density of approximately 10² c.f.u. per plate, and were incubated at 30 °C in air or in gas jars under an atmosphere of 0.2 to 1% (v/v) O₂ in N₂. Colony sizes of parent strain and mutants under low O₂ tension were similar, but in air, only the parent strain gave rise to colonies. When NH₄⁺ was supplied the mutants were able to form colonies in air. When acetate was supplied as an additional C source the apparent O₂ sensitivity of the mutants was 'corrected'. We call this phenotype Fos⁻ (inability to fix N₂ on sugars). In general, other utilizable carboxylic acids or ethanol would correct Fos⁻ strains. We exploited this feature to isolate further Fos⁻ strains; different carboxylic acid substrates gave rise to different classes of Fos⁻ strains (Table 1). Citrate is not usually utilized by azotobacters (see Thompson & Skerman, 1979) and at 2 mM it inhibited the growth of *A. chroococcum* MCD-1 on AM media. An unexpected observation was that most Fos⁻ mutants resisted up to 5 mM-citrate, thus providing a method for enriching Fos⁻ mutants by first selecting for citrate resistant (Cit^r) colonies. Approximately 10% of Cit^r clones showed aerosensitive diazotrophy. Following MNNG or TnI mutagenesis 278 independent mutants were isolated. The various groups of mutants are listed in Table 1.

Physiological properties of Fos⁻ mutants

We attempted to group the mutants according to various attributes such as growth rate, respiration characteristics, etc. Many of these characteristics would best be quantified in chemostat culture; however, with so many strains, this was not feasible and so batch culture methods were used throughout this study.

Growth of Fos⁻ mutants in liquid culture. In AM medium containing a combined-N source, most mutants had growth rates (doubling times 2.8 to 3.4 h) similar to the parent strain (2.8 h). However, some (e.g. Fos 250 and Fos 270) grew more slowly (doubling times 5 to 8 h), and two mutants, Fos 118 and Fos 260, grew significantly faster (2.3 to 2.7 h). Diazotrophic growth of the Fos⁻ mutants was only obtained provided a large inoculum (e.g. 50 to 100 Klett units) was used and the cultures were not aerated vigorously. Growth rates (doubling times 4 to 15 h) were always slower than the parent strain (3.5 h) under comparable conditions (Table 2). Cell yields, as determined by turbidity, were 40 to 80% of that of the parent strain.

Table 2. *Growth characteristics of A. chroococcum MCD-1 and Fos⁻ strains under N₂-fixing conditions with sucrose alone and sucrose plus carboxylic acids*

Sucrose-grown cells were diluted tenfold in medium supplemented with the indicated carboxylic acids to reach a cell density of 5 to 15 Klett units. The values are means of at least four independent determinations. SD for doubling times and lag periods were in the range of 10 to 20% and 10 to 30% of the given values, respectively.

Strain	Lag (h) with sucrose alone	Doubling time (h) with sucrose alone	Doubling time (h) with sucrose plus:			
			Acetate	Malate	Pyruvate	2-Oxoglutarate
MCD-1	0	3.5	2.3	2.0	2.3	2.2
Fos 35	3.4	7.8	3.3	3.1	3.5	3.9
Fos 39	3.2	6.3	3.2	6.9	3.2	3.4
Fos 102	2.9	5.3	2.3	2.3	3.3	3.3
Fos 118	6.0	4.4	2.3	2.3	2.3	2.7
Fos 187	3.5	5.0	2.5	8.5	2.7	2.4
Fos 189	2.0	7.5	2.4	2.2	7.0	2.3
Fos 250	10.1	7.0	7.0	4.5	6.5	4.0
Fos 252	5.7	11.5	9.2	5.6	10.0	3.5
Fos 258	22.0	12.0	9.0	6.0	11.5	4.8
Fos 260	5.8	4.3	2.3	2.4	2.6	2.2
Fos 270	2.1	5.5	6.0	6.0	7.1	5.8

A consistent property of batch cultures of the mutants was their sensitivity to dilution. When AM-grown populations were diluted tenfold into prewarmed AM, a growth lag was observed with the mutants but not with the parent strain. Fig. 1 shows the behaviour of Fos 102 as an example. When acetate was added immediately after dilution the lag was not observed (Fig. 1). Likewise, NH_4^+ addition eliminated the lag. These findings are consistent with the possibility that the lag is induced by increased aeration inhibiting N_2 fixation. An alternative explanation is that the mutants are hypersensitive to 'dilution shock'.

'Correction' of Fos⁻ phenotype by carboxylic acids. Most isolates (see Table 3 for a few examples) showed normal aerotolerant N_2 fixation when any of the substrates shown in Table 3 was provided in addition to sucrose in AM. No lag was induced upon dilution of such cultures and rates of growth in air were similar to those of the parent strain with the same carbon sources (see Table 2 and Fig. 1). However, some mutants could not be 'corrected' by certain carboxylic acids (Table 3). Fos 39 and Fos 187, which were not 'corrected' by malate, were isolated as able to grow diazotrophically in air on sucrose plus acetate. Other mutants which failed to grow diazotrophically in air with one or more carboxylic acids include Fos 189 to Fos 200, which were isolated as unable to grow in N_2 with pyruvate, and Fos 261 to Fos 264 which did not fix N_2 with malate. Some mutants showed an absolute requirement for a particular carboxylic acid in order to fix N_2 aerobically, e.g. Fos 250 to Fos 258 required 2-oxoglutarate. One of the Cit⁺ clones, Fos 270, was not 'corrected' by any of the carboxylic acids tested.

'Correction' of Fos⁻ phenotype by increased Ca^{2+} . Increasing the Ca^{2+} concentration in AM medium from 0.1 mM to 1 mM restored aerotolerant diazotrophy to all mutants tested except those not 'corrected' by pyruvate. Also, increasing the Ca^{2+} concentration shortened the growth lag induced by tenfold dilution of cultures, and increased the rate of growth in air. No changes in growth of the parent strain in the presence of 1 mM- Ca^{2+} were observed.

Respiratory characteristics of A. chroococcum MCD-1 and Fos⁻ mutants

Behaviour of MCD-1. When grown on AM or AM with glucose in place of sucrose, organisms were able to respire acetate. Acetate-grown organisms did not respire sugars without prior incubation. When both substrates were supplied together to sucrose-grown organisms, both were taken up. These results agree with data for *Azotobacter vinelandii* (Harris, 1945). We observed that O_2 uptake traces with sucrose were non-linear when the O_2 concentration was

Fig. 1

Fig. 1. Growth of *A. chroococcum* MCD-1 and Fos 102 on AM medium with and without acetate. AM-grown populations of MCD-1 (circles) and Fos 102 (triangles) were diluted tenfold to a density of 10 Klett units into fresh medium with 10 mM-acetate (filled symbols) or without added acetate (open symbols). Culture densities were determined at timed intervals.

Fig. 2

Fig. 2. Effect of acetate on respiration of *A. chroococcum* MCD-1 grown on AM medium with sucrose. AM-grown populations of MCD-1 were placed in a Clark-type O_2 electrode and consumption of O_2 was followed as described in Methods. To test the effect of acetate on respiration it was added to 10 mM at the time indicated in the figure using a new batch of organisms at the start of the experiment (.....).

Table 3. Ability of various compounds to 'correct' the *A. chroococcum* Fos⁻ phenotype

Cells were grown on AM medium supplemented with either 10 mM of the indicated carboxylic acid or 1 mM- $CaCl_2$. +, Diazotrophic growth in air; -, inability to grow on N_2 aerobically; ND, not determined.

Strain	Acetate	Fumarate	2-Oxoglutarate	Malate	Pyruvate	Succinate	Ca ²⁺
Fos 35	+	+	+	+	+	+	+
Fos 39	+	+	+	-	+	+	+
Fos 102	+	+	+	+	+	+	+
Fos 118	+	+	+	+	+	+	+
Fos 187	+	+	+	-	+	+	ND
Fos 189	+	+	+	+	-	+	-
Fos 217	+	+	+	+	+	+	+
Fos 250	-	+	+	+	-	+	-
Fos 252	-	+	+	+	-	+	-
Fos 258	-	+	+	+	-	+	ND
Fos 260	+	+	+	+	+	+	+
Fos 270	-	-	-	-	-	-	-
Fos 271	+	+	+	+	-	+	ND
Fos 275	+	+	+	+	+	+	ND

lower than 50 μM . The addition of acetate caused the O_2 uptake to become linear over a much greater range, although the maximum rate only increased slightly (Fig. 2). This effect was rapid and independent of the O_2 concentration at the time of addition of acetate. These findings indicate that the C source could influence the apparent affinity of the organisms for O_2 without changing the maximum respiration rate. We attempted to quantify the apparent $K_s(O_2)$ values with the different C substrates as described in Methods in order to compare the behaviour of MCD-1 with the Fos⁻ mutants.

Citrate at 2 mM inhibited the growth of MCD-1. Whilst the site of action of citrate is not known at this time, we observed that citrate addition caused a rapid inhibition of growth in AM with N_2 or NH_4^+ , and a progressive, and over 90 min total, inhibition of respiration in this strain.

Table 4. *Respiratory properties of A. chroococcum MCD-1 and Fos⁻ strains grown diazotrophically with sucrose alone and sucrose plus various additions*

Cells were grown on sucrose plus the indicated carboxylic acid (10 mM), and the maximum respiration rates, $V_{\max}$ [$\mu\text{mol O}_2$ consumed (mg protein)⁻¹ h⁻¹], and $K_s(\text{O}_2)$ (μM) were determined in the presence of these C sources. The values are means of four to ten determinations. SD for $V_{\max}$ and $K_s(\text{O}_2)$ were in the range 10 to 30% of the given values.

Category	Strain	No addition		Pyruvate		Acetate		Succinate		Malate	
		$V_{\max}$	$K_s(\text{O}_2)$	$V_{\max}$	$K_s(\text{O}_2)$	$V_{\max}$	$K_s(\text{O}_2)$	$V_{\max}$	$K_s(\text{O}_2)$	$V_{\max}$	$K_s(\text{O}_2)$
Wild-type	MCD-1	134	20	138	5	128	10	128	8	120	9
RI	Fos 118	125	20	130	6	110	7	133	5	119	10
	Fos 260	128	20	128	6	120	7	125	7	130	8
RII	Fos 39	70	24	100	8	102	7	140	6	40*	26
	Fos 102	50	22	101	10	85	9	118	10	125	8
	Fos 217	76	21	77	8	82	8	70	6	64	9
	Fos 250	80	23	50*	25	65*	27	90	5	92	8
	Fos 270	48	26	80*	30	84*	30	86*	25	78*	28
RIII	Fos 35	108	55	102	7	102	11	140	10	112	11
	Fos 189	121	45	110*	46	120	12	170	10	130	12

* Failure of this substrate to correct the Fos⁻ phenotype and failure of the cells to grow in N₂ with this substrate as the sole C source (see Table 3).

Surprisingly, citrate initially caused a response in the apparent $K_s(\text{O}_2)$ similar to that observed for acetate.

Behaviour of Fos⁻ mutants. Various Fos⁻ strains were grouped according to their respiratory properties when growing diazotrophically on sucrose as a C source under low aeration conditions (Table 4).

Group RI comprised two strains, Fos 118 and Fos 260, which were similar to the parent strain grown under comparable conditions with respect to maximum respiration rate and the apparent $K_s(\text{O}_2)$ when respiring sucrose or sucrose plus a range of carboxylic acids. These mutants also grew faster on NH₄⁺ than the parent (see above). To determine the possible lesion in these strains, organisms were pulse-labelled with ³⁵SO₄²⁻ both during growth and during the lag elicited by dilution.

The parent strain and six other Fos⁻ mutants (Fos 35, 39, 102, 189, 252 and 270) synthesized nitrogenase polypeptides during the dilution-induced lag, but the group RI mutants did not. The *Klebsiella pneumoniae nifA*^c high copy number plasmid pCK1, which overcomes O₂ and NH₄⁺ repression of *nif* expression in azotobacters (Kennedy & Robson, 1983), was introduced into the group RI strains, and restored the ability to synthesize nitrogenase polypeptides during the dilution-induced lag. Plasmid pCK1 also complemented the Fos⁻ phenotype in these strains. If the lag observed in this group of mutants is due to increased aeration, then they might be hypersensitive to O₂ repression of nitrogenase synthesis.

Group RII, of which only a few examples are listed in Table 4, contained mutants which exhibit low maximum rates of respiration, but values for apparent $K_s(\text{O}_2)$ that are similar to MCD-1. In this group, the ability of carboxylic acids to correct the phenotype was always correlated with an increased affinity for O₂ rather than increased respiration rate, as illustrated by the behaviour of some examples in Table 4. In the case of Fos 102, carboxylic acids increased both respiration rate and O₂ affinity, whilst in Fos 217 carboxylic acids caused only an increase in O₂ affinity. Fos 270 was not 'corrected' by any carboxylic acid tested, and though they all increased respiration rates significantly no change in the apparent affinity for O₂ was observed.

Group RIII contained strains which exhibited respiration rates similar to the parent but showed values for apparent $K_s(\text{O}_2)$ higher than the parent. In both the typical examples given in Table 4, the 'correcting' carboxylic acid lowered the value for apparent $K_s(\text{O}_2)$ without increasing the maximum respiration rate. In Fos 189, pyruvate neither 'corrected' nor altered the affinity for O₂.

The behaviour of both groups RII and RIII is consistent with the idea of 'correction' being correlated with increased respiratory activity at low O_2 tensions due to a change in the apparent affinity for O_2 .

Effect of Ca^{2+} . In representative mutants of the three groups, Fos 35, Fos 102 and Fos 118, and in the parent strain, increasing Ca^{2+} to 1 mM with sucrose-grown cells did not alter the maximum respiration rates with sucrose, but increased affinities for O_2 were observed (apparent $K_s(O_2)$ decreased from $> 20 \mu M$ to approx. $14 \mu M$).

Effect of citrate. Citrate at up to 5 mM did not inhibit respiration of the Cit^+ Fos^- mutants, although a decrease in the apparent $K_s(O_2)$ was observed, as with the parent strain, suggesting that citrate was taken up by the organisms.

*Respiratory activities of membranes from *A. chroococcum* MCD-1 and Fos^- mutants*

Membrane suspensions prepared from sucrose-grown or sucrose plus acetate-grown MCD-1 cells respired NADH [70 to $120 \mu mol O_2 (mg \text{ protein})^{-1} h^{-1}$], malate [36 to $66 \mu mol O_2 (mg \text{ protein})^{-1} h^{-1}$] and succinate [6 to $12 \mu mol O_2 (mg \text{ protein})^{-1} h^{-1}$]. Activities were additive with mixed substrates. The apparent $K_s(O_2)$ values were in all cases below $0.5 \mu M$, which was considerably below that observed in the intact organisms from which they were prepared. In representative mutants (Fos 35, 39, 102 and 118), no obvious differences were observed, indicating that the altered respiratory properties seen in such mutants may not be attributed to changes in the membrane-bound respiratory components.

DISCUSSION

Mutants of *A. chroococcum* which did not show characteristic tolerance to O_2 when fixing N_2 were distinguished from other Nif^- mutants by their ability to fix N_2 when the environmental O_2 concentration was considerably decreased. Mutants of this type have not previously been described. In general, they had several properties in common. (1) The mutant behaviour was observed when the organisms were grown on sugar but not on sugar plus carboxylic acids or on carboxylic acids alone. We called this phenotype Fos^- . (2) Growth on sugars with a combined N source was not impaired. (3) Increased Ca^{2+} in the medium substituted for the carboxylic acid requirement for aerobic N_2 fixation. (4) Mutants were resistant to the growth inhibition by citrate at 5 mM demonstrable in the parent (except those requiring 2-oxoglutarate). We were able to identify strains which did not exhibit one or more of these properties, and they give particular insights into the phenomenon of O_2 -tolerance.

Azotobacter mutants defective in O_2 -tolerance of N_2 fixation may bear mutations affecting one or more of the various contributory processes. Of these, we rule out aggregation (Bergersen & Turner, 1980), since the parent strain used in this study was a non-aggregating variant, and negative aerotaxis (P. Woodley & R. L. Robson, unpublished results) owing to the agitated culture conditions used to select the mutants. Mutants affected in conformational protection, respiratory protection or O_2 repression are likely. The properties of the Fos^- mutants suggested that they most probably belong in the second category. However, Fos 118 and Fos 260 were genetically complemented by the *nifA* gene product from *K. pneumoniae* and therefore seem to be affected in regulation of nitrogenase synthesis. One possibility is that these mutants are hypersensitive to O_2 repression, which normally is observed at greater aeration than is required to 'switch off' nitrogenase activity (Robson, 1979*b*; Postgate *et al.*, 1981). This may result from an altered repressor protein if O_2 regulation occurs in a manner similar to that observed in *K. pneumoniae*, where the *nifL* product mediates repression both by O_2 and by low levels of combined N (Hill *et al.*, 1981; Merrick *et al.*, 1982). Alternatively the lesion may lie in some general O_2 -sensing system. These Fos^- mutants grew faster than the parent strain on NH_4^+ , a phenomenon also observed for some *nifL* mutants of *K. pneumoniae* (MacNeil & Brill, 1980).

The general properties exhibited by the other Fos^- mutants can be discussed in relation to those growth conditions which elicit unusual O_2 sensitivity in N_2 -fixing wild-type organisms. C- and P-limitation caused *A. chroococcum* to be particularly O_2 -sensitive when fixing N_2 , at least when a sugar was provided as a C source (Dalton & Postgate, 1969*a, b*; Lees & Postgate, 1973).

Hence, mutations which lead to an 'intrinsic' limitation in either of these nutrients might give rise to O₂ sensitivity. It appears likely that the Fos⁻ mutants are intrinsically limited for some step in the metabolism of sugars, but not of carboxylic acids, so that growth with sugars and combined N is thus not affected, but respiratory protection at the expense of sugars is prevented.

The Ca²⁺ requirement of azotobacters is increased when they are grown in N₂ (Esposito & Wilson, 1958). The Ca²⁺ requirement can be replaced by supplying a carboxylic acid (Esposito & Wilson, 1958; Jakobsons *et al.*, 1962). These observations clearly relate to the behaviour of Fos⁻ mutants. Our data imply that in these earlier experiments Ca²⁺-starvation induced hypersensitivity to O₂ by interfering with the enhanced metabolism of sugars associated with respiratory protection. Thus the lesions in most Fos⁻ mutants lead to a physiological state which is analogous to Ca²⁺ starvation. The role of Ca²⁺ is not clear at present. Our results suggest that Ca²⁺ is involved in pyruvate metabolism because some mutants were not corrected either by Ca²⁺ or pyruvate. One such mutant, Fos 189, is deficient in phosphoenolpyruvate carboxylase (J. L. Ramos & R. L. Robson, unpublished data), which suggests that replenishment of the tricarboxylic acid cycle is important for O₂-tolerant N₂ fixation in *A. chroococcum*. Yet other mutants not corrected by Ca²⁺ indicate a role for this ion in the operation of the tricarboxylic acid cycle. Fos⁻ mutants which required 2-oxoglutarate for good growth even when supplied with NH₄⁺ resemble citrate synthase mutants (*gltA*) described in *E. coli* (see Weitzman, 1981).

Our observations on the influence of the C source on the apparent affinity for O₂ of whole organisms have not been reported before. Apparent K_s(O₂) values of approximately 20 µM for *A. chroococcum* grown in batch cultures on sucrose can be compared to the low affinity system (apparent K_s(O₂) 35 µM) reported by Bergersen & Turner (1980) for their most highly aerated chemostat cultures of *A. vinelandii* with glucose as the C source. They suggested that this system represented the terminal oxidase associated with the mechanism of respiratory protection. Whilst this system might be important at high O₂ concentrations, group RII Fos⁻ mutants, like the parent strain, exhibited low affinities for O₂ when grown in sucrose, and yet they were nevertheless O₂-sensitive. The ability of acetate to increase the apparent affinity for O₂ in sucrose-grown organisms may be the major factor in the correction of the Fos⁻ phenotype by this and other carboxylic acids. This possibility was supported by the observations that those carboxylic acids which corrected the various Fos⁻ mutants consistently increased the respiration rate at low O₂ tensions (i.e. below 50 µM), rather than the maximal respiration rate. However, if a rapid increase in the apparent O₂ affinity was a normal response to increased aeration then the parent strain should have exhibited a high affinity for O₂ with sucrose as the sole C source in the O₂ electrode experiments. That this was not the case suggests that the addition of carboxylic acids in general induces at least two rapid effects: the change in affinity for O₂, and a more cryptic change, as yet unidentified, which is important for respiratory protection and which is blocked in the Fos⁻ mutants grown on sucrose.

The mechanism of the rapid change in O₂ affinity is not understood but may have several explanations.

(1) If respiration of whole organisms becomes subject to respiratory control at low O₂ tensions in sucrose-containing media then the action of carboxylic acids may be analogous to uncoupling agents. The action of Ca²⁺ may be similar. Zimniak & Barnes (1983) have described an unusual dual Ca²⁺ transport system in *A. vinelandii*. One system is a 2H⁺/Ca²⁺ antiport similar to that found in other bacteria, and the other is an electrogenic Ca²⁺ porter. The two systems could together function as an uncoupling mechanism which might be regulated by the metabolic flux, or become increasingly more effective as extracellular Ca²⁺ is increased. The Ca²⁺ porter would then act in a manner analogous to the uncoupling protein of brown adipose mitochondria (see Nicholls, 1979).

(2) The fact that isolated membranes exhibited high affinities for O₂ with all physiological substrates tested suggests that the limitation which leads to a low O₂ affinity with sucrose *in vivo* is extrinsic to the membrane-bound respiratory-chain components. However, we cannot rule out the possibility that the real affinity for O₂ of a terminal oxidase, or the route of electrons in a distally branched electron transport chain with terminal oxidases of different affinities, can be modulated *in vivo* in a way which cannot be reproduced with isolated membranes. One likely

explanation of the decreased rate of respiration at relatively low O₂ tensions with sucrose is that oxidizable substrates are diverted to the formation of a poly- β -hydroxybutyrate which is favoured at high intracellular concentrations of NADH and acetyl-CoA (Senior & Dawes, 1973). Acetate addition would then be expected to change the metabolic status in favour of complete substrate oxidation. Another consequence of the addition of carboxylic acids could be changes in the levels of tricarboxylic acid cycle intermediates, resulting in an increased flux through those proximal limbs of the electron transport chain which are not coupled to energy conservation sites (Jones *et al.*, 1973*a*).

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