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A strain of *Arthrobacter* that tolerates high concentrations of nitrate

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Abstract

A gram-positive strain identified as *Arthrobacter globiformis* CECT 4500, tolerant to up to 1 M nitrate, was isolated from the grounds of a munitions factory. Under strict aerobic conditions, this bacterium used a wide variety of C-sources to obtain the energy required for growth, which took place when the nitrate concentration in the medium was below 150 mM. Cells of this bacterium growing in the absence of nitrate were seen as individual cells or forming pairs, whereas cells grown in the presence of nitrate formed short filaments. With ethylene glycol as the C-source, optimal conditions for the full nitrate removal by *Arthrobacter* were established under laboratory conditions with wastewaters from the synthesis of dinitroethylene glycol.

Introduction

Two important roles that nitrate reduction plays in bacterial physiology are nitrogen assimilation and anaerobic respiration (Stewart 1988; Zumft 1992). Assimilatory nitrate reduction involves the stepwise reduction of NO_3^- to NO_2^- in a 2-electron reaction mediated by nitrate reductase, and the subsequent reduction of NO_2^- to NH_3 involving 6 electrons in a reaction mediated by nitrite reductase (Guerrero et al. 1981). In the absence of oxygen, nitrate can serve as an alternative electron acceptor in respiratory chains in certain microorganisms (Zumft 1992). Some bacteria, such as *Klebsiella pneumoniae, Klebsiella oxytoca*, and *Pseudomonas aeruginosa*, are able to carry out both assimilatory and respiratory nitrate reduction (Riet et al. 1968; Bender & Friedrich 1990; Cali et al. 1989). Other bacteria, such as *Salmonella typhimurium*, grow anaerobically by using nitrate as a terminal electron acceptor (Magasanik & Neidhardt 1987). Many strict aerobic bacteria can use nitrate as the sole N-source after its assimilatory reduction (Ogawa et al. 1995; Nakano et al. 1995).

Wastewater with high levels of nitrate are generated in industrial processes involving the nitration of organic molecules to generate explosives, pharmaceuticals and fertilizers (Clarkson et al. 1991). Wastewaters from the cleaning of dairy fermentors and nuclear reactors also contain high nitrate loads (Walker et al. 1989; Pitt et al. 1981). Nitrate-utilizing microorganisms could be used to remove nitrate, but high nitrate concentrations are usually detrimental for cells and inhibit growth and lead to futile nitrate reduction (Krishnamachari & Clarkson 1993).

As part of a research project focused on the biological treatment of these wastewaters, we attempted to isolate microorganisms able to thrive on high concentrations of NO_3^-, owing to their ability to remove nitrate without accumulating nitrite. We report the isolation of a gram-positive strict aerobe strain identified as *Arthrobacter globiformis*, which can use nitrate as the sole source of nitrogen through the assimilatory pathway. This strain is able to tolerate concentrations of up to 1 M nitrate. The strain was grown in a specific mineral medium, which allowed us to identify reproducible growth conditions under laboratory conditions.

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Materials and methods

Microorganisms, growth media and growth conditions

Arthrobacter globiformis CECT 4500 was isolated in this study as able to grow with nitrate as an N-source, and with ethylene glycol as the sole C- and energy source. This clone was usually grown on M8 minimal medium (Abril et al. 1989) composed (per l water) of: Na_2HPO_4, 2.7 g; KH_2PO_4, 3 g; NaCl, 0.5 g; $MgSO_4$, 120.4 mg; ammonium ferric citrate; 6 mg; $ZnCl_2$, 0.125 mg; $MnCl_2 \times 4H_2O$, 0.075 mg; H_3BO_3, 0.75 mg; $CoSO_4 \times 7H_2O$, 0.5 mg; $CuCl_2 \times 2H_2O$, 0.025 mg; $NiCl_2$; 0.05 mg and $NaMoO_4$, 0.075 mg. This medium was supplemented with approximately 10 g l^{-1} ethylene glycol and 20 mM of KNO_3 from a commercial source or present in wastewater from the Unión Española de Explosivos factory in Quintanilla Sobresierra, near Burgos (Spain). The composition of these wastewaters was described previously (Ramos et al. 1996). When necessary the bacterium was grown on LB medium (Sambrook et al. 1989).

Arthrobacter globiformis CECT388, and *Bacillus subtilis* were from the Spanish Type Culture Collection and from our laboratory collection, respectively. These strains grow on the minimal medium described above with 20 mM NO_3^- as the sole N-source, and glycerol or glucose as the sole C-source.

Cultures were incubated in a rotary shaker at 200 strokes per min at 30 °C in conical flasks. Where indicated, bacteria were grown in a 2-1 bioreactor (Biostat B, Braun-Biotech) under the operational conditions given in the Results section.

Analytical methods

Compounds present in the culture medium were analyzed after the cells had been removed by centrifugation (5000 *g* for 14 min). Nitrite was measured by the method of Snell & Snell (1949); nitrate was determined by using a specific electrode and a potentiometer (micropH 2002, Crison). Ethylene glycol was determined by gas chromatography as described before (Ramos et al. 1996). Biomass was measured as dry weight (Egli et al. 1991).

Assay of nitrate reductase activity

Nitrate reductase was assayed in mixed alkyltrimethylammonium bromide (MATAB) permeabilized whole cells. Cultures of *A. globiformis* CECT 4500 containing 95–175 μg protein ml^{-1} were centrifuged at 12000 *g* for 1 min and resuspended at the same density in MOPS buffer (pH 7.0) supplemented with 2 mg MATAB ml^{-1}. After incubation for 30 min at 4 °C the cells had reached maximal permeabilization as shown by maximal nitrate reductase activity. Nitrate reductase activity was assayed in MOPS buffer basically as described previously (Manzano et al. 1976). One unit was defined as μmoles of nitrite produced min^{-1}.

Electron microscopy

Arthrobacter globiformis CECT 4500 was grown in LB or minimal medium with different initial concentration of NO_3^-. Cells were harvested by centrifugation and prepared for electron microscopy as described by Rodríguez-Herva et al. (1996).

Results

Isolation of A. globiformis *from the grounds of a munition factory*

We previously found that microbes in agricultural soils and sewage treatment plants are sensitive to high concentrations of NO_3^-. In contrast, bacteria tolerant to high nitrate levels were present in the ground of a munition factory (Piñar et al. 1997; Ramos et al. 1996). We set up classical enrichment cultures to isolate NO_3^--tolerant strains, in which minimal medium was supplemented with 100 mM NO_3^- as the N-source and 20–50 g l^{-1} ethylene glycol as the sole C-source. Six independent isolates able to grow on ethylene glycol were isolated. The clone that grew the fastest was kept for further analysis. This isolate was gram-positive and oxidase-negative. Analyses of the methyl ester derivatives of total phospholipids identified the strains as *Arthrobacter globiformis* and it was deposited in the Spanish Type Culture Collection (CECT) with the accession number 4500.

A. globiformis CECT 4500 used glycerol, glucose, fructose, sucrose, galactose, lactose, acetic acid and formaldehyde in addition to ethylene glycol as the sole carbon source. As a nitrogen source the strain use nitrate, ammonium and urea.

On LB medium this strain was sensitive to the following antibiotics (μg/disk): rifampicine (10), chloramphenicol (30), spectinomycin (20), streptomycin (25) and tetracycline (10). *A. globiformis* CECT 4500 tolerated nalidixic acid (10) and kanamycin (20).

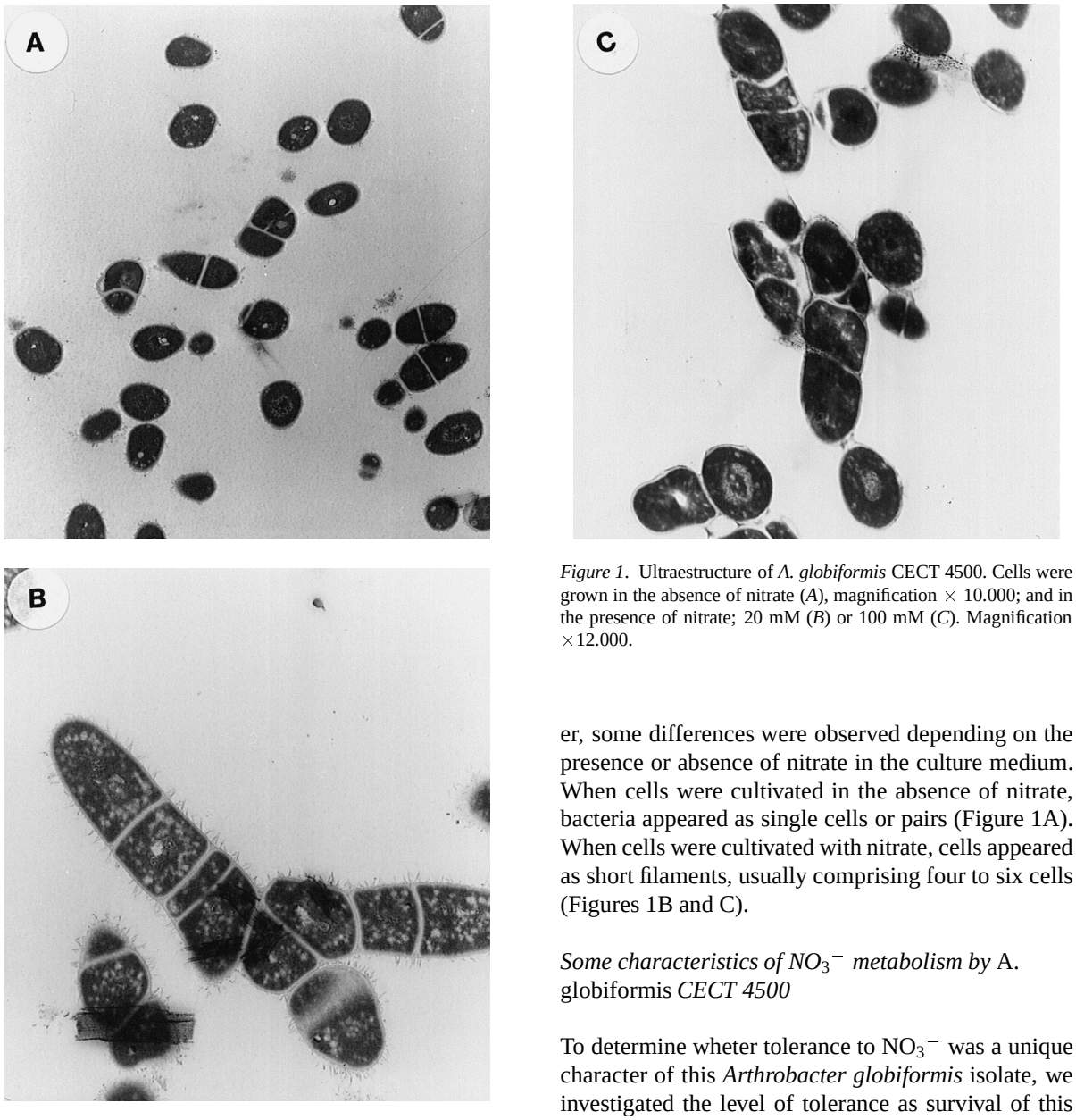

Figure 1. Ultraestructure of *A. globiformis* CECT 4500. Cells were grown in the absence of nitrate (*A*), magnification × 10.000; and in the presence of nitrate; 20 mM (*B*) or 100 mM (*C*). Magnification ×12.000.

Digested total DNA prepared from *A. globiformis* CECT 4500 and showed no sequence homologous to genes encoding nitrate assimilatory genes of gram positive *Basillus subtilis* (Ogawa et al. 1995) or gram negative *Klebsiella pneumoniae* (Lin et al. 1993).

The ultraestructure of *Arthrobacter globiformis* CECT 4500 was studied in cells growing in culture medium with and without 20 mM and 100 mM nitrate. Cells showed ultraestructural features typical of members of the genus *Arthrobacter* (Figure 1). However, some differences were observed depending on the presence or absence of nitrate in the culture medium. When cells were cultivated in the absence of nitrate, bacteria appeared as single cells or pairs (Figure 1A). When cells were cultivated with nitrate, cells appeared as short filaments, usually comprising four to six cells (Figures 1B and C).

Some characteristics of NO_3^- metabolism by A. globiformis *CECT 4500*

To determine wheter tolerance to NO_3^- was a unique character of this *Arthrobacter globiformis* isolate, we investigated the level of tolerance as survival of this strain after exposure to different concentrations of NO_3^-, *Bacilus subtilis* and *Arthrobacter globiformis* CECT 388 were used as controls in these assays.

Tolerance to nitrate in these strains was assayed as follows: cells were grown on minimal medium with 20 mM NO_3^- to about 10^8–10^9 CFU ml^{-1}. Then 0.1 ml was transferred to 1 ml of solution containing increasing NO_3^- concentrations ranging from 20 mM to 1 M. After incubation for 3 h, serial dilutions were spread on LB plates and the population of cells surviving the NO_3^- shock were counted. *A. globiformis* CECT 4500 tolerated up to 1 M NO_3^- without signif-

Table 1. Tolerance of *A. globiformis* CECT 4500, *A. globiformis* CECT 388 and *B. subtilis* to nitrate

Nitrate (mM)	Survival (% of initial CFU mL^{-1})		
	A. globiformis CECT 4500	*A. globiformis* CECT 388	*B. subtilis*
0	100	100	100
0.02	100	100	60
0.1	100	100	6
0.25	100	100	3
0.5	100	15	0.3
1.0	100	3	0.3

A. globiformis CECT 4500, *A. globiformis* CECT 388 and *B. subtilis* cells were grown on M8 minimal medium with 20 mM NO_3^- and 10 g/L ETG or glycerol for 24 h. Cells were harvested by centrifugation and resuspended to reach about 10^8 CFU/mL in 20 mM phosphate buffer pH 6.8 supplemented with the above indicated concentration of NO_3^-. After 3 h, cells were serially diluted and spread on LB plates to determine the number of viable cells (CFU) per mL. The survival data are given as percentage of the initial number of CFU/mL.

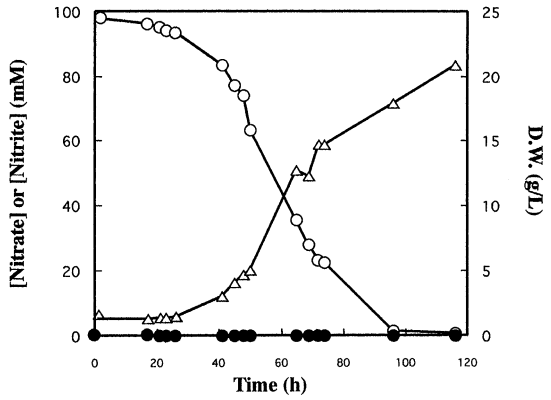

Figure 2. Growth of *A. globiformis* CECT 4500 in medium with 100 mM of nitrate. A 2-L fermentor with factory wastewater diluted to an initial concentration of about 100 mM NO_3^- and supplemented with 50 g/L ethylene glycol plus micronutrients was inoculated with *A. globiformis* CECT 4500 to reach the indicated initial cell biomass. Operational conditions were: air 1 L per L culture per min; pH 7.0 ± 0.5; agitation 600 rpm; temperature 30 °C. Nitrate (○), nitrite (●), and growth (△) were determined at the indicated times.

icant loss of cell viability, whereas *B. subtilis* and *A. globiformis* CECT 388 did not tolerate concentrations above 250 mM (Table 1). *B. subtilis* was more sensitive to nitrate than the *Arthrobacter* strains: at 100 mM NO_3^-, loss of viability was more than 95% of the initial cells. Survival of *Arthrobacter globiformis* CECT 4500 after incubation for 24 h in 1 M NO_3^- was 100%.

We also determined whether the initial NO_3^- concentration affected cell growth of this strain. Both the lag time before growth reassumed after 100-fold dilution and generation time in the exponential growth phase of this strain were influenced by the initial nitrate concentration. *Arthrobacter globiformis* CECT 4500 growing exponentially on minimal medium with 20 mM NO_3^- was transferred to the same medium with increasing NO_3^- concentrations (between 20 mM and 200 mM). The higher the initial NO_3^- concentration, the longer the lag phase was (between 6 h at 20 mM and 52 h at 200 mM), and the higher the generation time once growth reassumed (between 3.7 h at 20 mM and 18.7 h at 200 mM) (Table 2). When the initial amount of NO_3^- was below 100 mM, *A. globiformis* CECT 4500 consumed more than 99% of NO_3^- without any accumulation of nitrite (Table 2). When the NO_3^- concentration was increased to 200 mM, a significant proportion of NO_3^- (about 25%) was consumed without accumulation of NO_2^-. At concentrations above 200 mM no significant consumption of nitrate was observed (not shown). The yield of the cultures, determined as g dry weight per g of ethylene glycol used, was in the order of 0.53 ± 0.7, regardless of the initial nitrate concentration.

Enzymes of the NO_3^- assimilatory pathway in this strain were detected when the strain was grown on-NO_3^--containing medium; the level of nitrate reductase, the first enzyme of the NO_3^- assimilation pathway, was equally high in cells growing exponentially at different NO_3^- concentrations (20–100 mM), and was on the order of 140 ± 20 mU mg^{-1} cell protein.

Optimization of growth conditions in a 2 l reactor

We established the optimal operational conditions for the removal of nitrate by *A. globiformis* CECT 4500 present in wastewaters from the nitration of ethylene glycol. The operational conditions tested in a 2 l bioreactor were: agitation (rotational system) between 200 and 1000 rpm; temperature between 15 °C and 35 °C; and pH between 5.0 and 9.0. Air was bubled at a rate of 1 l per l of culture medium, and we used minimal medium with 20 mM NO_3^- from diluted factory wastewater and approximately 10 g l^{-1} ethylene glycol. Under these conditions optimal performance, defined as the shortest lag, fastest growth and full consumption of the C- and N-sources, were: agitation ≥ 400 rpm; temperature between 20 °C and 30 °C, and pH between 6 and 8. These conditions were found to hold when nitrate concentration was increased up to 160 mM. When nitrate was increased for each fraction of 20 mM nitrate, we

Table 2. Effect of initial nitrate concentration on growth of *A. globiformis* CECT 4500

NO_3^- (mM)		Lag	Generation	NO_2^-	ETG	D.W.	Y_{ETG}	Time for
I	F	(h)	time (h)	(mM)	(g/L)	(g/L)	(g/g EGT)	NO_3^- removal (h)
20	0.2	6	3.64	0	4	6.8	0.6	30
45	0.3	17	5.45	0	4	14	0.7	70
100	0.7	21	6.6	0	5	20	0.53	116
175	125	24	15.5	0	23	23.2	0.56	> 100
200	155	52	18.7	0.01	–	–	–	> 100

A. globiformis CECT 4500 was grown on M8 minimal medium with 20 mM NO_3^- until the late exponential phase, then diluted 100-fold in the same M8 culture medium supplemented with one of the initial concentrations of NO_3^- given above. The amount of EGT supplied was approximately 10 g/L for each 20 mM fraction of NO_3^-. The initial (I) and final (F) concentration of NO_3^- (mM) and the final concentration of NO_2^- (mM) and ethylene glycol (g/L) were determined at the time indicated in the column at the right of the Table. Yield of the culture is given as g of cell biomass/g of ethylene glycol. Generation time refers to the doubling time during exponential growth of the culture. Lag (h) refers to the time required before exponential growth started.

added approximately 10 g l^{-1} ethylene glycol. Figure 2 shows the performance of *A. globiformis* CECT 4500 under optimal conditions in a batch assay with an initial concentration of 100 mM NO_3^-. After an initial lag of about 21 h concomitant with cell growth, NO_3^- and ethylene glycol were consumed until falling below our detection limits. During the exponential growth phase, the rate of NO_3^- uptake in the exponential phase was estimated to be 0.57 ± 0.05 g NO_3^- per g cell protein per hour, while ethylene glycol was consumed at a rate of 3.5 ± 0.2 g per g cell protein per hour. The concentration of nitrite during growth remained below 10 μM.

The addition of nutrients represents a significant cost in the biotreatment of wastewaters. We therefore tested the effect of reducing the concentration of nutrients (between 1/2 and 1/100) in the medium (see the Materials and methods section for standard conditions). With *A. globiformis*, as long as pH remained between 6.5–7.5, phosphate was reduced to a concentration as low as 0.4–0.8 mM (1/100) with no significant effect on the efficiency of NO_3^- removal or the utilization of the C-source (not shown). Similar experiments were done for the rest of the nutrients supplied in the culture medium, such as Fe, $MgSO_4$, and trace elements. We found that reduction of these nutrients by as much as one-half in comparison with the amount supplied in the standard culture medium had no significant effect on the efficiency of N and C utilization (not shown).

Discussion

As the basis for this study, it was reasoned that environments which may have been exposed to transitorily high concentrations of NO_3^- could be colonized by microbes able to tolerate relatively high concentrations of nitrate. To test this hypothesis we looked for bacteria able to use ethylene glycol as the sole C-source and able to grow on 100 mM nitrate, a concentration restrictive for growth for many microbes (Francis & Makin 1991; Pitt et al. 1981). As a result we isolated a strain identified as *A. globiformis*, called CECT 4500, that was tolerant of up to 1 M NO_3^-, and was able to use this compound as the sole N-source at concentrations below 150 mM. This finding confirms that bacteria of the genus *Arthrobacter* constitute a large fraction of the aerobic microorganisms in soils, and are able to contribute to the different element cycles (Pipke & Amrheim 1988; Carter et al. 1995). Sensitivity to nitrate among bacteria has been found in nitrate-utilizing strains of *Anacystis*, *Klebsiella*, *Pseudomonas*, *Rhodopseudomonas* and *Azotobacter* (Pitt et al. 1981; Piñar et al. 1997; Ramos et al. 1982, 1996) and now in *Bacillus*. These microorganisms usually do not tolerate concentration of NO_3^- above 50–100 mM, which is 10- to 20-fold lower than the level of nitrate tolerated by the strain isolated in this study. Given that a culture collection strain of *A. globiformis*, CECT 388, also tolerated relatively high nitrate loads, many strains of this species may be relatively tolerant to nitrate. The mechanism(s) available for *A. globiformis* to adapt to the presence of high concentrations of NO_3^- is(are) unknown. We observed morphological

changes consisting of the formation of filaments when cells were grown in the presence of nitrate. This may be a way to reduce the surface exposed to nitrate, although the implications of this morphological change and tolerance to nitrate have not been elucidated. Given that the level of nitrate-reducing enzyme or nitrate uptake was independent of the concentration of nitrate, the rate of assimilation of this compound cannot be considered a factor involved in tolerance. Efforts are being made to determine wheter there exists an exclusion system of nitrate from the cytoplasm, such as those described for certain drugs (Nikaido 1996) and solvents (Isken & de Bont 1996; Ramos et al. 1997).

Although the mechanistic basis for NO_3^- tolerance in *A. globiformis* is not understood, the usefullness of this property for bioremediation of industrial wastes rich in NO_3^- was demonstrated by showing that this microorganism performed in a stream containing wastewaters from the synthesis of dinitroethylene glycol, which can contain up to 500 mM NO_3^-. Our results showed that under strict conditions, and as long as these waters were diluted to about 100–150 mM NO_3^- and an assimilable C-source was available, this microorganism eliminated all NO_3^-. We therefore conclude that *A. globiformis* CECT 4500 can be useful in removing NO_3^- from wastewaters produced in several industrial processes.

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