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Isolation of *ntrA*-Like Mutants of *Azotobacter vinelandii*

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A number of chlorate-resistant mutants of *Azotobacter vinelandii* affected in a general control of nitrogen metabolism were isolated. These mutants could not utilize dinitrogen, nitrate, or nitrite as a nitrogen source. The reason for this inability is that they were simultaneously deficient in nitrogenase and nitrate and nitrite reductase activities. They were complemented by a cosmid carrying a DNA fragment of *A. vinelandii* able to complement *ntrA* mutants of *Escherichia coli*, so they seemed to be *ntrA*-like mutants.

Nitrate and molecular dinitrogen are converted by nitrate and nitrite reductases and nitrogenase, respectively, to ammonia. Both processes, nitrogen fixation and nitrate assimilation, are regulated by the nitrogen source available in the growth medium.

The regulation of nitrogen fixation has been extensively studied in *Klebsiella* spp. The nitrogen fixation gene cluster of *Klebsiella* spp. contains at least 17 *nif* genes, arranged in several operons, which are required for the synthesis and activity of the enzyme nitrogenase. The expression of these genes is subject to "nitrogen control" at two levels. The *nifLA* operon is responsible for the production of an activator, the *nifA* product, and a repressor, the *nifL* product, which together regulate transcription from all *nif* promoters except that of the *nifLA* operon itself (3). Transcription of *nifLA* is in turn controlled by the nitrogen regulatory (*ntr*) genes *ntrA*, *ntrB*, and *ntrC*, which exert a general control on operons subject to nitrogen regulation in enteric bacteria (9). The *ntrA* product is also required for gene activation by the *nifA* product (12, 20). The *nifLA* promoter is therefore the primary target for the regulation of *nif* transcription in response to the level of fixed nitrogen in the growth medium.

Azotobacter vinelandii is a gram-negative soil bacterium that is able to reduce both dinitrogen and nitrate in aerobic conditions. The nitrogen fixation system of *Azotobacter* spp. has not been as studied as it has in *Klebsiella* spp. Nitrogenase synthesis is completely repressed by excess ammonium. Many Nif^- mutants have been characterized biochemically (17). There are indications that control may occur through a positive effector, perhaps analogous to the *nifA* gene product of *Klebsiella pneumoniae*. In fact, the *nifA* gene product of *K. pneumoniae* can activate the expression of *nif* genes in one mutant of *A. vinelandii* unable to synthesize any component of nitrogenase (5).

Compared to nitrogen fixation, rather little is known about nitrate assimilation from bacteria of the assimilatory nitrate-reducing type (14). Nitrate reductase activity levels in cell-free extracts from these bacteria are greatly influenced by the nitrogen source in the culture medium: nitrate reductase content is high in cells grown with nitrate or nitrite, thus indicating the apparently inducible nature of the enzyme (4). The nitrate reductase of *A. vinelandii* is inducible and binds to large enzyme particles (2). One chlorate-resistant (Chl^r) mutant lacking nitrate reductase has been described (21). At present there are no reports about regulatory nitrate reductase mutants in this organism.

The purpose of this work was to isolate regulatory mutants unable to fix dinitrogen and to assimilate nitrate. Here we report the isolation of mutants, selected on the basis of their chlorate resistance, that are affected in a general control of nitrogen metabolism.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the bacteria used, their genotypes or phenotypes, and their sources.

Media and culture conditions. Cultures of *A. vinelandii* were grown at 30°C under aeration in modified Burk nitrogen-free medium (4). The final EDTA and iron concentrations were 0.1 mM. To avoid the precipitation of iron, we mixed neutralized EDTA with the $FeSO_4$ solution and aerated the mixture vigorously overnight. When necessary, the nitrogen-free medium was supplemented with ammonium acetate (1 g/liter), potassium nitrate (0.8 g/liter), sodium nitrite (0.2 g/liter), or glutamine (1 g/liter).

Cultures of *Escherichia coli* were grown at 30°C in Luria-Bertani medium (10).

Enzyme assays. (i) Nitrogenase. Cultures grown to the late logarithmic phase in ammonium acetate medium were centrifuged, washed, and preincubated in nitrogen-free medium for 3 to 12 h to allow the derepression of nitrogenase synthesis. Nitrogenase activity was measured as acetylene reduction by intact cells as described previously (22).

(ii) Nitrate reductase. Cultures grown to the late logarithmic phase in ammonium acetate medium were centrifuged, washed, and suspended in nitrate medium. To determine basal nitrate reductase activity, we removed a sample and incubated the remaining culture for 5 h to induce nitrate reductase. Nitrate reductase activity was measured, by a modification of a procedure described previously (4), as nitrite formed either by toluenized or nontoluenized cells incubated at 30°C for 1 h. The reaction mixture contained, in a final volume of 1 ml, the following: morpholinepropanesulfonic acid (MOPS)-KOH (pH 7.0), 100 μmol; KNO_3, 10 μmol; methyl viologen, 0.15 μmol; KCNO 1 μmol; protein, 0.1 to 0.2 mg; and $Na_2S_2O_4$ in 95 mM $NaHCO_3$ 0.8 mg. Nitrite was estimated by adding diazocoupling reagents as described previously (8).

(iii) Nitrite reductase. Nitrite reductase activity was assayed, by a procedure described previously (24), as the disappearance of nitrite from intact cells previously induced by nitrate in the same way as for nitrate reductase.

(iv) Glutamine synthetase. Cells grown in ammonium acetate medium overnight were washed and incubated for 0 to 60 min in Burk medium to allow enzyme deadenylylation. Glutamine synthetase was measured in cells permeabilized by an alkyl trimethyl ammonium bromide mixture by a

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source
Strains		
E. coli		
1843	Pro^- Met^- Nal^r/pJB3JI	J. E. Beringer
5K	*resK modK* Thr^- Leu^- *tonA supE*(pCK1 or pCK3)	C. Kennedy
JC5466	Trp^- *recA* His^- *rpsE* Lac^-/pRK2013	C. Kennedy
HB101	*leuB proA thi-1* Str^r *hsdR hsdM recA supE lacZ*(pLV50 or pLV72)	C. Kennedy
ET8045	*rbs lacZ*::IS*1 gyrA hutCKA glnF*::Tn*10*	C. Kennedy
A. vinelandii		
UW	Wild type	W. Brill
UW6	$NifI^-$ $NifII^-$	W. Brill
UW (Rif^r)	Rif^r	C. Kennedy
AS22 and AS23	*ntrA* Chl^r Rif^r	This work
AS24 and AS25	*ntrA* Chl^r Rif^r	This work
AS27 and AS29	*ntrA* Chl^r Rif^r	This work
AS51 and AS52	*ntrA* Chl^r Rif^r	This work
AS54 and AS55	*ntrA* Chl^r Rif^r	This work
Plasmids		
pJB3JI	Tra^+ Cma^+ Tc^r Ap^r	J. E. Beringer
pCK1	Tra^- Sm^r (Km^r) *nifAKp p(nifB)* Km^r	C. Kennedy
pCK3	Tra^- Tc^r (Km^r) *nifAKp p(nifB)* Km^r	C. Kennedy
pRK2013	Tra^+ Km^r	C. Kennedy
pRK2073	Tra^+ Spc^r Sm^r Tp^r (Tn*7*)	C. Kennedy
PLV50	*ntrC* Tc^r	A. Toukdarian
pLV72	*ntrA* Tc^r	A. Toukdarian

transferase assay with and without 60 mM $MgCl_2$ as previously described (6).

Protein determination. Protein was estimated by a modification of the Lowry procedure (11).

Isolation of chlorate-resistant mutants. Chlorate-resistant mutants were selected, after mutagenesis of strain UW (Rif^r) with ICR 191 or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, on solid glutamine or ammonium acetate medium supplemented with potassium chlorate (100 mM).

Mutagenesis of pLV72. Cosmid pLV72 was mutagenized with Tn*5* by infecting HB101 (pLV72) with λ::Tn*5* (18) and selecting the transference of kanamycin resistance by using pRK2073 as a "helper plasmid" and strain ET8045 as a receptor.

Genetic analysis. (i) Complementation. In experiments of intrageneric conjugation, a derivative of R68-45, the Km^s plasmid pJB3JI, was used as a chromosomal transfer vector. In experiments of intergeneric conjugation, plasmids pCK1 and pCK3 and cosmids pLV50 and pLV72 were mobilized by the Tra^+ plasmid pRK2013 from *E. coli* 5K or HB101, respectively, to *A. vinelandii* strains. In all cases, biparental or triparental matings were performed on ammonium acetate plates at 30°C for 12 to 15 h. Nif^+ exconjugants were selected on solid Burk nitrogen-free medium. When necessary, the donor wild type was counterselected in medium containing rifampin (20 μg/ml). The transfer of pCK1 and pCK3 was selected by streptomycin (10 μg/ml) and tetracycline (10 μg/ml), respectively, on solid ammonium acetate medium. The expression of kanamycin resistance was determined by growth in medium supplemented with 1 μg of kanamycin per ml. The transfer of pLV50 and pLV72 was selected by tetracycline (10 μg/ml) on solid ammonium acetate medium.

(ii) Transformation. Transformation of chromosomal markers was done with crude DNA extracts as previously described (13).

RESULTS

Isolation of mutants defective in dinitrogen, nitrate, and nitrite assimilation. Chlorate-resistant colonies, selected as described in Materials and Methods, were tested for growth in nitrogen-free medium and in media supplemented with nitrate, nitrite, ammonium acetate, or glutamine. We isolated 20 mutants unable to use N_2, nitrate, or nitrite as a nitrogen source. The frequency of these mutants among the chlorate-resistant colonies was 1%. To rule out double mutants, we searched for spontaneous revertants and performed transformation experiments with wild-type DNA crude extracts. Revertants or transformants able to grow in nitrogen-free medium or in nitrate-supplemented medium were isolated, and their ability to excrete nitrite when grown in nitrate and their sensitivity to chlorate in nitrogen-free medium were tested. The frequency of spontaneous revertants was the same in nitrogen-free medium and in nitrate-supplemented medium: about 7×10^{-8} to 8×10^{-8} in 19 of the 20 mutants and 7×10^{-9} to 8×10^{-9} in the remaining 1 mutant. The majority of mutants, 15, behaved as single mutants, their revertants and transformants recovering the four wild-type characteristicts simultaneously: utilization of dinitrogen and nitrate as sources of nitrogen, nitrite excretion in nitrate medium, and chlorate sensitivity. We used 10 of these single mutants for later studies: AS22, AS23, AS24, AS25, AS27, AS29, AS51, AS52, AS54, and AS55.

Gene complementation. To classify the 10 mutants in complementation groups, we performed matings among all possible couples as described in Materials and Methods. The chromosomal transfer vector used was plasmid pJB3JI. This plasmid mobilizes *A. vinelandii nif* genes at frequencies ranging from 10^{-4} to 10^{-5}, similar to those reported previously for R68-45 (23). We obtained no Nif^+ exconjugants from all these matings. We made two kinds of controls. On the one hand, we crossed the wild-type strain as the donor with all the mutants as receptors, plated them in nitrogen-free medium, and obtained Nif^+ exconjugants in all cases, thus proving that the 10 independent isolated mutations are recessive. On the other hand, to verify whether mutations in different *nif* genes would give Nif^+ exconjugants, we mated the mutants with strain UW1. Nif^+ exconjugants were obtained in every case. Starting from these data, we assumed that the 10 mutants were affected in the same gene, and we chose 5 mutants for enzymatic characterization.

Enzymatic characterization of mutants. We measured nitrogenase, nitrate and nitrite reductase, and glutamine synthetase activities in five AS mutants (Table 2). Nitrogenase was not detected in any of the mutants. Basal nitrate reductase activities were partially reduced in the mutants, whereas induced nitrate reductase activities were strongly reduced, showing that there was no induction by nitrate of nitrate reductase in these mutants. In the same way, there was no induction of nitrite reductase. In contrast, the glutamine synthetase level was not changed. The adenylylated and deadenylylated activities measured immediately after transfer of the cells from ammonium acetate medium to nitrogen-free medium, as described in Materials and Methods, were similar in the mutants and in the wild type. There

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TABLE 2. Enzyme activities in Chl[r] mutants

Strain	Activity of:							
	Nitrogenase[a]	Nitrate reductase[a]		Nitrite reductase[b] (t = 5 h)	Glutamine synthetase[a]			
		t = 0 h	t = 5 h		Mn^{2+} (t = 0 h)	Mg^{2+} (t = 0 h)	$Mn^{2+}/Mn^{2+} + Mg^{2+}$	
							t = 0 h	t = 1 h
UW (Rif[r])	75	0.27	4.68	15	190	63.7	2.98	1.13
AS22	ND[c]	0.02	0.05	2.12	139	53	2.62	1.07
AS23	ND	0.07	0.1	1.68	115.5	43.3	2.67	1.01
AS24	ND	0.03	0.08	0.48	121	39.9	3.03	2.12
AS27	ND	0.02	0.27	0.83	99.7	43.2	2.31	1.4
AS29	ND	0.02	0.05	0.93	138	67.6	2.04	0.74

[a] Activity is reported as nanomoles of product formed per minute per milligram of protein.
[b] Activity is reported as nanomoles of nitrite disappeared per minute per milligram of protein.
[c] ND, Not detected.

were no significant differences between the mutants and the wild type in the ratio of adenylylated to deadenylylated activities, either at 0 or 60 min after the change of medium.

Complementation of mutants by pLV72 and pLV50. To determine whether our mutants should be classified as general regulatory nitrogen metabolism mutants (Ntr), we used two cosmids, constructed by A. Toukdarian and C. Kennedy at the Unit of Nitrogen Fixation in Brighton, England, which complement *ntr* mutations in *E. coli* and *K. pneumoniae* (C. Kennedy, personal communication). Cosmid pLV50 contains *A. vinelandii* DNA which corrects *ntrC* mutations, and cosmid pLV72 contains *A. vinelandii* DNA which corrects *ntrA* mutations. Neither pLV50 nor pLV72 is self-transmissible, but each was mobilized by the Tra[+] plasmid pRK2013. *E. coli* strains (HB101 and JC5466) and each of our 10 mutants were mated as trios on ammonium acetate agar plates. The transfer of pLV50 and pLV72 was selected as described in Materials and Methods. Transconjugants were replicated on nitrogen-free medium to check the restoration of the normal phenotype. The 10 mutants analyzed were complemented by pLV72. No mutants were complemented by pLV50. We measured nitrogenase and nitrate and nitrite reductase activities in the Nif[+] transconjugants obtained from the same five mutants analyzed in Table 2. The enzyme activities of the transconjugants were similar to the wild-type enzyme activities for the three enzymes assayed (Table 3). To definitively establish that the chlorate-resistant mutants were affected in an *ntrA*-like gene, we mutagenized cosmid pLV72 with Tn*5*. Four cosmids carrying Tn*5* insertions, obtained as described in Materials and Methods, lacked the ability to complement both *ntr*A mutants of *E. coli* and *ntrA*-like mutants of *A. vinelandii*.

TABLE 3. Enzyme activities in Chl[r] mutants carrying the pLV72 cosmid

Strain	Activity of:			
	Nitrogenase[a]	Nitrate reductase[a]		Nitrite reductase[b] (t = 5 h)
		t = 0 h	t = 5 h	
UW (Rif[r])	75	0.27	4.68	15
AS22(pLV72)	65.3	0.4	3.09	12.1
AS23(pLV72)	54.8	0.22	3.42	12.9
AS24(pLV72)	61.3	0.55	2.87	12.8
AS27(pLV72)	47.3	0.47	4.49	10.9
AS29(pLV72)	56.7	0.2	4.94	15.1

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *b*.

Effect of the *nifA* product in *ntrA* mutants of *A. vinelandii*. To study whether the *ntrA* product is required for the normal function of the *nifA* product in *A. vinelandii*, as previously described in other organisms, plasmids pCK1 and pCK3 were transferred to *ntrA A. vinelandii* mutants. These wide-host-range plasmids carry the *nifA* gene of *Klebsiella* spp. expressed from a constitutive promotor and a kanamycin resistance gene expressed from a *nifA*-dependent *nifB* promotor (5). They differ in that pCK1 is multicopy, whereas pCK3 has a low copy number. The transfer of pCK1 and pCK3 was selected as described in Materials and Methods. All transconjugants were Nif[−] and Km[s]. As expected, the transfer of pCK1 and pCK3 to *A. vinelandii* UW6, a molybdenum-iron protein nitrogenase mutant, give Km[r] transconjugants, whereas the transfer of these plasmids to *A. vinelandii* UW1, a regulatory nitrogenase mutant, gave Nif[+] and Km[r] transconjugants. From these results, we conclude that in *A. vinelandii* the *ntrA* product is required for the *nifA* product to activate *nif* genes.

DISCUSSION

A. vinelandii is a very interesting organism to study because it exhibits the peculiar ability of fixing dinitrogen in nonsymbiotic and obligatory aerobic conditions. However, the genetic analysis of *A. vinelandii* has been hampered by the lack of efficient gene transfer systems. Transformation, the first gene transfer system described in *A. vinelandii* (1, 13), cannot be readily used for large-scale analysis. This difficulty has been surmounted by the conjugal transfer of genes by means of promiscuous plasmids belonging to the P1 incompatibility group (23).

In *A. vinelandii* it is easy to isolate antibiotic- and antimetabolite-resistant mutants, but it has proved exceedingly difficult to isolate recessive nonselectable mutants (17). A possible reason for this difficulty is that *A. vinelandii* may have a minimum of 40 chromsomes per cell (19). Our strategy has been to search for mutants with altered nitrogen metabolism regulation from among a large collection of mutants isolated by their resistance to chlorate. This approach is based on the following rationale. Some forms of the enzyme nitrate reductase are known to reduce nontoxic chlorate to toxic chlorite (16); consequently, strains lacking nitrate reductase activity are resistant to chlorate. If nitrate reductase synthesis is subject to nitrogen metabolism regulation, some of the chlorate-resistant strains should be Ntr-defective mutants. These mutants would be easily identifiable as unable to assimilate nitrate, nitrite, and dinitrogen. Our results show that about 1% of chlorate-resistant strains are Ntr mutants. These mutants are simultaneously deficient

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for nitrate and nitrite reductase and nitrogenase activities but are normal for glutamine synthetase activity. This result is hardly surprising, since the synthesis of glutamine synthetase seems to be constitutive in *A. vinelandii* (7). More surprising is the fact that all the regulatory mutants belong to a single complementation group, the *ntrA* gene. By analogy with *Klebsiella* spp. it would be expected that *A. vinelandii* Ntr mutants should represent at least two different genes, *ntrA* and *ntrC*. In fact, the isolation of *Azospirillum brasiliensis* mutants that fail to grow either on dinitrogen or on nitrate and that are complemented by the *K. pneumoniae* *ntrC* gene has been reported (15). During the development of this work, others obtained *ntrC* mutants of *A. vinelandii* (A. Toukdarian and C. Kennedy, EMBO J., in press) that were Nif^+. However, we screened for a Nif^-, nitrate reductase-negative phenotype, and this may be the reason why we missed the *ntrC* class.

All regulatory mutants described in this work exhibit the three following characteristics: they do not complement each other in our gene transfer probe; they are complemented by an *ntrA* gene-carrying cosmid, and they fail to express the kanamycin resistance gene driven by the *nifB* promoter carried by the pCK1 and pCK3 plasmids. So, the assignment of these mutants to the *ntrA* gene seems to be a safe conclusion.

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