Applied Biochemistry and Microbiology, Vol. 38, No. 6, 2002, pp. 558–561. Translated from Prikladnaya Biokhimiya i Mikrobiologiya, Vol. 38, No. 6, 2002, pp. 649–652.

Reduction of Nitrates by *Azotobacter indicum* and *Azotobacter chroococcum* Cultures

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Received April 12, 2002

Abstract—The capacity for denitrification was studied in *Azotobacter* bacteria, which are free-living nitrogen-fixing obligatory aerobes. Data on nitrate reduction to nitrites and nitric oxide by *A. indicum* under anaerobic conditions were obtained for the first time for genus *Azotobacter.*

Various microorganisms can reduce nitrates. Both nitrite and nitrous oxide can be stable intermediate products. Denitrifying bacteria can switch between aerobic metabolism and anaerobic reduction of nitrate due to the close values of the redox potentials of oxygen and nitrate. Anaerobic reduction of nitrates is characteristic of microorganisms of different taxonomic groups [1, 2]. For example, we showed the diazotrophic microaerophilic bacteria *Rhizobium, Bradyrhizobium,* and *Azospirillum* to possess a capacity for dissimilating nitrate reduction related to a component of the nitrogen cycle, namely, nitrogen fixation [3–6]. Regarding obligatory aerobes, there are only fragmentary reports on the possibility of reduction of nitrates to nitrous oxide by aerobic *Azotobacter* bacteria [7].

We tested free-living nitrogen-fixing *Azotobacter aerobes, A. chroococcum* and *A. indicum*, for the ability to reduce nitrates under anaerobic conditions.

MATERIALS AND METHODS

The *A. indicum* strain 8 and *A. chroococcum* strain C_8 were used in the work. The collection *Azotobacter* strains were maintained on the Ashby medium containing (g/l) K_2HPO_4 (0.2), $MgSO_4$ (0.2), NaCl (0.2), $Na_2MoO_4 \cdot 2H_2O$ (0.006), $CaCO_3$ (5.0), sucrose (20), and agar (20). All liquid nutrient media were based on the modified Burk medium containing (g/l) $MgSO_4$ (0.4), $FeSO_4$ (0.01), Na_2MoO_4 (0.006), sodium citrate (0.5), $CaCl_2$ (0.1), $K_2HPO_4 \cdot 3H_2O$ (1.05), KH_2PO_4 (0.2), and glucose (20). In experiments with nitrates, KNO_3 (5 g/l) was added (if not indicated otherwise in Table 1).

The experiments shown in Table 1 were performed in 50-ml flasks. Each flask contained 25 ml of the medium. A 48-h culture of *Azotobacter* grown on the agar Ashby medium at 28°C was used as a plating material. In anaerobic experiments, the flasks were washed twice with argon, filled with a gas mixture containing C_2H_2 and N_2 (10 and 90%, respectively), and closed with rubber stoppers. In microaerophilic experiments, the flasks were filled with a mixture of the following composition (%): C_2H_2 (10), N_2 (80), O_2 (10), and CO_2 (0.03). Table 2 and Figs. 1 and 2 show the results of the experiments in which the biomass initially (at the first stage) was grown aerobically on the Burk or Ashby medium in the presence or in the absence of nitrates under the conditions of nitrogen fixation. Then (at the second stage), the biomass of the cultures (prepared by centrifugation under aseptic conditions) was placed under either anaerobic or aerobic conditions on the nitrate-containing Burk medium and incubated at 28°C in triplicate. To determine the nitrate quantity, 1 ml of the suspension resulting from centrifugation at 8000*g* for 15 min was supplemented with 0.5 ml of sulfanilic acid and 0.5 ml of *N*-(1-naphthyl)ethylenediamine. After the 15 min necessary for the staining development, the nitrate quantity was determined spectrophotometrically at 548 nm [8]; the nitrogen-fixing activity was determined by the acetylene method [9]. The ethylene production was recorded using a Khrom-3

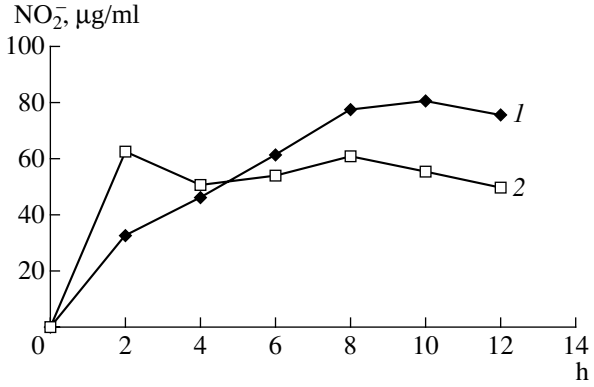

Fig. 1. Nitrate reduction to nitrites under anaerobic conditions by a suspension of *Azotobacter indicum* 8 cells growing (*1*) under aerobic conditions in the presence of 5 g/l KNO_3 or (*2*) under the conditions of nitrogen fixation in the absence of nitrate. The medium contained KNO_3 (5 g/l).

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Table 1. Effects of oxygen and nitrate concentrations on the growth* and nitrogen fixation in two *Azotobacter* strains

Strain	Variant		Biomass, OD_{550} after 48 h	C_2H_4, nM/mg dry biomass	Residual O_2, %
	O_2, %	NO_3^-, g/l			
A. indicum 8	0	0	0.20	364	0.0
	20	0	0.30	809	4.0
	0	5	0.20	333	0.0
	20	5	0.50	11	0.4
	10	2.5	0.20	304	0.4
A. chroococcum C_8	0	0	0.25	252	0.0
	20	0	0.40	580	3.2
	0	5	0.25	31	0.0
	20	5	0.60	4	2.8
	10	2.5	0.25	29	0.9

* Optical density (OD) was measured in a 1-mm cuvette; the initial OD_{550} values of *A. indicum* 8 and *A. chroococcum* C_8 were 0.20 and 0.25, respectively; the growth duration was 48 h.

gas chomatograph (Czech Republic) equipped with a flame-ionization detector on a column with ASK silica gel (column length, 120 cm; internal diameter, 5 mm; evaporator temperature, 80°C; temperature in the constant-temperature cabinet, 50°C). The gas carrier (nitrogen) expenditure was 40 ml/min. Nitrous oxide (N_2O) was assayed using an LKhM-8MD gas chromatograph (Soviet Union) with a heat-conductivity detector (detector temperature, 40°C), on a column with Porapac Q (the column length, 150 cm; the internal diameter, 2.5 mm) at room temperature. The gas carrier (hydrogen) expenditure was 40 ml/min. The gases O_2 and N_2 were analyzed using an LKhM-8MD gas chromatograph with a heat-conductivity detector (detector temperature, 40°C), on a column with a 13X molecular sieve (column length, 120 cm; internal diameter, 5 mm) at room temperature. The gas carrier (argon) expenditure was 40 ml/min. In all cases, the separation was performed at room temperature. Nitric oxide (NO) was assayed using an ECOPHYSYC CLD 500 AL NO analyzer (Germany). The content of poly-β-butyrate was determined by means of IR spectrophotometry [10]. The culture growth was monitored nephelometrically. The protein content was determined by the Lowry method [11]. For assay of the molybdenic cofactor (MoCo), the cells were destroyed by grinding in liquid nitrogen and centrifuged at 15 000 *g* to prepare a cell-free extract. The MoCo assay included isolation of the cofactor from MoCo-containing enzymes by heating, subsequent complementation of MoCo with an imperfect nitrate reductase apoprotein of the *Neurospora crassa* nit-1 mutant, and the assay of the reconstructed nitrate reductase [12]. The activity of NADH-dependent nitrate reductase was measured in 1 ml of a reaction mixture of the following composition (mM): potassium phosphate buffer (pH 7.0) (50), KNO_3 (10), Na_2MoO_4 (2.5), NADH (0.3), and PAD (0.01). The reaction was initiated by addition of NADH to the reaction mixture. After incubation for 20 min at 30°C, the nitrite content was estimated as described above.

The activity of nitrate reductase provided with electrons by methyl viologen reduced with dithionite was measured in 1 ml of a reaction mixture of the following composition (mM): sodium phosphate buffer (pH 7.0) (40), methyl viologen (0.8) or bromophenol (0.5), $NaNO_3$ (2.5), and dithionite (4.6). The reaction was initiated by addition of dithionite to the reaction mixture. Then, the reaction mixture was shaken until decoloration and supplemented with the reagents to assay nitrite as described above.

Table 2. Production of nitrites and nitric oxide by *A. indicum* 8 and *A. chroococcum* C_8 cell suspensions under anaerobic conditions at different nitrate concentrations in the medium*

KNO_3, mg/ml	*A. chroococcum* C_8		*A. indicum* 8	
	NO_2^-, μg/mg dry weight	NO, ppmv	NO_2^-, μg/mg dry weight	NO, ppmv
0.5	25.0	0.7	545.0	57.3
1	11.7	0.7	408.0	31.2
2	15.0	0.7	341.2	31.2
5	32.5	1.0	767.5	26.4
7.5	10.0	0.9	608.0	81.4
10	15.0	1.1	305.0	73.6

* The exposure time was 48 h. OD_{550} of the cell suspension in a cuvette with an optical path of 1 mm was 0.40. The cell suspension was prepared after the growth of bacterial cultures for 48 h on the Ashby medium under the conditions of nitrogen fixation (in the absence of nitrate).

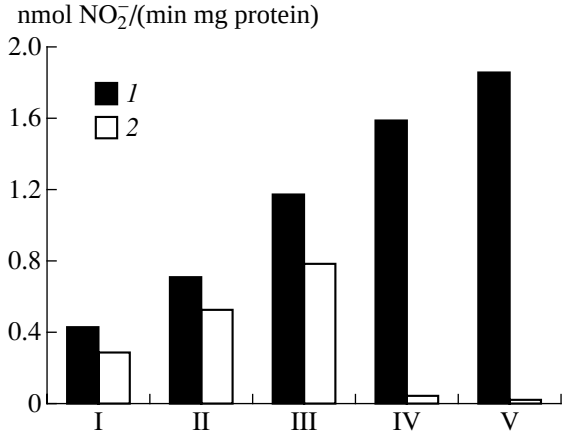

Fig. 2. The activities of nitrate reductase (nmol NO_2^-/(min mg protein)) in the presence of different electron donors: (II) methyl viologen, (III) bromophenol, (IV) NADH, and (V) NADPH; (I) the content of molybdenum cofactor (MoCo) in the *Azotobacter indicum* 8 growing (*1*) in the presence and (*2*) in the absence of nitrate.

RESULTS AND DISCUSSION

In diazotrophs, there is a close relationship between the energy-consuming processes of nitrogen fixation, respiration, and synthesis of reserve biopolymers [13, 14]. From the collection of *Azotobacter* strains, two strains were chosen that were the most different in the parameters specific for diazotrophs: the *A. chroococcum* strain C_8, capable of hypersynthesis of poly-3-oxybutyrate (as much as 85% of the cell dry weight) and with a low activity of nitrogenase, and the *A. indicum* strain 8, with a low content of poly-3-oxybutyrate (5% of the cell dry weight) but capable of active fixation of molecular nitrogen.

Table 1 shows the effects of oxygen concentration in the gas phase and of nitrates on the growth of cultures and on the nitrogenase activity. Both *A. chroococcum* C_8 and *A. indicum* 8 displayed the maximum growth under aerobic conditions (20% O_2) on the medium containing nitrate at a concentration of 5 g/l. If the oxygen level was decreased to 10% and the nitrate content to 2.5 g/l, both cultures did not grow, nor was growth observed in any culture under anaerobic conditions. Not considering in detail the data on nitrogen fixation (they are typical of nitrogen-fixing microorganisms) and on the nitrate-induced inhibition of nitrogenase, note that the nitrogen fixation was increased under anaerobic conditions. This was especially pronounced in the case of *A. indicum*: the nitrogen-fixing activity in the presence of nitrates under aerobic conditions and under anaerobic conditions was 11 and 333 nmol C_2H_4 per mg dry weight, respectively.

Because both cultures did not grow under anaerobic conditions, the subsequent experiments were performed with cell suspensions growing aerobically on nitrate-containing media or under the conditions of nitrogen fixation in the absence of nitrates.

Table 2 shows the data on intermediate products of nitrate reduction by suspensions of *A. indicum* 8 and *A. chroococcum* C_8 cells growing under the conditions of nitrogen fixation. Both cultures reduced nitrate to nitrite under anaerobic conditions. The nitrite amount produced by the *A. indicum* 8 culture was an order of magnitude higher than the amount produced by *A. chroococcum* C_8.

Table 2 shows that a nitrate concentration of 5 g/l was optimal for nitrate reduction to nitrite for both *A. chroococcum* and *A. indicum.* At this concentration of nitrate, *A. chroococcum* C_8 and *A. indicum* 8 produced 32.5 and 767.5 μg NO_2^- per mg dry weight, respectively. Higher concentrations of nitrate decreased the reducing ability of bacteria but were not inhibiting. Nitrous oxide (N_2O) was found in none of the experiments. The NO_3^- reduction to nitric oxide in *A. indicum* is of interest (Table 2): the maximum production of NO (81.37 ppmv) was observed in the medium containing 7.5 g/l KNO_3. It seems that, in *A. indicum*, a detoxification mechanism of the nitrite produced is switched on, with a subsequent induction of nitrite reductase and then with a further reduction of NO_2^- to NO, which is characteristic of denitrifying bacteria. The cells were likely to use this mechanism to ensure their survival during anoxia when oxygen was replaced by another electron acceptor (NO_3^-).

Figure 1 shows the reduction of nitrates by *A. indicum* growing in the presence and in the absence of nitrate. A successive reduction of NO_3^- to NO_2^- was specific for the culture growing in the presence of nitrate. After exposure for 10 h, the nitrite level in the medium was stabilized, which was apparently connected with the nitrite reductase induction. The cells growing in the absence of nitrate actively reduced NO_3^- to NO_2^- during the first 2 hours (up to 60 μg NO_2^- was produced per mg dry weight), and the level of nitrite in the medium somewhat decreased later. The comparison of changes in the NO_2^- accumulation by cells growing in the presence and in the absence of nitrate (the lag phase was absent in both cases) suggested that nitrate reductase in this culture should be constitutive.

The nitrate reductase activity was estimated using different electron donors in *A. indicum* growing in the presence and in the absence of nitrates, and the nitrate reductase of this strain growing in the presence of nitrate had a complete chain of electron transport (Fig. 2), which included both the diaphorase and terminal components (NADH, NADPH, and methyl-viologen- and bromophenol-dependent activities). In the case of cell growth under the conditions of nitrogen fixation, only the terminal component of the *A. indicum* nitrate reductase electron-transport chain (the methyl-viologen- and bromophenol-dependent activities) was active. *A. vine-*

landii growing in the presence of nitrates had a shortened nitrate reductase (with methyl viologen as an electron donor) [15]. Molybdenic cofactor is the active site of nitrate reductase. The concentrations of MoCo differed little in the *A. indicum* cells growing in the presence and in the absence of nitrates (Fig. 2).

Thus, the reduction of NO_3^- to NO_2^- occurred in both cultures studied (*A. indicum* 8 and *A. chroococcum* C_8). In *Azotobacter indicum*, the nitrate reductase activity leading to NO_2^- production was followed by induction of the nitrate reductase and nitric oxide formation. The nitrate reductase of *A. indicum* seems to be an enzyme with a complete chain of electron transport, unlike the nitrate reductases of the majority of *Azotobacter* bacteria, which have a shortened chain of electron transport, have no diaphorase component of the enzyme, and use reduced ferredoxin as an electron donor.

The ability of *Azotobacter indicum* to reduce nitrates under anaerobic conditions is interesting from the biochemical and biotechnological viewpoints.

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