Soil Biology & Biochemistry 100 (2016) 1–8

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Reduction of nitrate to nitrite by microbes under oxic conditions

Constance A. Roco [a], Linda L. Bergaust [b], James P. Shapleigh [a], Joseph B. Yavitt [c, *]

[a] Department of Microbiology, Cornell University, Ithaca, NY 14853, USA
[b] Department of Chemistry, Biotechnology and Food Sciences, Norwegian University of Life Science, NO-1432, Ås, Norway
[c] Department of Natural Resources, Cornell University, Ithaca, NY 14853, USA

ARTICLE INFO

Article history:
Received 18 February 2016
Received in revised form
10 May 2016
Accepted 14 May 2016

Keywords:
Aerobic nitrate reduction
Bacterial genome
Dissimilatory nitrate reductase
Nitrite production

ABSTRACT

Human activities have greatly increased the input of nitrate to natural and managed ecosystems, but the fate of excess soil nitrate is still unclear. Many studies assume that dissimilatory reduction of nitrate to nitrite is an anaerobic process, but this first step of denitrification can occur in some bacteria at oxygen concentrations that are high enough to repress downstream reduction of nitrite to gaseous products. Here, we examine whether dissimilatory reduction of nitrate under aerobic conditions is an additional, underappreciated fate of nitrate in soil. Aerobic nitrate reduction occurred in soils when provided with both nitrate and a carbon source, with the greatest nitrite accumulation in the wetland sites. The addition of a nitrification inhibitor did not significantly reduce aerobic nitrate reduction activity, nor did an assimilation repressor. Average nitrite production in soils with added carbon, nitrate, and nitrification inhibitor ranged from 7.5 to 50% of added N-nitrate in a five-hour incubation. Bacteria capable of aerobic nitrate reduction were readily isolated from these soils, comprising approximately 35% of the isolates retrieved. Sequencing16S rDNA of these isolates revealed both gram-negative and gram-positive bacteria, with the majority being gram-negative proteobacteria. In six of the isolates, onset of nitrate reduction occurred at 45–86% of atmospheric oxygen concentrations. Reduction of nitrate under aerobic and semi-aerobic conditions did not result in significant enhancements in carbon dioxide production or total electron flow rate to electron acceptors. The genomes of these six isolates were sequenced and targeted RT-qPCR revealed a wide diversity of regulatory controls on the nitrate reductase(s). The results suggest that aerobic nitrate reduction can occur in diverse bacteria, have multiple types of physiological controls, and can occur independently of the gas-forming reactions of denitrification. Thus, it is an unappreciated fate of nitrate in soil.

1. Introduction

Anthropogenic nitrogen (N) production has led to new challenges in understanding how excess N cycles in natural and managed ecosystems (Schlesinger, 2009; Fowler et al., 2013). A particular problem is that excess nitrate can potentially be transferred from soil to small streams and shallow groundwater, to rivers, and ultimately to coastal marine ecosystems where it fuels eutrophication (Carpenter et al., 1998). This flow from soil to aquatic ecosystems can be mitigated to some extent, since nitrate can be assimilated by plants and bacteria or reduced to either ammonium (DNRA) or gaseous end products through microbial dissimilatory processes. Microbial denitrification, the stepwise reduction of nitrate to dinitrogen via nitrite, nitric oxide, and nitrous oxide, can potentially alleviate the negative effects of nitrate leaching from soils.

Denitrification is frequently viewed as a tightly coupled pathway whose activity is inversely correlated with oxygen (O_2) levels, and thus has been studied mostly in soils with very low O_2 concentrations. The typical denitrifier is a facultative anaerobe that preferentially utilizes O_2 as an electron acceptor and restricts electron flow towards N-oxides as long as O_2 is available (Shapleigh, 2012; Bergaust et al., 2011). However, studies on model denitrifiers have shown that different steps in the pathway can be differentially controlled by O_2, and the reason that O_2 quenches the process may lie as much with chemical constraints as with physiological necessities (Martinez-Espinosa et al., 2011; Shapleigh, 2011; Bateman and Baggs, 2005). For example, the rapid chemical reaction of NO with O_2 may be the reason why the production and

* Corresponding author. Department of Natural Resources, Cornell University, Ithaca, NY 14853-3001, USA.
E-mail address: jby1@cornell.edu (J.B. Yavitt).

http://dx.doi.org/10.1016/j.soilbio.2016.05.008
0038-0717/

reduction of NO is the part of the pathway most tightly regulated in the presence of O_2 (Bergaust et al., 2008; Hartsock and Shapleigh, 2010; Watmough et al., 1999). In contrast, nitrate and nitrite do not readily react with O_2, and thus reduction of nitrate to nitrite is adjusted to best fit physiological necessities (Dalsgaard et al., 2014; Hartsock and Shapleigh, 2011; Sears et al., 2000; Martinez-Espinosa et al., 2011).

Given that the multiple enzymes in the denitrification pathway do not necessarily have tightly linked expression, it is reasonable to postulate that specific steps in the pathway function independently. More specifically, there is no reason to assume that the first step in the pathway is obligatorily linked to any of the remaining three steps, and thus, this step could occur under less stringent O_2 control resulting in an underappreciated source of nitrite. For example, Su et al. (2011) and Isobe et al. (2012) showed circumstantially that nitrite production in acidic soils is necessary to balance the budget of the chemically converted nitrous acid (HONO) compound found in the atmosphere (Isobe et al., 2012; Su et al., 2011). Oswald et al. (2013) provided evidence that neutral-to-basic soils in arid and arable areas show relatively high emission fluxes of HONO and NO in nitrite rich soils, accounting for up to 50% of the reactive N release from soil (Oswald et al., 2013). Chemodenitrification of nitrite has also resulted in the production of gaseous N-oxides, with the reactive free radical NO as the major product (Thorn and Mikita, 2000; Davidson, 1992; Stuven et al., 1992). As chemodenitrification occurs when soil pH is <5 (Chalk and Smith, 1983), this process is likely significant in soils of the northeastern USA (Kulkarni et al., 2013). Another possible abiotic fate of nitrite is the binding to soil via the so-called nitrosation process (Smith and Tiedje, 1979; Matocha et al., 2012). This fate could explain nitrate fixation onto soil organic matter that has been suggested as a sink for added anthropogenic N in some forest ecosystems (Davidson et al., 2003).

The production of nitrite from nitrate in soils under oxic conditions has been shown by a few other studies. Previous work by Carter et al. (1995) has described heterotrophic bacterial isolates from soil capable of nitrate reduction in the presence of atmospheric oxygen, which has been referred to as aerobic nitrate reduction (ANR) and will be the term used here (Carter et al., 1995). Also, Vega-Jarquin et al. (2008) demonstrated ANR in soil from an alkaline lake bed in Mexico when incubated in 100% atmospheric O_2 and in the presence of acetylene, a nitrification inhibitor (Vega-Jarquin et al., 2008).

There is still limited understanding of the bacteria capable of performing ANR. Here, we examine ANR in a range of soil types using an oxidized carbon source, succinate, with the addition of nitrification and nitrate assimilation inhibitors in order to provide a more detailed understanding of the eco-physiological context of ANR, including the determination of the O_2 concentration at the onset of nitrate reduction in bacterial isolates demonstrating the ANR phenotype. With these bacterial isolates we also investigated the diversity of regulation of the dissimilatory nitrate reductase(s), which include the membrane-associated nitrate reductase (Nar) and a periplasmic enzyme (Nap). The results of this work suggests that ANR is a common, yet underappreciated trait, and this pathway may make a significant but still unclear contribution to the soil nitrogen cycle.

2. Methods

2.1. Experimental sites and soils

Soils were collected in September 2010 from four sites near Ithaca, NY (Table 1). The soil at the agricultural field site and second-growth forest site at McGowan Farm are classified as an Alfisol (mesic Glossoboric Hapludalf; USDA Classification) derived from sandy outwash with a fine loamy texture (Beatty and Stone, 1986; Castrillo et al., 2007). The agricultural field site and second-growth forest site at Smith Woods are Alfisol (mesic Psammentic Hapludalf; USDA classification) with a sandy texture (Beatty and Stone, 1986; Marks et al., 1999). Two forested wetland sites, Sapsucker Woods and Stewart Park, were also sampled. In both sites, trees occur on elevated hummocks surrounded by water-filled pits, which are 0–40 cm deep. A fragipan soil horizon about 1 m deep prevents drainage and maintains a high water table. The soil at the Sapsucker Woods site is classified as an Inceptisol (mesic Mollic Endoaquept; USDA Classification), which has a fine-loamy texture and is non acidic (Miller et al., 2004). Soil at the Stewart Park site is also classified as an Inceptisol (mesic Fluvaquentic Endoaquept; USDA Classification). At both of the forested wetland sites soils were collected separately from the hummocks and pits. In July 2011 samples were taken from a forested site at the Hubbard Brook Experimental Forest (HBEF) within the White Mountain National Forest, West Thornton, NH. Soils at this second-growth forest site are classified as Spodosol (Typic Haplorthod; USDA Classification) derived from granitic glacial till with sandy-loam to loamy-sand texture (Adams et al., 2008).

At each of the sites described above, soil samples (roughly 200 g each) were collected from three randomized locations from the 0–10 cm depth interval. Samples were stored for <60 min in plastic bags before being assayed. Soil pH was measured with a glass electrode in a 1:5 soil-water slurry. Soil moisture content was calculated as ((wet weight-dry weight)/wet weight) × 100.

2.2. Assay for ANR

Analysis of ANR activity began within 60 min of soil collection. Soil (0.5 g fresh-weight) was added to a 1 ml solution in a 1.5 ml microcentrifuge tube. The solutions added to the soils were: 1) deionized water (W), 2) deionized water with 0.03 mg N-KNO_3 g^{-1} fresh-weight soil (W+N), 3) Sistrom's medium (Lueking et al., 1978; SIS); 4) SIS plus 0.03 mg N-KNO_3 g^{-1} fresh-weight soil (SIS+N); 5) SIS+N and 300 mM dicyandiamide (DCD) (SIS+N+DCD), and 6) SIS+N and 113 mM ammonium sulfate (SIS+N+NH_4). Succinate, a relatively oxidized carbon source found in soils, was the carbon source in the Sistrom's medium and was used to provide the approximate redox level to cell biomass (McKinlay and Harwood, 2011), where Carter et al. (1995) previously used a reduced carbon source of butyrate to demonstrate ANR. DCD was used as a nitrification inhibitor (Giltrap et al., 2010; Davies and Williams, 1995) and excess ammonium sulfate was used to ensure nitrite production was not a consequence of the assimilatory nitrate reductase (Giltrap et al., 2010; Vega-Jarquin et al., 2008).

All incubations were done in triplicate and agitated continuously by vortex under atmospheric conditions for up to five hours. Nitrite accumulation was measured after 30 min, 60 min, three hours, and five hours of incubation. Nitrite was determined by adding 50 μl of the soil slurry to 900 μl phosphate buffer and 1 mL of Griess reagents A and B (Roussel-Delif et al., 2005). Intensity of colorimetric change was measured on the spectrophotometer at an absorbance of 540 nm.

2.3. Classification and identification of isolates

Bacterial isolates were isolated under well-aerated, heterotrophic conditions from the soils collected from the various sites. Bacteria capable of ANR were isolated as described in Roco et al. (2016). In brief, isolates from soil were incubated aerobically for 24 h at 30 °C in a 96-well plate on Sistrom's agar medium supplemented with 113 mM $(NH_4)_2SO_4$ and 10 mM KNO_3 before being