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Isolation and Characterization of Aerobic Denitrifiers from Agricultural Soil

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Abstract: Denitrification is generally considered an anaerobic process. However, in recent years it has been shown that bacteria can also reduce nitrate to nitrite under aerobic conditions. The characterization of biologically available nitrogen forms and their biological cycling mechanisms is important for ecological and agricultural implications. In this study, aerobic nitrate reducers were isolated from greenhouse soil. Using a nitrate reduction assay, it was found that 39 out of 60 isolates can reduce nitrate to nitrite under aerobic conditions. Five of those 39 isolates were selected for further characterization of their aerobic nitrate reduction activity. Four of those 5 isolates were found to have periplasmic nitrate reductase activity using MV^+ as a non-biological electron donor. Nitrite production of these isolates under aerobic conditions was determined using different nitrogen forms as sole nitrogen sources. It was found that nitrite accumulation in all isolates was higher when nitrate was the sole nitrogen source than when nitrate+ammonium was the sole nitrogen source. No nitrite accumulation was observed when ammonium was the sole nitrogen source. This study suggests that when ammonium exists together with nitrate, aerobic nitrate reduction does not lead to high nitrite accumulation.

Key Words: denitrification, aerobic denitrification, aerobic nitrate reduction, periplasmic nitrate reductase.

Tarım Toprağından Aerobik Denitrifikasyon Bakterilerin İzolasyonu ve Karakterizasyonu

Özet: Denitrifikasyon genelde anaerobik proses olarak kabul edilir. Ancak, son yıllarda aerobik koşullarda da nitratı nitrite indirgeyen bakteriler gösterilmiştir. Azot formlarının ve azotun biyolojik dönüşüm mekanizmalarının bilinmesi ekolojik ve tarımsal önem taşır. Bu çalışmada, tarım toprağından aerobik denitrifikasyon yapan bakteriler izole edildi. 60 izolattan 39'nun aerobik koşullarda nitratı nitrite indirgeyebildiği tespit edildi. 39 izolattan beşi aerobik nitrat redüksiyon aktivitesinin belirlenmesi için seçildi. Biyojik olmayan elektron vericisi MV^+ kullanarak beş izolattan dördünün periplazmik nitrat redüktaz aktivitesine sahip olduğu bulundu. Bu izolatların farklı azot kaynaklarında, aerobik koşullarda nitrit üretme özellikleri belirlendi. İzolatların nitratlı ortamda, amonyak + nitratlı ortama kıyasla kültürlerinde yüksek seviyede nitrit biriktiği, azot kaynağı amonyak olan ortamda ise, izolatların hiçbirinin nitrit biriktirmediği gözlendi. Sonuç olarak, aerobik nitrat redüksiyonunda nitratla birlikte amonyağın varlığı yüksek seviyede nitrit birikimine neden olmamaktadır.

Anahtar Sözcükler: Denitrifikasyon, aerobik denitrifikasyon, aerobik nitrat redüksiyonu, periplasmik nitrat redüktaz.

Introduction

Denitrification is widely considered to be the conversion of nitrate to its reduced forms and its final products are usually in gas form. For this reason, denitrification is considered to be a process that removes bioavailable nitrogen forms from the Earth's surface (1). Since the loss of nitrate from agricultural soil reduces crop yield as well as affecting the fauna and flora on and in the soil, denitrification is not a desirable process from an agricultural point of view. Moreover, excess nitrate or nitrite in soil or water ecosystems leads to environmental and health problems (2). This excess nitrate in water or soil can be converted to its acid forms, which consequently reduce soil and water quality (3,4). In addition to ecological problems, excess nitrate also stimulates denitrification, resulting in the loss of bioavailable nitrogen used by plants in the form of the gaseous nitrogen compounds N_2O and N_2 (5). Despite these detrimental effects, denitrification is beneficial in sewage treatment because it converts NO3 to its gaseous forms, thus reducing the amount of available nitrogen in the sewage.

Denitrification is originally described as an anaerobic respiratory process in which the first step is catalysis by membrane-bound nitrate reductase (1,6). However, recently it has been shown that denitrification can also take place under aerobic conditions and a periplasmic nitrate reductase seems to be the major enzyme in aerobic denitrification (7,8).

There are 3 nitrate reductases in bacteria that can perform the reduction of nitrate to nitrite under different physiological conditions. Membrane-bound respiratory nitrate reductase (NAR) can generate metabolic energy by using nitrate as a terminal electron acceptor during anaerobic denitrification. Cytoplasmic assimilatory nitrate reductase (NAS) can convert nitrate to ammonium, which can then be incorporated into biomolecules. The third enzyme is a dissimilatory nitrate reductase (NAP), which exists in the periplasm of some Gram-negative bacteria and can reduce nitrate to nitrite under both aerobic and anaerobic conditions (9 -12).

NAP has been isolated from many Gram-negative bacteria (8, 13-17) and its gene has been identified in all members of proteobacteria, predominantly in γ-enterobacteria (18). Isolations from soil and fresh water indicated that aerobic nitrate reduction is widespread in nature (19).

NAP activity is not repressed by either ammonium or oxygen. The NAP system contains 4 to 7 genes, clustered in *nap* operon, and the *napABC* genes are essential for *in vivo* nitrate reduction. In some bacteria the *nap* genes are located on endogenous plasmids (e.g., *Rhodobacter capsulatus*) and others on chromosomes (e.g., *Escherichia coli*). *Nap A* gene analysis of the pseudomonas isolates indicated that some strains that have NAP genes and express activity are incapable of aerobic nitrate reduction (20).

Different physiological functions have been proposed for this enzyme. The NAP seems not to be primarily involved in nitrate assimilation or anaerobic respiration, although nitrite generated by NAP can be used as a nitrogen source or as a substrate for anaerobic denitrification depending on the organisms (12). It is more likely that NAP is involved in aerobic denitrification and/or the maintenance of an optimal redox potential. The physiological function of NAP may vary in different organisms or even in the same bacterium under different metabolic conditions.

Since enzymes responsible for nitrate reduction are located in different parts of the cell, the activity of nitrate reductases is determined using non-physiological electron donors such as benzyl viologen (BV^+) and methyl viologen (MV^+) dyes. Both the membrane-bound (NAR) and the periplasmic nitrate reductase (NAP) can utilize reduced BV^+ and MV^+ as electron donors. However, the cell membrane is permeable to BV^+, whereas MV^+ is relatively incapable of crossing the cell membrane. Therefore, BV^+ and MV^+ are used to detect the activity of nitrate reductase enzymes whose active sites are located in the cytoplasm or in the periplasm, respectively (8,14,17,21,22).

Understanding the conversion of nitrate in agricultural soil is of ecological and economical importance. Since nitrite production is usually considered to be an anaerobic process and the extent of aerobic nitrate reduction is not fully known, the effect of aerobic nitrite production on the global nitrogen cycle, environment and agriculture is not known. The aim of this study was to isolate and characterize aerobic nitrate reducers from agricultural soil.

Materials and Methods

Isolation of Bacteria

Soil samples were taken from the area around vine tree roots (approximately 20-30 cm diameter area of the body) in 4 randomly chosen sections of a greenhouse at Akdeniz University Agricultural Faculty. A total of 12 samples were collected from 5 cm below the surface of the soil using a sterile metal cylindrical tool (45 cm in length and 5 cm in diameter). All soil samples were collected in a sterile glass container and delivered to the laboratory within 20 min.

Soil samples were sieved using a sterile sieve with pores of 6 mm. Ten grams of sieved sample was dissolved in 100 ml of dissolving buffer (0.85% NaCl and Tween 80). After appropriate dilutions, samples were inoculated to both defined agar medium (30 mM succinate, 10 mM NH_4Cl, 14 g K_2HPO_4, 2.7 g KH_2PO_4, 2.5 g NaCl, 5ml 2% $MgSO_4$ solution, 4 mM $FeSO_4$ and 50 mM fungicide, pH 7.2) (19). Plates were incubated at 30 °C for 4 days. The number of bacteria in the soil sample was calculated by counting colonies forming in the defined agar plates (23). The morphological and biochemical characterization of isolates was performed according to Bergey's Manual of Bacteriology (24).

Nitrate Reduction Assay

The ability of colonies to reduce nitrate to nitrite was determined using the nitrate reductase assay modified from 19. The isolates were grown on defined medium plates for 2 days at 30 °C. One hundred micro liters of 1% sodium nitrate was added to the colonies on plates. After 10 min incubation at room temperature, 100 µl of the nitrate detection reagent (containing 2 parts 4% w/v sulfanilamide in 20% conc. HCl and 1 part 0.08% w/v napthylethylenediamine in 1% conc. HCl) was added and the occurrence of a reddish pink color indicated the existence of nitrite, and therefore aerobic reduction of nitrate to nitrite by bacteria.

Nitrite Assay

The nitrite concentration in culture supernatants was estimated by colorimetric determination (22,25). Isolates were grown in defined liquid medium with different nitrogen sources (either 10 mM of NO_3 or NO_3 and NH_3 or NH_3) by shaking at 150 rpm at 30 °C for 12-16 h depending on the time needed for the isolates to reach their late logarithmic phase. Cells were harvested by centrifugation at 6000 rpm for 3 min and 1 ml of supernatant was taken into 3 ml cuvettes. Then 0.02 ml of nitrite determination reagent was added to the same cuvettes that were left at room temperature for 20 min and the color was measured spectrophotometrically at 540 nm against the blank.

Periplasmic Nitrate Reductase Activity

Nitrate reduction activity in whole cells was measured by following the oxidant-dependent oxidation of dithionite-reduced methyl viologen (MV^+) (7,19). A single colony of the isolates was inoculated in nutrient broth medium and grown overnight at 30 °C under shaking. The cultures were harvested and the pellets were washed twice with 10 mM phosphate buffer (pH 7.4). The cell suspensions were used to inoculate 50 ml of defined medium containing 10 mM $NaNO_3$ in 250 ml conical flasks and were incubated in shaken flask culture at 30 °C for 12-16 h depending on the time needed for the isolates to reach their late logarithmic phase. For the periplasmic nitrate reductase activity, the isolates were washed twice with 10 mM phosphate buffer (pH 7.4) to avoid any nitrate contamination from the medium. The cells of the isolates were diluted in 2 ml of the same buffer in a 3 ml cuvette and the concentration of cells in this cuvette was adjusted to give an O.D.= 0.4 at 600 nm. Two milliliters of the cell suspension adjusted to final conc. was injected into a 3 ml rubber sealed cuvette. After adding 20 µl of 100 mM methyl viologen to the same cuvette using a Hamilton syringe, air from the cuvette was removed with oxygen-free nitrogen over 10 min. Aliquots of a freshly prepared, concentrated solution of sodium dithionite were added to the cuvette with the Hamilton syringe until a steady-state absorbance at 600 nm of 1.5-2.0 was obtained. The reaction was started by injecting nitrate through the rubber septum to a final conc. of 10 mM. The resulting absorbance decrease was measured at 600 nm and recorded every 5 s. All assays were performed in a water jacketed chamber maintained at 30 °C. The enzyme activity was determined using the formula: $\varepsilon = A / c \times d$ cm mol^{-1} (ε for MV^+ is 13 mM^{-1} cm^{-1}) (26).

Results and Discussion

The number of bacteria grown on defined agar plates at 30 °C for 2 days was 1 x 10^6 cfu/g soil. Sixty isolates, which formed colonies on defined agar plates, were selected for nitrate reductase activity. Thirty-nine of the 60 tested isolates were found to reduce the added nitrate to nitrite under aerobic conditions in the test system according to the formation of a reddish pink color on the plates. In the same test system, no nitrite was observed in 21 out of those 60 tested isolates. Nitrate reduction in the test system by isolates is attributed to the activity of periplasmic nitrate reductase enzyme (9,19) because under these experimental conditions, 1) activity of NAS is repressed by ammonium that already exists in the growth medium (27); 2) NAR cannot reduce nitrate to nitrite under oxic conditions (9,28,29). However, nitrite seen on the test system might be the result of heterotrophic nitrification of ammonium (30) that exists on the growth medium but the absence of color formation on the control plates rules out this possibility.

The morphological and biochemical properties of those 39 isolates found to reduce nitrate were characterized and 5 of those 39 characterized isolates' biochemical properties are given in Table 1. Since the physiological function of the periplasmic nitrate reductase in nitrogen fixing bacteria (*Rhizobium* spp. and

Table 1. Biochemical and morphological properties of isolates.

Isolates/properties	A11	C2	C8	D3	E2
Gram	-	-	-	-	-
Catalase	+	-	-	+	-
Oxidase activity	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Nitrogen fixation	-	+	+	-	+
Chemolithotroph	-	+	+	+	+
Starch hydrolysis	-	-	-	-	-
Arginine dihydrolase	+	-	-	+	-
Gelatin hydrolysis	-	-	-	-	-
Fluorescent pigment	-	-	-	+	-
Denitrification	-	+	+	-	+

Azospirillum brasilense Sp245) was recently found to be uniquely different from its general assumed functions (8,17), 3 of 5 isolates were selected from the nitrogen fixing bacterial group. Although these nitrogen fixing isolates seem to be the same according to their properties given in Table 1, their colony morphology on defined agar medium and growth on aerobic nitrogen fixing liquid medium indicate that they differ from each other.

The 5 isolates selected were grown aerobically in a defined liquid medium with different nitrogen sources (either 10 mM of NO_3 or NO_3 and NH_3 or NH_3) as a sole nitrogen source. It was found that nitrite began to accumulate after 2 and 8 h of incubation of the isolates when NO_3 or NO_3 and NH_3 was the sole nitrogen source. Maximum nitrite accumulations for the isolates are given in Table 2.

When NO_3 is the sole nitrogen source in the growth medium, bacteria with assimilatory nitrate reductase can reduce nitrate to nitrite and then nitrite is reduced to ammonium. Therefore, nitrite accumulation in the NO_3 growth medium can be attributed to NAS activity. On the other hand, NAP may be responsible for the nitrite accumulation in these cultures as it is an oxygen insensitive periplasmic dissimilatory enzyme and its product can be readily excreted from cells. In the light of this study, it is not clear whether NAS can produce this excess amount of nitrite or whether NAP contributes significantly to nitrate reduction, but nitrite accumulation in these cultures deserves to be characterized.

Nitrite accumulation in the NO_3 and NH_3 growth medium can be attributed to neither NAS nor NAR as they are repressed by ammonium and oxygen, respectively. Therefore, nitrite accumulation under oxic conditions suggests that nitrite produced by the isolates A11, C2, C8, D3 and E2 is likely to be a result of their NAP activity (Table 2).

No nitrite was determined at any stage of growth when NH_3 was the sole nitrogen source. Some denitrifying bacteria can produce nitrite by oxidation of ammonium through heterotrophic nitrification (30). Although it is not known whether the isolates can produce nitrite by heterotrophic nitrification, it is clear that nitrite does not accumulate as a result of this mechanism.

In order to show that the isolates have NAP activity, reduced MV^+ was used as a non-biological electron donor. Table 3 shows that A11, C2, C8 and E2 have NAP enzyme activity. No NAP activity was observed in the isolate D3. This result suggests that either the isolate D3 has no NAP enzyme or its NAP activity cannot be

Table 3. Whole-cell MV^+ dependent periplasmic nitrate reductase activities.*

Isolate	nmol/min/mg
A11	8
C2	16
C8	16
D3	0
E2	20

* Units are nanomoles of MV+ electron donor oxidized per minute per milligram cell of dry weight.

Table 2. Maximum nitrite accumulation for the isolates when they were grown aerobically in NO_3 or NH_3 and NO_3 medium as a sole nitrogen source.

Isolate	NO_3 medium (nitrite µM)	NH_3 + NO_3 medium (nitrite µM)	Nitrite accumulation Assimilation/Dissimilation
A11	1000	5	200
C2	1400	300	5
C8	15	5	3
D3	220	30	7
E2	450	10	45

determined with this assay. Three of the isolates that showed NAP activity belong to free aerobic nitrogen fixing bacteria. Recently, Bedzyk et al. (1999) and Steenhoudt et al. (2001) have shown that nitrogen fixing bacteria, *Rhizobium* spp. and *Azospirillum brasilense* Sp245, also have the NAP enzyme.

NO_3 and NO_2 are 2 important nitrogen forms for organisms and their environment. Denitrifying bacteria are used to remove nitrate from polluted waters (31). The aerobic nitrogen fixer chemolithotrophic isolates (C2, C8 and E2) found to reduce nitrate to nitrite under aerobic conditions can also reduce nitrate under anaerobic conditions (Table 1). Therefore, these isolates can be used in the treatment of waters that are poor in organic materials.

The study suggests that when ammonium exists together with nitrate, aerobic nitrate reduction does not generally lead to high nitrite accumulation. However, when nitrate exists as the sole nitrogen source, nitrite can accumulate in large amounts in aerobic zones of the soil. Since nitrite is not a preferred nitrogen form for plants and leads to soil acidification and, consequently, loss of cations, the use of nitrate-only fertilizers may not be as beneficial as expected in terms of soil productivity. Furthermore, nitrite production as a result of aerobic denitrification suggests that where crop yield is concerned, it is also important to consider the loss of NO_3, which is readily available to plants, from agricultural soil by aerobic nitrate reduction, as well as by anaerobic denitrification.

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