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Regulation of Nitrogen Fixation in *Azotobacter vinelandii* OP: the Role of Nitrate Reductase

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A number of chlorate-resistant mutants were selected, and one of these, clr68-5, was studied in detail. This mutant cannot utilize nitrate in vivo to overcome the effect of nonmetabolizable repressors of nitrogenase. The reason for this inability was that strain clr68-5 lacked nitrate reductase. Nitrate inhibited the activity of nitrogenase but did not act as a corepressor of nitrogenase in strain clr68-5 as it does in the wild type. Ammonia seemed to act as corepressor of nitrogenase in both strains.

Molecular nitrogen and nitrate are converted by nitrogenase and nitrate reductase plus nitrite reductase, respectively, via convergent metabolic pathways to ammonia. It is of interest to know whether nitrate regulates nitrogenase.

Nitrate has been shown to inhibit nitrogen fixation by whole cells of *Azotobacter vinelandii* (12, 27), *A. agile* (2, 12), *A. chroococcum* (12, 28), and *Anabaena cylindrica* (5), and by root nodule symbiotic systems (17, 25).

Stumbo et al. (24) found that certain strains of *A. chroococcum* would lose their nitrogen-fixing capacity after being cultured for 105 days or more on nitrate.

It is not clear from the above whether nitrate inhibits nitrogenase activity per se or nitrogenase formation, or both. Nitrate is metabolized; consequently, a serious ambiguity is introduced: Does nitrate itself have any effect on nitrogenase? Lee and Wilson (12) and Wilson, Hill, and Burris (27) noted that nitrate inhibited fixation to a greater extent in cells pregrown on nitrate than in cells previously unexposed to the compound.

This observation was taken to imply that a product of nitrate metabolism, rather than nitrate itself, was inhibiting nitrogen fixation (26). At the time that these observations were made, it would have been very difficult to test this hypothesis directly because the assays for nitrogenase and nitrate reductase in cell-free preparations were not available. No differentiation was made between inhibition of nitrogenase activity and nitrogenase formation, and no consideration was given to the possibility that there might be an adaptive "nitrate-permease" capable of bringing nitrate in contact with the nitrogen-fixing apparatus.

Present methodology allows a reexamination of this problem, and the use of nitrate reductaseless mutants should obviate the complications introduced by metabolic conversion of nitrate. Some forms of nitrate reductase are now known to reduce nontoxic chlorate to toxic chlorite (9, 18, 19). Consequently, strains lacking this species of enzyme should be resistant to chlorate. Mutants deficient in at least some form of nitrate reductase have been selected on this basis in several species of bacteria and in one strain of yeast (9, 20).

The purpose of this investigation was to determine what effect, if any, nitrate might have on the activity in vitro and on the formation of nitrogenase. This communication reports the isolation of nitrate reductaseless mutants, selected on the basis of their chlorate resistance, and the observation that nitrate inhibits but does not appear to repress nitrogenase in these mutants.

MATERIALS AND METHODS

Bacterial strains. *A. vinelandii* strain OP (3), kindly provided by P. W. Wilson, was used as the wild-type strain. Chlorate-resistant mutants clr68-5 and clr68-20 were isolated in separate experiments from strain OP in this laboratory in 1968.

Culture conditions. The media, modified Burk's nitrogen-free medium (DN_2) with 2% glucose as carbon source and with or without added nitrogen sources, the growth conditions, and the conditions for induction of nitrogenase were described previously (21). Growth was followed turbidimetrically by use of a Klett-Summerson colorimeter and a no. 54 filter. One Klett unit is equivalent to approximately 2.4×10^6 cells per ml. Chlorate and methylamine solutions were sterilized by filtration through disposable units (Falcon Plastics Co., Los Angeles, Calif.).

Nitrogenase was induced as follows. From a minute

solid inoculum, cultures were grown at 27 C with shaking into late exponential phase, in a medium containing 5 g/liter of Casamino Acids digest (Difco). The bacteria were then harvested by centrifugation and a part of the harvest was resuspended in nitrogen-free medium, with or without additions (induction medium), and incubated as before for 12 to 14 hr. During the latter treatment, cells underwent approximately three divisions. The final yield of cells in all the pertinent experiments reported herein was between 3.0 and 3.3 g (wet weight) of bacteria per liter of induction medium.

Extraction of enzymes. Nitrogenase was extracted as described previously (21). Nitrate reductase was prepared as follows. Cells were harvested at 4,000 × *g* in a refrigerated centrifuge (model RC2B, Servall, Inc., Norwalk, Conn.) for 10 min, washed once with 1% NaCl in 0.01 M potassium phosphate buffer (*p*H 7.3) to remove nitrite, and centrifuged as before for 20 min. The resulting pellet was suspended in three volumes (v/w) of 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (*p*H 7.0) containing 0.001 M Na_2 ethylenediaminetetraacetate, and incubated with 2 mg of lysozyme per ml for 20 min at 30 ± 1 C, and subsequently for another 10 min at 30 ± 1 C with 0.2 mg of deoxyribonuclease per ml. At the beginning of the latter incubation, $MgCl_2$ was added at a final concentration of 0.02 M. The resulting brei was kept on ice until used within 2 hr. Microscopic examination of this preparation revealed only cell fragments, and the yield of enzyme activity was greater than 90% of that measured in living cells.

Enzyme assays. Nitrogenase was assayed by a modification of the procedure of Bulen et al. (1), as described previously (21). One unit of nitrogenase is defined as a difference of 1 nmole of ammonia formed per min between a reaction mixture incubated under a nitrogen atmosphere and its otherwise identical control incubated under an argon atmosphere. Above 3.2 units, activity was strictly proportional to the amount of cell-free extract in the reaction mixture. The specific activity of nine extracts of fully induced cells used in nine randomly chosen experiments reported herein was 3.0 ± 0.1 units of nitrogenase activity per mg of protein. Measurements are recorded as units per milliliter of extract.

Glucose-6-phosphate dehydrogenase (G6PD) was measured by a modification of the procedure of Glock and McLean (6), as previously described (21). One unit of activity is defined as the glucose-6 phosphate-dependent reduction of 1 μmole of nicotinamide adenine dinucleotide phosphate ($NADP^+$) per min. All measurements were made in a range of extract concentration where the specific activity (activity per milligram of protein) was constant.

G6PD, recorded as units of activity per milliliter of extract, served as the control enzyme. The nitrogen source in the culture medium has but a small influence on the level of G6PD compared to its effect on nitrate reductase or nitrogenase. The specific activities of G6PD (units of G6PD per milligram of protein) in a sample of 12 experiments were 0.52 ± 0.03, 0.52 ± 0.03, and 0.66 ± 0.04 in extracts of cells grown or induced on DN_2, DN_2 plus various levels of nitrate, and DN_2 plus various levels of ammonium ions, respectively.

The ratio of nitrogenase (N) or nitrate reductase (NR) to G6PD was used as an index of repression in preference to the ratio of N or of NR to milligrams of protein. In one kind of ratio, two active enzymes are being compared; in the other, an active enzyme is being compared to total protein, which could be active, inactive, denatured, or even partly degraded.

NR was measured by a modification of the procedure of Lowe and Evans (13). The reaction mixture contained in a final volume of 1.05 ml: 120 μmoles of $Na_2P_4O_7$ (*p*H 7.0); 20 μmoles of KNO_3; 0.14 μmoles of Benzyl viologen; 2.3 μmoles of $Na_2S_2O_4$ in 0.2 M $Na_2P_4O_7$ hydrochloride (*p*H 7.0) previously flushed for 30 min with argon; and 0.05 to 0.20 ml of extract. The reaction was started by the addition of the $Na_2S_2O_4$, incubated in a water bath (model 82, Thelco, Chicago, Ill.) at 30 ± 1 C for 15 min, and stopped by vigorous shaking on a Vortex mixer (Scientific Products, Evanston, Ill.) until the Benzyl viologen was completely oxidized. A 3-ml amount of 95% ethyl alcohol was then added to precipitate large molecules, and after vigorous shaking the mixture was centrifuged at approximately 5,000 × *g* for 10 min in a small Angle centrifuge (model A, Ivan Sorvall, Inc., Norwalk, Conn.). The alcoholic supernatant fluid was then decanted and assayed colorimetrically for nitrite as described earlier (22). One unit of activity is defined as the nitrate- and reductant-dependent production of 1 nmole of nitrite per min under the above conditions. Activity was proportional to extract concentration in the reaction mixture between 1.3 and 8.0 units and is recorded as units per milliliter of extract.

Protein was measured by the Biuret method (4).

Selection of mutants. Cells from cultures in stationary phase were plated on DN_2 solid medium (21) containing 7 or 10 g of KCO_3 per liter. After 1 week, the resistant colonies were picked and purified by isolation of single colonies at least three times on nonselective media and were then checked periodically in liquid medium for chlorate resistance.

Chemicals and reagents. Glucose-6 phosphate, $NADP^+$, phosphocreatine, creatine phosphokinase, and adenosine triphosphate were purchased from the Sigma Chemical Co., St. Louis, Mo.; desoxyribonuclease and lysozyme from the Worthington Biochemical Corp., Freehold, N.J.; benzyl viologen from Mann Assayed Biochemicals, New York, N.Y.; $KClO_3$ and $Na_2S_2O_4$ (purified grade) from Fisher Scientific Co., Fairlawn, N.J.; argon and nitrogen (purified grade) from Canadian Liquid Air, Ltd., Hamilton, Ontario, Canada; 2-methylalanine from Eastman Organic Chemicals Co., Rochester, N.Y.; and methylamine from J. T. Baker Chemical Co., Phillipsburg, N.J: Casamino Acids (vitamin-free) were from Difco.

RESULTS AND DISCUSSION

Isolation and characterization of nitrate reductaseless mutants. Strain clr68-5 is one of a series of mutants that is more resistant to chlorate than is the wild type, but it is not completely

insensitive to this compound (Fig. 1). A simple interpretation of the above observation is that strain clr68-5 is resistant but permeable to chlorate and, therefore, presumably permeable to nitrate as well.

Methylamine and methylalanine were shown in a previous paper (21) to repress nitrogenase. Since they cannot serve as nitrogen sources, growth on nitrogen-free medium is inhibited by these compounds. If an alternative source of nitrogen, such as nitrate or ammonia, is supplied under these conditions, growth is restored because nitrogenase is not required for the assimilation of these alternate nitrogen sources (21).

One would predict that in a nitrate reductaseless strain the inhibition by methylamine and methylalanine would be relieved by ammonia, but not by nitrate. Strain clr68-5 behaves like a nitrate reductaseless mutant in this regard (Fig. 2, 3).

As expected, NR activity was not detected in extracts of strain clr68-5 (Table 1). Since nitrate is not metabolized by strain clr68-5, it should be possible to examine its effect on nitrogenase formation and activity in this strain.

Effect of nitrate on nitrogenase activity in vitro and on its induction in vivo. The results summarized in Fig. 4 indicate that nitrate inhibits nitrogenase. Potassium chloride was found to be much less inhibitory at equivalent concentrations, indicating that the inhibition by nitrate was not merely an effect of ionic strength.

Nitrate in the induction medium had much less effect on the level of nitrogenase in extracts of strain clr68-5 than on the level of this enzyme in extracts of the wild-type strain (Fig. 5). Another chlorate-resistant mutant, strain clr68-20, isolated from a culture independent of that used to obtain strain clr68-5, behaved identically to strain clr68-5 in this regard. The simplest interpretation of these results is that ammonia, the metabolic product of nitrate reduction, is repressing nitrogenase in the wild-type strain, and that nitrate itself represses very little, if at all.

The residual effect of nitrate on nitrogenase in strain clr68-5 is parallel to the effect of nitrate on nitrogenase in vitro. The concentration of nitrate-nitrogen in the enzyme assay mixture required to give 50% inhibition of nitrogenase activity is between 4.2 and 5.6 mM. The concen-

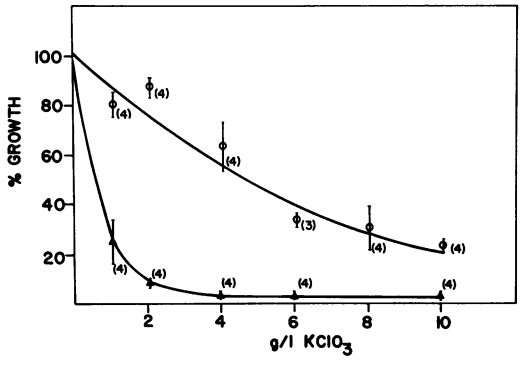

FIG. 1. *Effect of filter-sterilized potassium chlorate on growth of strains OP (△) and clr68-5 (○) in nitrogen-free medium (DN_2). Bars represent standard deviations from the mean, numbers in parentheses the number of experiments in the sample. Growth (100%) was between 228 and 272 Klett units and refers to the turbidity of a culture of cells growing in the absence of chlorate. All cultures that grew were in the exponential phase at the time of measurement.*

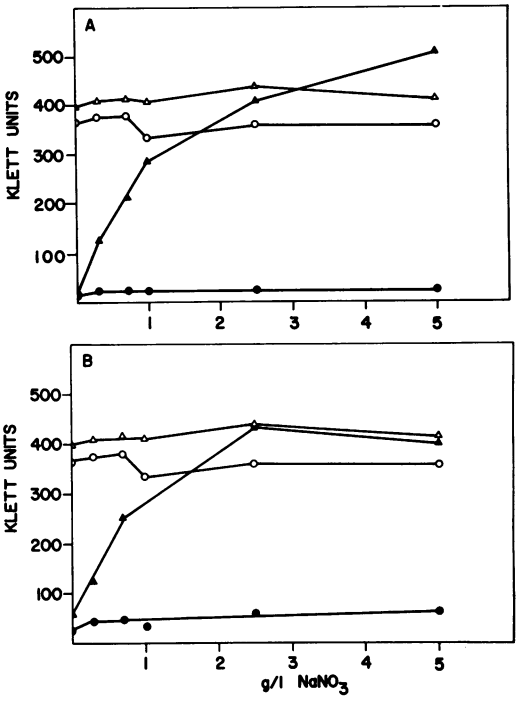

FIG. 2. *Effect of nitrate on growth on DN_2 of strains OP (▲, △) and clr68-5 (●, ○) in the presence of no inhibitor (△, ○), and of 1 g of inhibitor per liter (▲, ●). Flasks containing DN_2 were inoculated with each strain and subsequently incubated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 27 C for 40 hr. Under the conditions just described, all cultures that grew were in the exponential phase at the time of sampling. The lag phase lasted between 24 and 28 hr for all growing cultures. Each experiment is representative of two trials. In Fig. 2A, the inhibitor was methylalanine and in Fig. 2B, methylamine.*

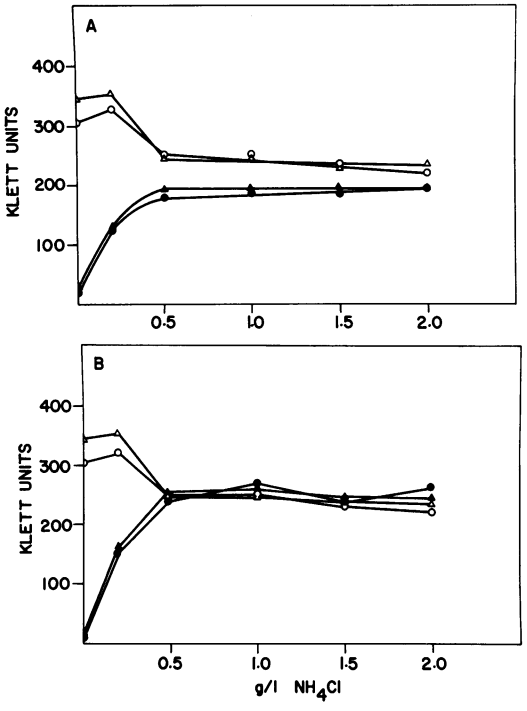

FIG. 3. *Effect of ammonium ions on growth of strains OP (△, ▲) and clr68-5 (●, ○) on DN_2 in the presence of no inhibitor (○, △) and of 1 g of inhibitor per liter (●, ▲). Details are as described for Fig. 2, except the growth period which was 32 hr. Under the conditions described, wild-type and clr68-5 cultures were in comparable stages of growth at the time of sampling: not growing in the presence of inhibitors and without NH_4^+–N; in exponential phase in the presence of inhibitor and less than 1.5 mM NH_4^+–N or in the absence of inhibitor; in early stationary phase in the presence of inhibitor and 1.5 mM NH_4^+–N or higher. The lag phase was approximately the same in all growing cultures. Each experiment is representative of three trials. In Fig. 2A, the inhibitor was methylalanine and in Fig. 2B, methylamine.*

TABLE 1. *NR in extracts of strains OP and clr68-5*[a]

Strain	Units of enzyme activity per milliliter of extract		Ratio of NR to G6PD (R)
	NR	G6PD	
OP	21, 26, 22	10, 10, 9	2.4 ± 0.1
clr68-5	0	10	0
clr68-5	0	10	0
clr68-5	<1	6.5	Negligible

[a] Cells were either pregrown in DN_2 (21) containing 5 g of added Casamino Acids per liter and subsequently induced in DN_2 containing 2.5 g of added KNO_3 per liter, or grown from a minute solid inoculum on DN_2 containing 2.5 g of added KNO_3 per liter. In three additional experiments under widely different conditions of growth, R varied in extracts of OP but was consistently negligible in extracts of clr68-5.

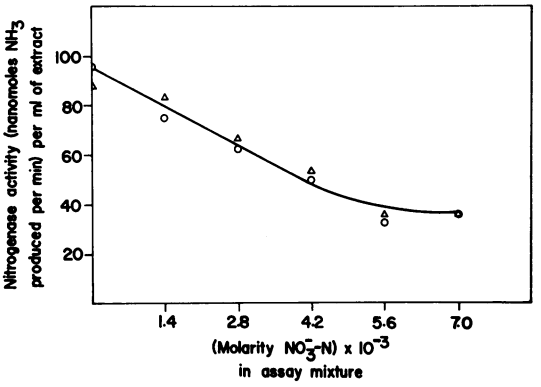

FIG. 4. *Effect of nitrate on nitrogenase activity in vitro. Extracts of fully induced cells of strains OP (△) and clr68-5 (○) were used. The assay mixture of nitrogenase contains 0.28 ml of double-distilled water. This was replaced with the desired volume and concentration of a solution of KNO_3 in double-distilled water. The ratios of nitrogenase to G6PD activities per ml of extract were 11.8 and 12.5 for strains OP and clr68-5, respectively. This experiment is representative of two trials.*

tration of nitrate-nitrogen in the induction medium which gives a 50% reduction in the level of nitrogenase in extracts of strain clr68-5 is between 4.2 and 5.6 mM. However, the final concentration of nitrate in the assay of nitrogenase in the two types of experiments is entirely different. When cells are extracted and the extract is diluted by the assay mixture, the maximal final concentration of nitrate in the enzymatic reaction medium is 15-fold less than it was in the induction medium.

There are two simple ways to interpret the above observations: (i) that *A. vinelandii* cells do not concentrate or exclude nitrate from the medium and that nitrate causes an irreversible inhibition of nitrogenase, and (ii) that *A. vinelandii* concentrates nitrate 15-fold from the medium. The latter interpretation seems unlikely. The former interpretation might help explain the observation of Stumbo and Gainey (24) that prolonged exposure to nitrate resulted in the loss of the ability of *Azotobacter* to grow on a nitrogen-free medium.

If strain clr68-5 were constitutive for nitrogenase, one might expect the formation of the enzyme to be insensitive to repression by nitrate.

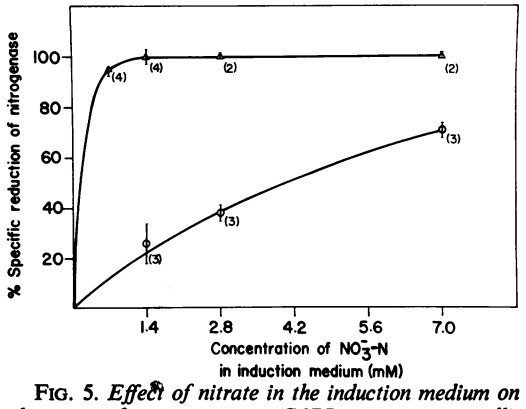

FIG. 5. *Effect of nitrate in the induction medium on the ratio of nitrogenase to G6PD activity per milliliter of extract of strains OP (△) and clr68-5 (○). Cells were grown at 27 C from a small solid inoculum into late lag phase on DN_2 containing 5 g of added Casamino Acids per liter, with moderate agitation on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). They were harvested and transferred to DN_2 containing the desired concentration of added potassium nitrate (induction medium) and incubated as before for 12 to 14 hr (induced). Per cent specific reduction of nitrogenase (P) is defined as follows: R = units of nitrogenase activity per ml of extract/units of G6PD activity per milliliter of extract; R_0 = R of an extract of cells induced in DN_2; R_x = R of an extract of cells induced in DN_2 + $xMNO_3$-N; $P = 100 - 100 (R_x/R_0)$. R_0 for strain OP was 10.1 ± 0.5 (10 experiments); for strain clr68-5 it was 11.0 ± 1.2 (9 experiments). Bars represent standard deviations from the mean; figures in parentheses represent the number of experiments from which the mean was calculated.*

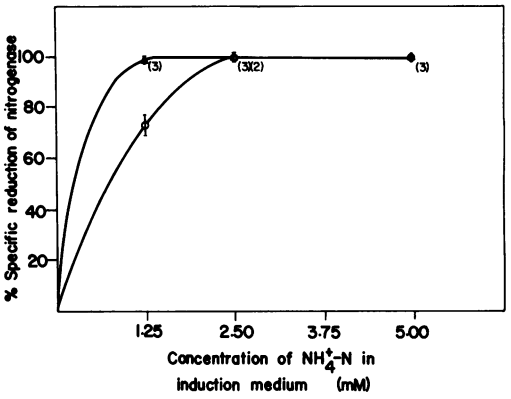

FIG. 6. *Effect of ammonia in the induction medium on the ratio of nitrogenase to G6PD activity per milliliter of extract in cell-free preparations of strains OP (△) and clr68-5 (○). Details are as described in the legend to Fig. 5. The induction media contained NH_4Cl where indicated.*

If such were the case, nitrogenase induction should be similarly resistant to the effect of ammonia, widely accepted as a repressor of this enzyme (2, 7, 10, 11, 14–16, 23). There is a small difference between the effects of ammonia on nitrogenase formation in the wild-type strain and in strain clr68-5 (Fig. 6). The difference between the comparable effects of nitrate on the wild type and on strain clr68-5 is much greater (Fig. 5). It was not possible to examine the effects of concentrations of ammonia-nitrogen in the induction medium lower than 1 mM, because at these low levels the repressive effect is confused by a stimulation of nitrogenase formation in both the wild-type strain and strain clr68-5. This stimulatory effect occurs with different nitrogen sources at low concentrations; thus, it may be due to a general stimulation of protein synthesis.

The simplest interpretation of the foregoing experiments is that nitrate inhibits the activity of nitrogenase but not its formation. Consequently, nitrate cannot be considered a corepressor of nitrogenase.

The inhibition of nitrogenase in strain clr68-5 by nitrate from the induction medium can be easily understood if nitrate is able to enter the cells of this strain.

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