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Regulatory Mutation That Controls *nif* Expression and Histidine Transport in *Azospirillum brasilense*

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Mutagenesis of *Azospirillum brasilense* with nitrosoguanidine and selection on ethylenediamine yielded prototrophs which fixed nitrogen in the presence of ammonia. Nitrogenase activity in mutant strains exceeded that of the wild type three- to sixfold. The same mutants were also constitutive for histidine transport. Enzyme activities involved in ammonia assimilation were not affected by the mutation. The data suggest that the mutation occurred at a site which regulates *nif* and histidine transport functions.

The genus *Azospirillum* comprises two species, *Azospirillum brasilense* and "*Azospirillum lipoferum*," which live in close association with the roots of various grasses (21). Many papers have been published on the physiology and ecology of *Azospirillum* spp. (4, 15, 16), whereas few have been published on gene regulation and manipulation in this genus. One of the more prominent features of *Azospirillum* species is their high nitrogen fixation efficiency. Nitrogen fixation activity in *Azospirillum* spp., as in other nitrogen-fixing bacteria, takes place in the absence of ammonia or at a concentration that does not exceed 0.2 mM. The assimilation of ammonia by *Azospirillum* spp., as in a variety of enteric bacteria, proceeds via glutamine synthetase (GS) or glutamic dehydrogenase (GDH), depending on ammonia availability (3, 7). *Azospirillum* mutants altered in GS activity fail to derepress nitrogenase in the absence of ammonia (3) and resemble the *asm* mutants described for *Klebsiella pneumoniae* (14). It has been suggested, therefore, that GS plays a regulatory role in nitrogenase biosynthesis in *Azospirillum* spp. (3, 7). *Azospirillum* mutants which express *nif* functions as a result of mutations in loci apparently involved in regulation of general ammonia utilization are described here for the first time.

A. brasilense grown in minimal salts medium (15) was mutagenized in 50 mM maleic buffer (pH 5.6) supplemented with nitrosoguanidine to a final concentration of 100 μg ml^{-} and incubated without shaking for 1 h at 35°C. The mutagenized culture was washed twice with 10 mM KP_i buffer (pH 6.8) and suspended in 50 ml of succinate minimal medium. After incubation for 24 h at 35°C without shaking, the culture was washed with KP_i buffer, suspended in 1 ml of the same buffer, and plated on nitrogen-free minimal agar plates supplemented with ethylenediamine to a final concentration of 0.05%. Colonies which acquired resistance to ethylenediamine were screened for constitutive nitrogen fixation in the presence of 5 mM NH_4Cl.

Nitrogenase was assayed by the acetylene reduction method on whole cells described previously by Postgate et al. (18). Cultures were grown in semisolid nitrogen-free medium for 18 h. Bottles (30 ml) containing 10 ml of medium were sealed with a rubber stopper, and 10% of the headscape gas was replaced with acetylene. Cultures were incubated for 1 h before the ethylene was measured by using a Gowmac gas chromatograph with a Porapak N column at 80°C. Specific activity was expressed as nanomoles of ethylene produced $hour^{-1}$ milligram of $protein^{-1}$. Two predominant phenotypes were found among the resistant colonies. Group 1 consisted of mutants that acquired resistance to ethylenediamine that did not affect the regulation of nitrogen fixation. Group 2 consisted of mutants that acquired resistance to ethylenediamine and were able to fix nitrogen in the presence of ammonia. Three representatives of group 2, designated BTG-182, BTG-200, and BTG-1130, were chosen for further experimentation.

The nitrogenase activities in the mutant and parent strains are shown in Table 1. In the absence of ammonia, all three mutants possessed a five- to sixfold higher nitrogenase activity than did the wild-type strain. In the presence of 2 mM or more NH_4Cl in the growth medium, no nitrogenase activity was detectable in the parent strain, whereas the three mutants were capable of reducing acetylene at different levels and were influenced by NH_4Cl concentration. At 10 mM NH_4Cl, strains BTG-182 and BTG-200 expressed 58 and 45%, respectively, of the activity observed in the absence of ammonium chloride. Under identical conditions, BTG-1130 retained only 14% of the activity obtained with no NH_4Cl. Except for BTG-1130, the nitrogenase activity observed in the presence of 10 mM NH_4Cl was about threefold higher than that of the parental strain under derepressed conditions. When NH_4Cl was replaced by $NaNO_3$, nitrogenase specific activity was 111 and 87.5 for BTG-182 and BTG-200, respectively, independent of the amount of $NaNO_3$ present. No activity was observed in BTG-1130 and in the parental strain, even at 2 mM $NaNO_3$ (data not shown).

Because it has been suggested that nitrogen fixation in *Azospirillum* spp. is regulated by a number of enzymes involved in nitrogen assimilation, the levels of GS, L-glutamate:2-oxoglutarate aminotransferase (NADPH oxidiz-

TABLE 1. Nitrogenase specific activities of the various strains in the absence and presence of ammonium chloride[a]

NH_4^+ concn (mM)	Sp act (nmol of ethylene reduced h^{-1} mg of $protein^{-1}$) for strain:			
	ATCC 29729 (wild type)	BTG-182	BTG-200	BTG-1130
0	107	551	601	580
2		540	495	307
5		670	398	95.6
10		323	275	81.6

[a] Cultures were grown in succinate semisolid minimal medium with and without added NH_4Cl.

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TABLE 2. Specific activities of enzymes involved in nitrogen metabolism in the various mutants at limiting and excess concentrations of NH_4^+[a]

Strain	NH_4^+ concn (mM)	Sp act (μmol γ-glutamyl hydroxymate min^{-1} mg of $protein^{-1}$) of GS				Sp act (μmol oxidized min^{-1} mg of $protein^{-1}$)	
			Transfer assay				
		Biosynthesis	Mg^{+2}	Mn^{+2} + Mg^{+2}	Unadenylated	GOGAT (NADPH dependent) NADPH	GDH (NADH dependent) NADH
ATTC 29729 (wild type)	1	0.071	0.440	0.36	0.59	0.064	0.037
	20	0.015	0.217	0.083	0.37	0.061	0.101
BTG-182	1	0.146	0.900	0.840	0.93	0.044	0.030
	20	0.027	0.360	0.174	0.49	0.060	0.126
BTG-200	1	0.144	1.240	0.770	0.62	0.080	0.054
	20	0.021	0.360	0.126	0.35	0.090	0.170
BTG-1130	1	0.068	0.650	0.420	0.60	0.051	0.022
	20	0.014	0.260	0.086	0.33	0.049	0.164

[a] Cultures were grown in succinate minimal medium overnight in the presence of the NH_4Cl concentration indicated. Fresh medium was inoculated with the overnight cultures and allowed to grow for 3 h before harvest.

ing) (GOGAT; EC 1.4.1.13) and L-glutamate:NAD^+ oxidoreductase (GDH; EC 1.4.1.2) were determined in low and high concentrations of NH_4Cl.

For enzyme assays, cultures were grown overnight in succinate minimal medium with 2 mM NH_4Cl. A 5-ml sample of this culture was diluted into 50 ml of fresh medium with appropriate concentrations of NH_4Cl and allowed to grow for 3 h before processing. Crude cell extracts were prepared from 10 ml of washed culture in 2 mM Tris hydrochloride buffer (pH 7.5) and disrupted by sonication, and the clear supernatant was used to determine enzyme activities.

L-Glutamate:ammonialigase (GS; EC 6.3.1.2) was estimated by measuring A_{540} of γ-glutamyl hydroxamate (19) or by the glutamyl transferase assay in the presence of γ-glutamyl hydroxamate produced $minute^{-1}$ milligram of $protein^{-1}$.

GDH was assayed by monitoring the oxidation of NADH at 340 nm as described by Kenealy et al. (8).

GOGAT was assayed as for GDH except that 5 mM glutamine was substituted for 80 mM NH_4 (8). Specific activities were expressed as micromoles of NADH or NADPH oxidized $minute^{-1}$ milligram of $protein^{-1}$ for GDH and GOGAT, respectively.

Enzyme specific activities in crude cell extracts are shown in Table 2. The data show that GS specific activity (forward reaction) was three- to fivefold lower in the presence of 20 mM NH_4Cl in all strains. At 1 mM ammonia, a two- to threefold difference in the transfer assay and the state of the adenylated fraction was observed between the wild-type and mutant strains. As expected, GDH specific activities in cell extracts prepared from cultures grown in high NH_4Cl concentrations were considerably higher than activities in cultures grown in low concentrations of NH_4Cl. It is obvious that the physiological response to ammonia concentration was not altered in these mutants. GOGAT activities determined in cell extracts of the different mutants and the wild type were quite similar regardless of ammonia concentration during growth. The data indicate that GOGAT is not affected by ammonium ions and confirm previous findings (3).

The constitutive expression of *nif* functions in the mutants described here may result from (i) a mutation within a *nif* regulatory region similar to *nifLA* described for *K. pneumoniae* (5) or (ii) a mutation in a regulatory gene (or genes) which control the expression of a number of genes involved in general nitrogen utilization. If the first possibility is true, we would expect no function other than *nif* expression to be altered in these mutants. If the alternative is correct, we would expect additional functions associated with nitrogen utilization to be affected. To discriminate between these two possibilities, we chose to test histidine transport function, because histidine transport in *Salmonella* spp. has been shown to be under the control of a general nitrogen regulatory system (10).

Histidine transport and incorporation into proteins was determined as described by Ames (1). Cultures were grown in succinate minimal medium overnight, washed with 10 mM KP_i buffer (pH 6.8), and diluted into fresh minimal medium with 5 or 0.1 mM ammonium chloride to an optical density at 600 nm (OD_{600}) of 0.01 in a final volume of 100 ml. Cultures were grown for 90 min at 33°C, and [^{3}H]histidine (1 mCi ml^{-1}; specific activity, 11,700 mCi $nmol^{-1}$) was added to yield a final concentration of 5 nM. Samples (1 ml) were removed at 1-min intervals and processed further as described by Ames (1). Rates were expressed as picomoles of [^{3}H]histidine incorporated into trichloroacetic acid-precipitable protein OD_{600} U^{-1} $minute^{-1}$ at 33°C. Protein was determined by the method of Lowry et al. (11). The results of histidine transport experiments are shown in Fig. 1. In the parental strain, histidine transport was fully repressed in the presence of 5 mM NH_4Cl, whereas under the condition of limiting ammonia concentration the histidine transport system was derepressed. A rate of 2.8 pmol of histidine min^{-1} $OD_{600}{}^{-1}$ was obtained for the wild type. Under identical conditions, BTG-200 and BTG-1130 cultures were fully derepressed for histidine transport both in the presence of 5 mM NH_4Cl and when the ammonia concentration was low. The transport rate as calculated from the slope of the line shown in Fig. 1 is 7 pmol min^{-1} $OD_{600}{}^{-1}$ for the two mutants. BTG-182 differed from BTG-200 and BTG-1130 in that it lacked the ability to fully derepress histidine transport at low concentrations of NH_4Cl. It also differed from the wild type in failing to fully repress transport in the presence of ammonia. The calculated rate of histidine transport in low ammonia concentrations was 0.9 pmol min^{-1} $OD_{600}{}^{-1}$, which is one-third the activity observed in the wild type under identical conditions. In the presence of 5 mM NH_4Cl, a

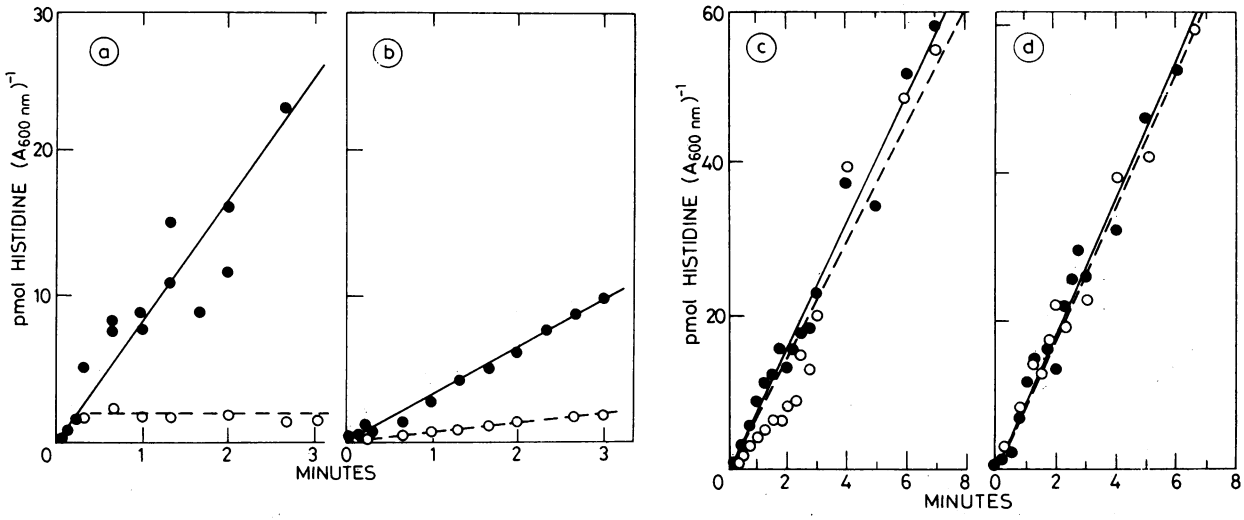

FIG. 1. Histidine transport activity in mutant and wild-type strains in cultures grown in limiting and excess concentrations of NH_4Cl. Cells were grown and assayed for transport as described in the text. (a) ATCC 29729 (wild type); (b) BTG-182; (c) BTG-1130; (d)BTG-200. ○, With 5 mM NH_4Cl; ●, with 0.1 mM NH_4Cl.

transport rate of 0.3 pmol of histidine min^{-1} OD_{600}^{-1} was obtained. This rate is 27-fold lower than that of the other two constitutive mutants and far greater than the rate observed for the parental strain in the presence of NH_4Cl. The three mutants described here fall into two classes. BTG-200 and BTG-1130 were constitutive for histidine transport at a high rate and BTG-182 was constitutive for histidine transport at a low rate. Our observations suggest that a *nifLA*-like regulatory mutation is not likely, because *nif* expression was not the sole function affected.

The phenotypes of the mutants described in this paper follow a pattern of regulation which may be in close analogy to those described in the enteric bacteria for the *ntrA ntrB* and *ntrC* gene products involved in the regulation of GS *nif* expression and histidine transport (2, 5, 6, 9, 10, 12, 13, 17, 20).

BTG-182 seems to carry a mutation in the *ntrC*-like gene product, as both repression and activation are impaired for histidine transport. Accordingly, *nif* expression in strain BTG-182 was expected to follow a similar pattern. Contrary to expectation, *nif* expression was as high in BTG-182 as in strains BTG-200 and BTG-1130. This high-constitutive expression of *nif* function suggests positive complementation for activity between a normal *ntrA* and a faulty *ntrC*-like gene product that activates *nif* and histidine transport functions to different degrees.

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