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EXPERIMENTAL ARTICLES

Activity of Nitrate Reductase in *Desulfovibrio vulgaris* VKM 1388

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Abstract—Evidence was obtained of the inhibitory effect of nitrate on the metabolism of *Desulfovibrio vulgaris* 1388. Nitrate is reduced only at low concentrations and in the presence of sulfate in the medium. Genetic data suggest that the genome of *D. vulgaris* 1388 contains the information about the γ subunit and possibly the NarG catalytic subunit of the membrane-bound nitrate reductase.

Key words: Desulfovibrio vulgaris, nitrate reduction, membrane-bound nitrate reductase.

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Nitrate reduction is not believed to be a common feature of the genus *Desulfovibrio*. However, cultivation conditions may affect their ability to exhibit of the activity of nitrate reductase [1]. In sulfate-reducing bacteria (SRB), various pathways of the regulation of nitrate reduction have been described. For example, in suspensions of *D. desulfuricans* C4S cells, nitrate is an electron acceptor preferred to sulfate; however, inducible nitrate reductase activity was not detected in the cells grown with excess of sulfate in the medium [2]. Four *Desulfovibrio* strains with constitutive nitrate reductase activity were capable of nitrate reduction only in the media containing low amounts of sulfate [3]. *D. desulfuricans* DT101 reduced nitrate and sulfate simultaneously [4]. In *D. desulfuricans* FBA 20a, nitrate reductase was induced in the presence of nitrate and repressed in the presence of sulfate [5], while in *D. desulfuricans* Essex 6 nitrate repressed sulfate reduction [6].

This diversity in the regulation of nitrate reduction within one genus results probably from the differences in the structure of nitrate reductases or from their combinations in different bacteria. All bacterial nitrate reductases belong to three groups, differing in their functions and intracellular localization, namely cytoplasmic assimilatory, periplasmic dissimilatory, and membrane-bound nitrate reductases [7]. The latter groups contain the catalytic subunits (NapA and NarG, respectively), which contain a *bis*-molybdopterin guanidine nucleotide (*bis*-Mo-MGD) and [4Fe-4S] clusters, as well as the subunits responsible for electron transfer from the quinole pool to NarG or NapA. The results of biochemical and spectroscopic investigation of bacterial nitrate reductases demonstrate that membrane-bound and periplasmic nitrate reductases may perform different physiological functions [8].

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Molecular genetic data exist only for two SRB nitrate reductases, NAP from *D. desulfuricans* ATCC 27774 [9, 10] and the γ subunit of *D. desulfuricans* G20 (GenBank accession no. NC007519). Investigation of dissimilatory nitrate reductases from other *Desulfovibrio* species is therefore important.

The goal of the present work was investigation of nitrate reduction and the search for its genetic determinants in *D. vulgaris* 1388.

MATERIALS AND METHODS

D. vulgaris 1388 (previously *D. desulfuricans* 1388) was obtained from the All-Russian Collection of Microorganisms (Pushchino, Russia). The strain was grown anaerobically in 15-ml penicillin vials (for growth experiments) or in 500-ml bottles (to obtain biomass); the SRB medium was used [11]. A trace elements solution [11] (1.0 ml) and yeast extract (0.1 ml) were added to the medium. Sulfate and nitrate were introduced in the amounts determined by the experimental conditions. Bacterial growth was assessed as OD_{600} on a Specol 211 spectrophotometer.

Bacterial protein was determined by the modified Lowry method [12].

Nitrate and sulfate were determined by the standard procedures [13, 14].

All the biochemical and spectrometric data are averages of the results of two independent experiments with three repeats each.

DNA isolation was carried out by the standard phenol–chloroform method followed by DNA precipitation with ethanol in the presence of 3 M potassium acetate [15].

Polymerase chain reaction (PCR) was carried out with the oligonucleotide primers developed basing on the nucleotide sequences flanking the *D desulfuricans*

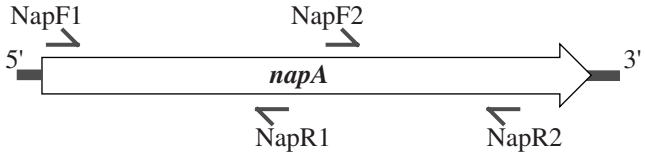

Fig. 1. Localization of the oligonucleotide primers constructed on the basis of the *napA* gene sequence from *D. desulfuricans* ATCC 27774.

ATCC 27774 *napA* gene (no. NC002937 GenBank) (Fig. 1): F1: 5'-TTTTGCCATGTCCGC-3'; R1: 5'-TGCCCATACACCAGAG-3'; F2: 5'-GCCATTC-CCAACGCCAAAC-3'; R2: 5'-TGCCCGTCATGGT-GCCGT-3'; the oligonucleotide primers complementary to the γ subunit of the membrane-bound nitrate reductase from *D. desulfuricans* G20: F3: 5'-CCGTTC-CTTTCCGCATCCC-3'; R3: 5'-TACTTCTTGGGC-GGGTTCC-3'; and the primers complementary to NarG: F4: 5'-CACCCATGGCGTCAACT-3'; R4: 5'-GAGGCAAATCGGCGTAC-3'. PCR was carried out in a Tertsik thermal cycler (DNK Technologiya, Russia). The volume of the reaction mixture was 25 μl. The programs were as follows: for NapA, 95°C, 3 min (1 cycle); 95°C 20 s (25 cycles); 60°C 30 s (25 cycles); 72°C 40 s (25 cycles); 72°C 10 min (1 cycle); for the γ subunit: 95°C 3 min (1 cycle); 95°C 20 s (25 cycles); 62°C 20 s (25 cycles); 72°C 30 s (25 cycles); 72°C 10 min (1 cycle); for NarG: 95°C 3 min (1 cycle); 95°C 30 s (25 cycles); 59°C 30 s (25 cycles); 72°C 30 s (25 cycles); 72°C 5 min (1 cycle). The PCR products were separated by electrophoresis in 2% agarose in Tris–acetate buffer (pH 8.0–8.2). GeneRules DNA Ladder (Fermentas, Lithuania) was used as a molecular mass marker. The amplicon sizes were calculated using the DNASIS v. 3.0 software package; the nucleotide sequences were analyzed with the Vector NTI 7.1 software package (InforMax, Inc.).

RESULTS AND DISCUSSION

We have previously demonstrated nitrate formation in the course of nitrocellulose transformation by desulfovibrios [16]. Its subsequent reduction to ammonium indicated a dissimilatory type of nitrate reductase activity. In the present work, reduction of nitrate per se by *D. vulgaris* 1388 was investigated. The cells were grown on lactate (32 mM) in the presence of sulfate (20 mM) to the late exponential phase and collected by centrifugation under oxygen-free conditions (5000 *g*, 4°C). The cells were then washed twice with an argon-saturated medium without electron acceptors and resuspended in the same medium; protein concentration was 1.31 mg/ml. Addition of sulfate and nitrate (at ~7 mM each) to the cell suspension resulted in simultaneous reduction of both acceptors (Fig. 2). This was an indication of the constitutive nature of nitrate reductase in *D. vulgaris* 1388. No significant difference was

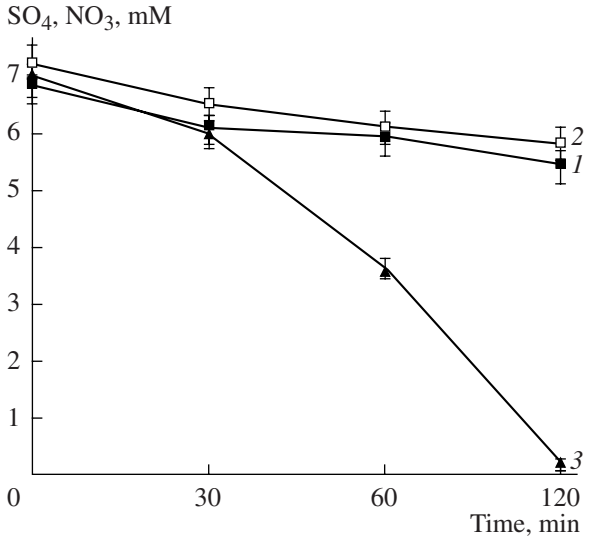

Fig. 2. Concentrations of nitrate and sulfate in the medium with *D. vulgaris* 1388 cell suspension in the absence of sulfate (*1*); in the presence of sulfate (*2*); sulfate consumption (*3*).

detected between the rates of nitrate reductions in the presence and in the absence of sulfate (0.72 ± 0.036 and 0.70 ± 0.032 μmol NO^{3-} ml^{-1} h^{-1}, respectively, was reduced). The rate of nitrate reduction was, however, significantly lower than the rate of sulfate reduction (3.4 ± 0.17 μmol SO_4^{2-} ml^{-1} h^{-1}). After 120 min, only ~20% of nitrate was reduced; by this time, sulfate was almost completely removed from the medium.

Nitrate reduction by growing cultures was studied in the media containing nitrate (10 mM) in the presence or absence of sulfate (10 or 10 mM). After 10 days, no growth and removal of nitrate or sulfate was observed. This concentration of nitrate suppressed sulfate reduction, while nitrates were not utilized as alternative electron acceptors.

In subsequent experiments, the nitrate concentration was decreased, and nitrate reduction was observed, albeit at a low rate (Fig. 3). Although the culture was grown under sulfate limitation (10 mM), the additional electron acceptor (nitrate) did not cause an increase in biomass. Moreover, increased nitrate concentrations resulted in its increased inhibitory effect on bacterial growth. The causes of such an effect of nitrate on *D. vulgaris* 1388 growth are unclear. According to the published data, nitrate was either not utilized by SRB cells and had no inhibitory effect on cell growth (as in the case of *D. vulgaris* Hildenborough [17]) or supported bacterial growth, acting as an electron acceptor and competing with sulfate.

The physiological function of nitrate reductase in *D. vulgaris* 1388 is therefore unclear. Nitrate reduction by *D. vulgaris* 1388 is evidently not energetically profitable. At low nitrate concentrations, nitrate reductase probably plays a protective role. Unlike sulfate, nitrate

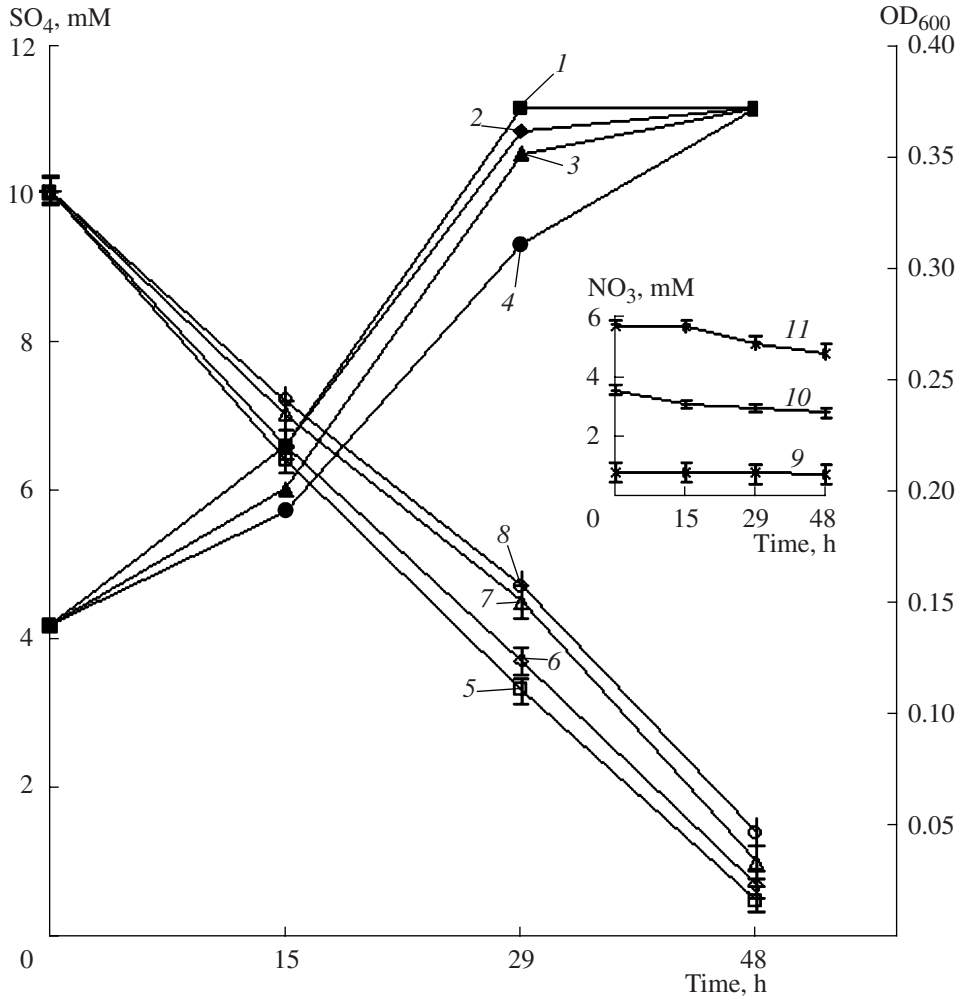

Fig. 3. Residual concentrations of sulfate and nitrate in the course of *D. vulgaris* 1388 growth: OD_{600} (*1*) and sulfate (*5*) in the control, without nitrate; OD_{600} (*2*) and sulfate (*6*) in the presence of 0.74 mM nitrate (line *9* in the insert); OD_{600} (*3*) and sulfate (*7*) in the presence of 3.48 mM nitrate (line *10* in the insert); OD_{600} (*4*) and sulfate (*8*) in the presence of 5.63 mM nitrate (line *11* in the insert).

is known to create a higher redox potential [18]; this may cause intracellular damage affecting bacterial metabolism. Interestingly, addition of nitrate to the final concentration of ~4 mM in the mid-exponential growth phase did not result in nitrate reduction or changes in the growth rate. Thus, the physiological state of the culture or some other factor determined its insensitivity to nitrate.

As for the genetic determinants encoding nitrate reductaseactivity in *D. vulgaris* 1388, we suggested that it may be determined by the presence of the genes encoding either the periplasmic (NAP) or the membrane-bound nitrate reductase (NAR). The presence of a periplasmic nitrate reductase in *D. desulfuricans* ATCC 27774 was initially reported by Bursakov et al. [19]. Analysis of the genomes of *D. vulgaris* Hildenborough (NC002937, GenBank) and *D. desulfuricans* G20 (NC007519, GenBank) revealed the presence of a NAR γ subunit; other subunits, however, were not present.

Our previous experiments revealed that the periplasmic fraction is responsible for only ~15% of the activity of nitrate reductase in *D. vulgaris* 1388 [16].

PCR analysis of *D. vulgaris* 1388 DNA with the primers complementary to the 5' terminus of *D. desulfuricans* ATCC 27774 *napA* revealed three major signals. This was an indication of several homological sites or of the nonspecificity of the primers used. The primers to the 3' terminus of the gene revealed an amplicon of 1600 bp, significantly higher than the expected size calculated *in silico* (787 bp).

PCR analysis of *D. vulgaris* 1388 DNA with the primers complementary to the γ subunit of *D. desulfuricans* G20 revealed a 730-bp PCR product (Fig. 4); this value is in good agreement with the theoretically calculated one (748 bp). Considering the differences in the amino acid sequences of the periplasmic and mem-

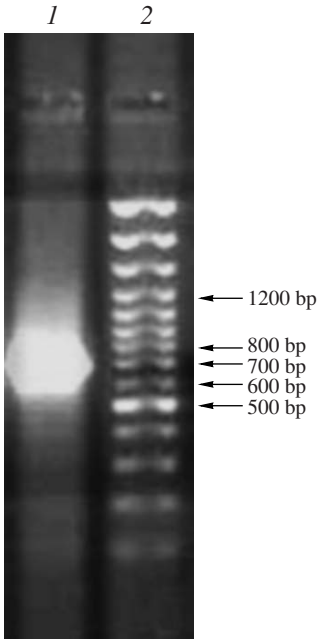

Fig. 4. Electrophoresis of the PCR products of *D. vulgaris* 1388 genomic DNA with the primers complementary to the γ subunit of *D. desulfuricans* G20: PCR product of the *D. vulgaris* 1388 genomic DNA (*1*); DNA marker (*2*).

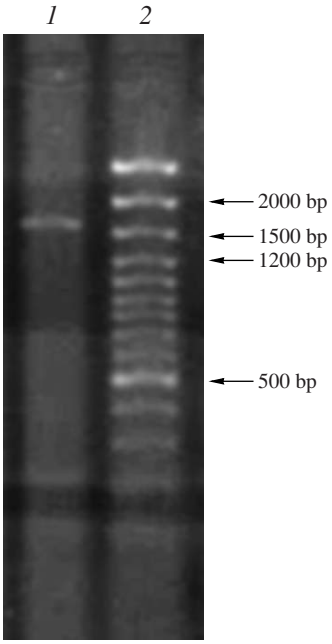

Fig. 5. Electrophoresis of the PCR products of *D. vulgaris* 1388 genomic DNA with the primers complementary to the *narG* nucleotide sequence of some prokaryotes: PCR product of the *D. vulgaris* 1388 genomic DNA (*1*); DNA marker (*2*).

brane-bound nitrate reductases [7], we attempted identification of the catalytic subunit of *narG*, the membrane-bound nitrate reductase revealed in the genome of *D. vulgaris* 1388. Numerous *narG* sequences of prokaryotic nitrate reductases were analyzed in the course of primer development. The ~1600-bp PCR product was obtained using the *D. vulgaris* 1388 genomic DNA and the primers constructed for *narG* (Fig. 5).

Our results suggest that NAR is present in the cells of *D. vulgaris* 1388. However, complete sequencing of the *narG* subunit is required for the final conclusions.

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