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Denitrifying *nirK*-containing alphaproteobacteria exhibit different electrode driven nitrite reduction capacities

Ariadna Vilar-Sanz [a], Narcís Pous [b], Sebastià Puig [b], M. Dolors Balaguer [b], Jesús Colprim [b], Lluís Bañeras [a,*]

[a] Molecular Microbial Ecology Group, Institute of Aquatic Ecology, University of Girona, Facultat de Ciències, Campus Montilivi, Girona, Spain
[b] LEQUIA, Institute of the Environment, University of Girona, Facultat de Ciències, Campus Montilivi, Girona, Spain

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ABSTRACT

Biocathodic electrode-driven denitrification has been proved experimentally in complex biofilms. However, experimentation with isolated bacteria in pure culture is still limited. In this paper, six *Alphaproteobacteria* (*Rhizobiales*), found to be dominant in a denitrifying biocathode, have been characterized bioelectrochemically. Bacteria were isolated using strict autotrophic conditions in the presence of nitrate or nitrite. Six representative isolates were selected and proven able to denitrify under autotrophic and heterotrophic conditions in liquid media. Bioelectrochemical reduction of nitrate, nitrite and nitrous oxide was tested using cyclic voltammetry. Electrode-driven nitrite reduction was only detected in four of the six isolates. However, no electrode-driven nitrate or nitrous oxide reduction could be detected for any of them. In the presence of nitrite, estimated midpoint potentials for bioelectrocatalyzed reactions ranged from −500 to −534 mV *vs.* SHE. Two of the isolates exhibited midpoint potentials at −450 and −486 mV *vs.* SHE when incubated in the absence of any external nitrogenous electron acceptor. These redox peaks were attributed to electrode-driven hydrogen production in the biofilm. We have proven that electrode-driven nitrite reduction is feasible in monospecific biofilms. However, significant variability in relation to electrode-driven nitrogen reduction processes was observed in closely related species, confirming a strain-specific behavior.

1. Introduction

Denitrification is a microbial facilitated four-step reduction process in which electrons are sequentially used to reduce nitrate to nitrogen gas. Denitrification has been efficiently exploited for wastewater treatment, and has recently been coupled to electrically mediated systems to promote nitrate reduction in waters of low organic matter content (carbon to nitrogen ratio < 2) [1]. In electrically mediated reactors, such as bioelectrochemical systems (BES), complex bacterial communities are formed in the cathode and the anode compartment, leading to the coexistence of many metabolic reactions [2–4]. However, electrotrophic and electrogenic bacteria have a preponderant role in BES and they contribute to the electron transfer in the electrode-to-cell or cell-to-electrode directions due to the activation of certain gene clusters [5,6]. In addition, the simultaneous presence of different bacterial species within biofilms allows the formation of complex intrinsic relationships between cells causing electrons to be transferred rather efficiently between bacteria [7]. When this is the case, oxidation and reduction moieties of a redox reaction can occur at relatively distant positions, and may combine for a common physiological process [8]. At least theoretically, the same holds true for biological step-sequence reducing reactions, such as denitrification, which may result as a cooperative behavior of different bacteria, rather than from the activity of a single species. Unfortunately, few experimental studies exist in which the electron harvesting capacity of denitrifying bacteria on biocathodes has been analyzed using pure cultures [7,9].

Cyclic voltammetry (CV) has been widely used to characterize electrochemically dependent reactions. One of the most interesting features of CV is its usage for the estimation of formal reduction potentials ($E^{\circ\prime}$), which indicates the actual potential at which an oxidation-reduction reaction is taking place. At least theoretically, CVs for biologically catalyzed reactions can be performed both using pure extracts of enzymes suspected to perform a redox reaction, or using intact whole cells attached to an electrode. Characterizations of pure protein extracts by CV have been obtained for most of the enzymes involved in denitrification, *i.e.* nitrate reductase [10], nitrite reductase [11], nitrous oxide reductase [12], and cytochromes [13]. In general, cathode potentials of pure protein extracts in cyclic voltammetry experiments did not match exactly those obtained with whole intact cells. Shifts in formal

Abbreviations: BES, bioelectrocatalytic system; BMM, basal mineral medium; CV, cyclic voltammetry; CA, chronoamperometry; Ec_1, mid-point potentials at turnover conditions; Ec_2, mid-point potentials at non-turnover conditions; MFC, microbial fuel cell.

* Corresponding author.
E-mail address: lluis.banyeras@udg.edu (L. Bañeras).

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cathodic potentials were probably caused by the occurrence of complex interactions between different proteins and shuttle molecules that may modify the detection of electron-receiving enzymes in intact cells [14]. Complexity of these unpredictable reactions increases in biofilms formed by different bacterial species. In this sense, the development of single-species biofilms composed of active bacteria is necessary to elucidate electrotrophic activities of denitrifiers, and to fix effective electrode potential in living systems. We hypothesize that providing a precise formal potential for living cells may increase electron use efficiencies in biocathodic denitrification. For this, isolation of denitrifiers and growth on mono-specific biofilms is necessary.

Isolation of bacteria in pure cultures using strictly electrode-derived electrons is technically difficult and no reliable method exists for this purpose [7]. Alternatives to this method using other reducing power sources may include strictly autotrophic conditions, *i.e.* mineral medium composition supplied with carbon dioxide, and anaerobic conditions in the presence of nitrate and nitrite as electron acceptors. Most common inorganic electron donors for the isolation of autotrophic denitrifiers are hydrogen, iron (II) and reduced sulfur species, *e.g.* sulphide or thiosulphate [15,16]. Moreover, combined bioelectrochemical systems with autotrophic denitrification in the presence of sulfide or hydrogen have been proven effective for the removal of nitrate in contaminated low-organic carbon underground water [17], which may indicate shared metabolic possibilities for the existing denitrifiers in bioelectrochemical systems.

In the present study we aimed at deciphering the electrotrophic capacities of alphaproteobacteria showing facultative autotrophic denitrification capacities. Denitrifying alphaproteobacteria were isolated in pure cultures and those showing high similarities to dominant bacteria found in biocathode selected. Selection of isolates was made according to 16S rRNA and *nosZ* genes sequence similarities. Cyclic voltammetries were used to test for the bioelectrochemical reduction of nitrate, nitrite, and nitrous oxide in a three-electrode arrangement using strict anaerobic conditions. The tested strains showed different abilities in their bioelectrochemical performance thus increasing knowledge of cathode-driven bacterial reactions for pure cultures of denitrifiers.

2. Materials and methods

2.1. Biofilm sampling of denitrifying microbial fuel cells

Biofilm samples were taken from two denitrifying microbial fuel cells (MFCs) operated for >50 days. The cathodes of the two cells were inoculated with the effluent of a parent MFC treating synthetic wastewater mainly composed of sodium acetate and a buffer solution. This parent MFC was inoculated with activated sludge from a urban wastewater treatment plant. The new denitrifying MFCs consisted on a double-chamber MFC treating acetate-enriched synthetic wastewater at the anode and nitrate-enriched synthetic wastewater at the cathode. A maximum denitrification rate of 76 ± 12 $gN \cdot m^{-3} \cdot d^{-1}$ was obtained. Cathodes were fed with an enriched mineral medium containing: 0.49 g/L $NaHCO_3$, 0.2 g/L $NaNO_3$, 0.92 g/L $NaH_2PO_4 \cdot 2H_2O$, 5.6 mg/L $CaCl_2 \cdot 2H_2O$, 0.04 g/L $MgSO_4 \cdot 7H_2O$, 5.2 mg/L KCl, and 0.1 mL/L of an oligoelements solution (SL10). More details on media composition and MFC operation and performance have been described earlier [18,19].

Graphite rods (6 × 38 mm; Alfa Aesar, Germany) were used as electrodes in the cathode and anode. Two graphite rods were aseptically collected and maintained at 4 °C. Less than 2 h after collection, samples were processed for bacteria enrichment and isolation. Biofilm cells were dislodged from graphite rods using three consecutive sonication rounds (30 s) in an ultrasonic bath operated at maximum intensity (Selecta, Spain). Viability analyses confirmed that these conditions ensured cell detachment while minimizing cell lysis.

2.2. Media composition

A basal mineral medium (BMM) amended with inorganic electron donors was used for the enrichment and isolation of autotrophic denitrifiers. BMM was prepared using the same composition of the cathode feeding solution with the addition of 1 mL of a filter-sterilized seven vitamin solution. pH was adjusted to 6.8 ± 0.1. Solid BMM was prepared by the addition of 12.5 g/L of high quality agar (Merck®, Germany). Three electron donors were used: thiosulfate was directly added to the medium before autoclaving (5 g/L, final concentration), sulfide was provided from an acidified (0.1 M HCl) thioacetamide solution, hydrogen was supplied in the gas phase after purging the jar with a gas mixture containing either 5% or 32% H_2. Media compositions were named according to the electron donor used as BMM_H_2, BMM_H_2S and BMM_Na_2S2O_3. BMM agar plates were prepared and incubated overnight in a Coy Lab anaerobic chamber before being used for inoculation (Coy Laboratory products, Inc., Grass Lake, Michigan, EUA). Cultures were incubated at 30 °C in N_2 purged (BMM_H_2S and BMM_Na_2S2O_3) or H_2 purged (BMM_H_2) anaerobic jars containing AnaeroGen bags (Oxoid, Hampshire, United Kingdom).

2.3. Isolation of nosZ containing bacteria

Cell suspensions were serially diluted in isotonic solution and inoculated in triplicates on petri dishes containing BMM_H_2, BMM_H_2S and BMM_Na_2S2O_3 media. A total of 991 colonies selected after 30 days of incubation and were aseptically transferred to 96 deep well microplates containing 1.2 mL of liquid BMM. After growth, microplates were centrifuged (Eppendorf Centrifuge 5804R, Hamburg, Germany) for 30 min at 3000 ×*g*. DNA was extracted using a freeze-thaw method (3 consecutive cycles of 10 min at 85 °C followed by 10 min at −80 °C). Blank controls containing sterile water were added to all plates to check for cross contamination during extraction. No cross contamination was assumed if no DNA could be detected above detection limits of PCR using bacterial universal primers in blank wells. Microcultures were screened for the presence of nitrous oxide reductase genes (*nosZ*) by PCR (Section 2.4) using primers *nosZ*-F and *nosZ*1622R [20].

nosZ containing microcultures were subsequently inoculated on agar plates with the aim to obtain pure cultures of denitrifiers. For every microculture, the same electron donor used in the enrichment process was used. Bacterial colonies were sequentially re-isolated on selective media for at least 4 times until pure isolated cultures were obtained.

2.4. Genetic characterization of bacterial isolates

Bacterial isolates were characterized for the presence of denitrifying genes by PCR amplification. DNA was extracted directly from colonies using Chelex® 100 Resin (Bio-Rad laboratories, Inc., Hercules, California, EUA) following the manufacturer specifications. The following primer combinations were used in the genetic characterization, 1960F/2650R for *narG* nitrate reductase, V67/V17 for *narG* nitrate reductase, cd3aF/R3cd for *nirS* nitrite reductase, F1aCu/R3Cu for *nirK* nitrite reductase, nosZ-F/nosZ1622R for *nosZ* nitrous oxide reductase, and 27F/1492R for the 16S rRNA gene. PCR conditions have been published elswhere [18].

Sequences of *nosZ* and 16S rRNA genes were obtained (Macrogen, the Netherlands) and used for identification of isolates. Neighbor-joining trees were constructed with the pair-wise deletion method using kimura2 or amino-Poisson corrections for nucleotide or deduced amino acid sequences using MEGA v.5.0 software. Tree topology was evaluated by bootstrap analysis using 1000 replicates.