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Nitrification and simultaneous denitrification by *Azospirillum brasilense* 12S

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Abstract. *Azospirillum brasilense,* an associative diazotrophs from sorghum roots grows autotrophically on NH_4^+ and $CaCO_3$. NH_4^+ a is also oxidized to NO_2^- and then denitrified. Addition of malate to the autotrophic medium enhances both NH_4^+ oxidation as well as NO_2^- dissimilation. The incomplete nitrification linked denitrification results in a rapid loss of nitrogen from the growth medium. The bacterium also shows assimilatory NO_3^- and NO_2^- reductases and fixes nitrogen at < 50 μg N/ml of NH_4^+, NO_3^- or NO_2^-.

Keywords. *Azospirillum brasilense;* autotrophic; heterotrophic; ammonia; nitrate; nitrite; nitrification; denitrification; nitrogenase.

Introduction

Azospirillum spp. are important associative nitrogen fixing bacteria of roots of grasses and cereals (Veeger and Newton, 1984). In recent years, nitrogen metabolism of these organisms has been studied by many workers (Okon *et al.,*1976; Neyra *et al.,* 1977; Magalhaes *et al.,* 1978; Scott *et al.,* 1979; Berlier and Lespinat, 1980). The azospirilla strains are known to dissimilate NO_3^- and NO_2^- under oxygen limiting conditions (Magalhaes *et al.,* 1978; Nelson and Knowles, 1978). In malate- NH_4^+ medium, the process is hastened (Okon *et al.,* 1976; Burris *et al.,* 1978). However, it is not known whether under such conditions these bacteria can also use NH_4^+ as an electron source and can dissimilate NO_2^- to gaseous nitrogen. Therefore an associative diazotroph (*A. brasilense* 12S) of sorghum was studied for its behaviour with nitrogen on compounds under autotrophic and heterotrophic conditions.

Materials and methods

A. brasilense 12S was isolated from rhizoplane of field grown sorghum (*Sorghum vulgare* L.) Var. HC 136. The culture is maintained on malate medium (Kundu *et al.,* 1985) slants by regular transfers. To determine the ability to oxidize ammonia, the culture was grown in either autotrophic medium A(g/1) $CaCO_3$ 5·0; K_2HPO_4 1·0; $MgSO_4$. $7H_2O$ 0·2; NaCl 0·1; $(NH_4)_2SO_4$ 1·0; (mg/ml) $MnSO_4$ 4·4; Na_2MoO_4 4·0; KI 0·75; $CuSO_4$. $5H_2O$ 0·25; $ZnSO_4$ $7H_2O$ 1·5 and $CoCl_2$. $6H_2O$ 0·25, medium B $CaCO_3$ was replaced with $CaCl_2$. $2H_2O$ (200mg/l) or heterotrophic medium C (medium A containing 20 mM sodium malate). Media (10 ml each) were dispensed in 30 ml tubes and sterilized. Fresh culture grown at 28°C in NH_4Cl-malate broth for 48 h having a cell population of 10^8 cells/ml was inoculated (0·1 ml/tube). The tubes were incubated at 28°C for 6 days under stationary conditions.

Abbreviation used: ARA, Acetylene reduction activity.

NO_2^-, NH_4^+ and total nitrogen were estimated at intervals of 2 days. NO_2^- and NH_4^+ nitrogen were determined in the cell free supernatant (centrifuged at 8500 *g* for 30 min). NO_2^- was estimated by sulfanilic acid method (Prince, 1945) and NH_4^+ by Nesslerization (Jackson, 1958). Total N in the samples was determined by micro-Kjeldahl method (Markham, 1942). Total bacterial numbers in the broth after 6 days of growth were determined by dilution plating on ammonium malate medium.

Nitrogenase activity was determined on malate medium slants (medium C) containing 0, 50, 100, 200 and 400 μg N/ml as $(NH_4)_2SO_4$, NH_4C1, KNO_3 or KNO_2. Five ml of the media were dispensed into 15 ml assay tubes. The slants were inoculated with fresh culture and incubated at 28°C. Acetylene reduction activity (ARA) was determined using gas chromatography at 3 day intervals upto 9 days of growth using Nucon 5500 gas Chromatograph (Hardy *et al.,* 1968).

Results and discussion

The bacterial culture 12S from sorghum roots showed typical spiral movement, expressed nitrogenase in malate solid, semi solid and liquid media. It was identified as *A. brasilense* by following the scheme suggested by Tarrand *et al.* (1978).

Table 1 shows NO_2^-, NH_4^+ and total nitrogen in the broth culture upto 6 days of growth. NH_4^+ was first oxidized to NO_2^- by the bacterium in both autotrophic (medium A) and heterotrophic (medium C) conditions of growth. However, the relative concentration of NO_2^- was higher in medium C. The NO_2^- levels after 2 days of growth in either of the media did not change significantly. Neither NO_2^- was detected nor any loss of NH_4^+ occurred in medium B since the organism did not grow in the absence of a carbon source. To determine whether NO_2^- is further oxidized to NO_3^-, the level of NO_3^- in the culture medium was also determined. However, NO_3^- was not detected, This showed that the bacterium nitrified NH_4^+ to NO_2^- both under autotrophic as well as heterotrophic conditions but the concentration of NO_2^- did not increase above 0·9 μg NO_2^-/ml of the medium. The level of NH_4^+ nitrogen in the medium decreased from 0·21 mg/ml to 0·13 and 0·07 mg/ml in medium A and C, respectively. This decrease in NH_4^+ was disproportionate to either NH_4^+ assimilation or NO_2^- accumulation, since total N (0·22 mg/ml) in both the media also decreased significantly (0·16 and 0·10 mg/ml, respectively). The loss of nitrogen therefore, appeared to be due to simultaneous NO_2^- respiration. To verify this, the cotton plugs of the tubes were replaced with suba seals at 2, 4 and 6 days of growth and incubated

Table 1. Oxidation of NH_4^+ by *A. brasilense* in malate and autotrophic media upto 6 days.

Incubation time (days)	NH_4^+ (mg/ml)		Total N (mg/ml)		NO_2^- (μg/ml)	
	A	C	A	C	A	C
0	0·21	0·21	0·22	0·22	0·00	0·00
2	0·15	0·13	0·19	0·14	0·26	0·76
4	0·13	0·11	0·17	0·12	0·28	0·84
6	0·13	0·07	0·16	0·10	0·26	0·83

A, Autotrophic medium; C, heterotrophic medium.

further for 24 h. The gas phase in the tubes was then analysed for NO and N_2O by gas chromatography using TCD and porapak column. Both NO and N_2O were detected in the gas phase suggesting denitrification of NO_2^- formed from NH_4^+.

To establish this, cells from medium C were collected after 4 days of growth by centrifugation and resuspended in 0·2 M phosphate buffer (pH 6·5) containing 1·0 μg/ml of KNO_2. After 4 h at 28°C- NO_2^- level in the supernatant was estimated. The N level decreased from 1·0–0·5 μg NO_2^- /ml indicating active respiration by the bacterium. Although NO_2^- dissimilation is known in Nir^+ strains of *A. brasilense* (Nelson and Knowles, 1978), the oxidation of NH_4^- to NO_2^- with its subsequent dissimilation suggests an unusual respiratory mechanism in this bacterium.

To confirm autotrophic growth in NO_3^- medium, total viable cell counts were made after 6 days of growth. The counts were 14×10^6,12×10^4 and 50×10^7 cells/ml in media A, B and C, respectively, indicating that NO_3^- supports growth with NH_4^+ serving both as N_2 as well as an electron donor in medium A. Autotrophic growth was slower than in heterotrophic medium. Since the strain showed incomplete nitrification associated with NO_2^- dissimilation, growth and nitrogenase induction studies were carried out in media containing variable concentration of NH_4^+, NO_3^- and NO_2^- in malate medium. $(NH_4)_2SO_4$ or NH $_4$C1 (50 μg N/ml) did not affect the ARA. However, at this concentration of KNO_3 or KNO_2 nitrogenase induction was delayed upto 6 days (table 2). The higher ARA (16·0 n mol C_2H_4/h/tube) was recorded with $(NH_4)_2SO_4$. The increased nitrogenase activity was found at 6th day of incubation. No activity was detected at 100, 200 and 400 μg N/ml of $(NH_4)_2$ SO_4, NH_4Cl, KNO_3 or KNO_2 even upto 9 days. The bacterium showed growth on slants with different nitrogen sources which confirmed the presence of assimilatory enzymes for all these compounds.

Table 2. Effect of nitrogen sources on nitrogenase activity of *A. brasilense* in malate medium.

Incubation time (days)	Nitrogenase activity (n mol C_2H_4/h/tube)				
	NO–N	NH_4Cl	$(NH_4)_2SO_4$	KNO_3	KNO_2
0	0·0	0·0	0·0	0·0	0·0
3	3·0	2·6	2·3	0·0	0·0
6	10·0	14·2	16·0	11·0	3·8
9	8·2	8·9	6·5	2·5	3·0

N concentration 50 µg N/ml.

Azospirilla are considered to have a potential in cereals as N_2 fixers. Present studies have exposed a major limitation of these bacteria in plant nitrogen economy. As these crops are generally raised with inorganic nitrogenous fertilizers, the assimilatory enzymes in these bacteria could be of ecological advantage for the initial rapid multiplication in the plant rhizosphere. However, the unusual metabolism of NH_4^+, where NH_4^+ is oxidised to NO_2^- which is then simultaneously dissimilated can cause loss under microaerophilic conditions.

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