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Minimal NOx emission by *Lysinibacillus sphaericus* in nutrient poor soil

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Abstract

The aim of this study was to determine whether nitrogen dioxide emissions by Lysinibacillus sphaericus exist in nutrient poor soil. First, we evaluated the presence of two genes involved in denitrification (nosF and *nosD) by PCR screening of five strains of L. sphaericus (III (3)7, OT4b.49, OT4b.25, OT4b.31* and *CBAM5). We then applied a bacterial consortium made up by L. sphaericus III (3)7* and *OT4b.49 into closed microcosms of soil* and *with minimum salts medium (MSM) supplemented with ammonia to measure the concentration of produced nitrogen dioxide over time. The assays with closed microcosms showed a minimum level of nitrogen dioxide over time. The nosF* and *nosD primers amplified the expected fragment for the five strains* and *the sequenced nosF* and *nosD PCR product showed an ATPase domain* and *a copper-binding domain respectively, which was consistent with the function of these genes. The basal emission of nitrogen dioxide by L. sphaericus in soil is coupled to its ability to enhance the nitrogen bioavailability for soils deficient in nutrients. Therefore, our results indicate that this microorganism can be considered as a good c*and*idate to validate the low emission of NOx in field* and *in the future as an alternative for biofertilization.*.

Keywords: *Lysinibacillus sphaericus*, nitric oxide, nitric dioxide, biofertilization, denitrification

Introduction

Nitrogen constitutes one of the most important nutrients for the sustainability of life on Earth. Unfortunately, the balance of this element transformation has been altered by anthropogenic sources, mainly by combustion and chemical fertilization processes (Fowler et al. 2013). One of the fundamental losses of nitrogen to the atmosphere happens through nitrogen oxides ($NO - NO_2 - N_2O$). These have adverse effects on the environment due to their implication in the formation of smog, acid rain, global warming and ozone layer depletion (World Health Organization., 2006; Portmann *et al.,* 2012).

The main source of these compounds is anthropogenic as they are produced from fossil fuel combustion. However, bacterial nitrification and denitrification also contribute largely to the emission of nitrogen oxides (Tortoso and Hutchinson., 1990; Kool *et al.,* 2011). It is known that bacterial nitrification is carried out only by autotrophic organisms that belong to β subclasses of Proteobacteria, such as *Nitrospira*, *Nitrobacter* and *Nitrosococcus spp* (Levy-Booth *et al.,* 2014). On the other hand, denitrification by bacteria is performed by a wide range of organisms such as *Pseudomonas spp* and *Paracoccus denitrificans* (an aerobic denitrifier), which is the most representative bacteria of the Proteobacteria group (Hayatsu *et al.,* 2008).

However, it has been found that heterotrophic bacilli are also involved in nitrification and denitrification (Verbaendert *et al.,* 2011). (Kim *et al.,* 2005) characterized the presence of *Bacillus* strains (*Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis*) in these processes for wastewater treatment. Also, there is genomic evidence for genes related to the nitrogen cycle for gram-positive bacilli (Lin *et al.,* 2010). Particularly for *Lysinibacillus sphaericus*, the presence of genes such as *nifU* for nitrogen fixation (Hu *et al.,* 2008; Peña-Montenegro and Dussán., 2013), nitrate reductase (Peña-Montenegro *et al.,* 2015) and n*osD*, *nosF*, and *nosL* involved in denitrification (Rey *et al.,* 2016b; Gomez-Garzón *et al.,* 2016) suggests that this microorganism could also be involved in these processes.

It was also found that *L. sphaericus* can fix nitrogen gas as ammonium and perform *in vitro* nitrification (Dussán., 2016) where the strains of *L. sphaericus* III (3)7 and OT4b.49 were the most efficient in this process. Thus, this study aims to evaluate whether *L. sphaericus* contributes to nitrogen dioxide production when it is added to a nutrient poor soil from the Eastern Llanos basin, Colombia.

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Materials and Methods

Bacterial strains and growth conditions

The five strains of *L. sphaericus* used in this study are listed in Table 1. They were obtained from the bacteria collection at the Center of Microbiological Research (CIMIC). Nutrient agar was the growth medium used for the microbial consortium (*L. sphaericus* III (3)7 and OT4b.49), it was incubated at 30°C for 48 hours and used for the closed microcosms assays.

Amplification of nosF and nosD genes

PCR screening was performed for five strains of *L. sphaericus* (III (3)7, OT4b.49, OT4b.25, OT4b.31 and CBAM5). The set of primers used to amplify a partial codification region of *nosF* were: nosFf (Forward primer) 5'-TTGAAGCGTGTTTCCTTGTG-3' and nosFr (Reverse primer) 5'-AATTTCAGTGATCGGACCAGA- 3'. The amplification procedure was: 95°C for one minute, then 30 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, followed by one cycle of 72 °C for 5 minutes.

For the partial coding sequence of *nosD* amplification procedure, the following primers were obtained: nosDf (Forward 92 primer) 5'- TGACACTCAACAGGCAAAGG-3' and nosDr (Reverse primer) 5'-CCATCCATTGACCAAAGCTC- 3'.The PCR procedure involved one step of 95°C for one minute, then 30 cycles of 95 °C for 30 s, 49 °C for 30 s, and 72 °C for 30 s, followed by a final step of 72 °C for 5 minutes. The primers used for the amplification of both genes were designed using the sequences annotated from the genomes

Table 1: Bacterial strains used in this study

Strain	Isolation source	Reference
L. sphaericus CBAM5	Subsurface soil of oil well explorations	(Peña-Montenegro *et al*., 2015)
L. sphaericus OT4b.25	Beetle larvae	**(**Rey *et al*., 2016)
L. sphaericus OT4b.31	Beetle larvae	**(**Peña-Montenegro & Dussán, 2013**)**
L. sphaericus III (3)7	Soil	**(**Rey *et al*., 2016)
L. sphaericus OT4b.49	Beetle larvae	(Gómez-Garzón *et al*., 2016)

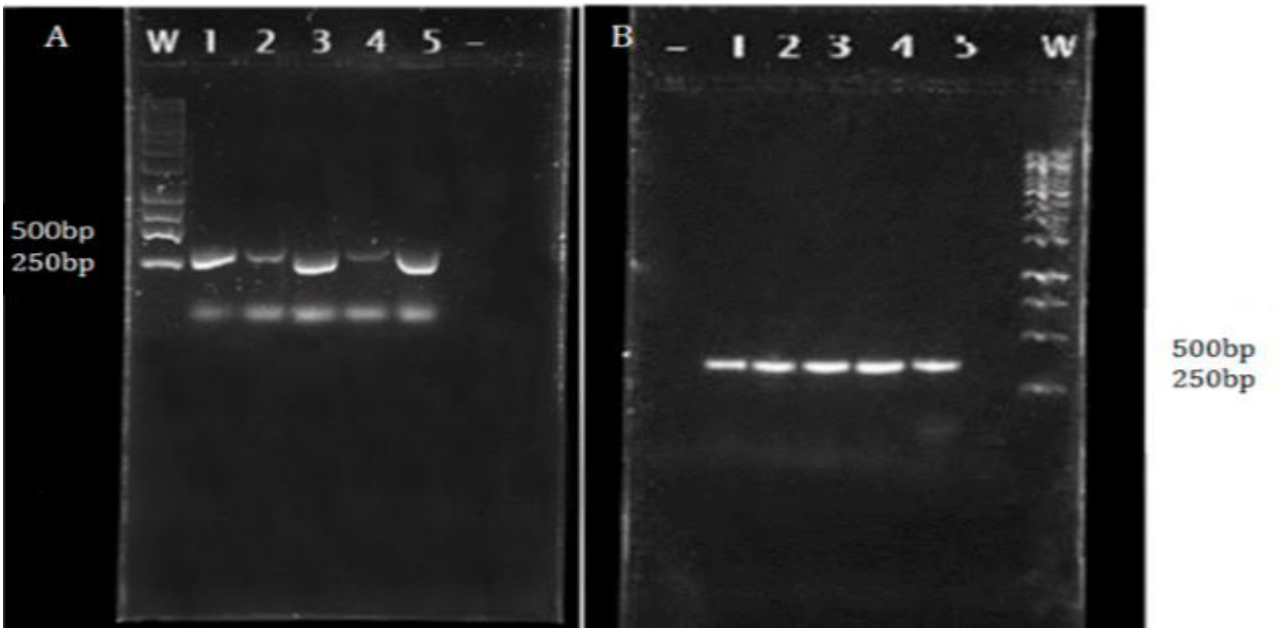

Figure 1: 1.5 %agarose gels with the PCR products of *nosD* and *nosF* amplification for *L. sphaericus* strains A). The expected band size for *nosD* is of 320 bp. Lane order is as follows: W (Weight marker Gene Ruler 1kb DNA ladder (Invitrogen), 1 (strain CBAM5), 2 (strain OT4b.25-positive control), 3 (strain OT4b.31), 4 (strain III (3)7), 5 (strain OT4b.49) and – (negative control). B) The expected band size for *nosF* is of 309 bp. Lane order is as follows: – (negative control), 1(strain CBAM5), 2 (strain OT4b.25-positive control), 3 (strain OT4b.31), 4 (strain III (3)7), 5(strain OT4b.49) and W Weight marker Gene Ruler 1kb DNA ladder (Invitrogen).

of *L. sphaericus* OT4b.25, III (3)7 and OT4b.49 (Rey *et al.,* 2016a; Gómez-Garzón *et al.,* 2016). For both PCR procedures this strain was used as positive control. The amplification products were visualized in 1.5 % agarose gel at 90V for 70 minutes, purified and sequenced by the DNA Sequencing Laboratory (Universidad de los Andes).

Sequence analysis

Primary sequences were cleaned with CLC Main Workbench and then the nucleotides obtained and their corresponding deduced amino acid sequences were compared against GenBank database using BLASTN from the National Center for Biotechnology Information. We also carried out a conserved domains analysis with the amino acids sequences using Pfam and Interpro (EMBL-EBI).

Closed microcosms assays

The substrates used for these assays were nutrient poor soil (low N and P) from Casanare, Colombia and minimal salts medium (Hartsman *et al.,* 1992) supplemented with 0.5g L^{-1} of ammonia.

Nitrogen oxides emission measurements were performed using the Williams and Fehsenfeld (1991) chamber method with the following modifications: three treatments of 30 g of mixed soil (1 part remediated: 3 parts of clean soil) with another 3 treatments of 30 mL of minimum salts medium supplemented with ammonia were set up in closed recipients. Following this, a bacterial mix (with 10^9 UFC/g as a final concentration for both strains) or bacterial mix with 0.22 g of fertilizer (NPK) was inoculated into both media. Each treatment was maintained at 30ºC and it was subjected to 2 replicas. Samples of 10 mL of air were taken with a syringe on day 0, 7, 14, and 21. These samples were added into closed recipients with an aqueous solution of sodium hydroxide (pH 9) and then used to indirectly measure the nitric oxide and nitrogen dioxide quantity using SpectroquantNova60A®.

This indirect measurement relies on the aerobic conversion of nitrogen oxide into nitrogen dioxide and oxidation of nitrogen dioxide into nitrite and nitrate according to the following reaction (Kuropka., 2011):

$$2NO_2 + 2NaOH \rightarrow NaNO_2 + NaNO_3 + H_2O$$

Statistical analysis using R project for Statistical Computing was performed using the Kruskal Wallis test (p-value of 0.05).

The amplification results for both denitrification genes showed a band of approximately 300 bp that is consistent with the expected PCR product for all the five strains studied (Figure 1). For *nosF* and *nosD*, the sequences obtained showed 99 % identity with the reference genome for *L. sphaericus* C3-41 (Table 2). The conserved domain analysis for NosF resulted in an ATPase domain, which is consistent with the function that it performs in a NosZ assembly. Furthermore, for NosD, a comparative analysis showed a copper-binding domain for all five sequences according to the domains present for the protein encoded by this gene. Few findings for gene characterization involved in denitrification for Gram-positive bacteria are currently available. (Liu *et al.,* 2008) described functional characteristics of *Geobacillus thermodenitrificans* NG80-2 *nosZ* cluster revealing that Gram positive and Gram-negative bacteria have conserved the molecular mechanism related to the final step of denitrification. However, further studies on the *nosZ* sequence for *L. sphaericus* along with PCR screening and confirmation of gene expression for all the denitrification genes are required. The presence of atypical *nosZ* cluster does not necessarily indicate denitrification activity as most of bacteria and archaea do not have other denitrification genes (Sanford *et al.,* 2012). It is also necessary to evaluate the activity of these enzymes and their importance related to nitrogen oxides production, as Beaumont *et al.,* (2002) found that the disruption of nitrite reductase in *Nitrosomonas europaea* is not sufficient to stop nitric and nitrous oxide production.

Figure 2 shows that there are significant differences between treatments for day 14, with bigger concentrations for treatments inoculated with the bacterial mix (soil with NPK and MMS) which coincides with the possible biogenic emissions of nitric oxide converted rapidly to nitrogen dioxide by these bacteria (Davidson *et al.,* 2000). As MMS is supplemented with ammonia as nitrogen source, the results obtained indicate that *L. sphaericus* could perform nitrification and denitrification. Both processes generate nitrogen oxides as intermediates (Hayatsu *et al.,* 2008). The measures of nitrite were not included as the results were similar to the detection level. Although the expected ratio of nitrites and nitrates was equal to the unity (Aoki *et al.,* 1982; Kuropka 2011), the excess nitrogen dioxide led to the oxidation of nitric oxide, and the temperature conditions and volume of the microcosm contributed to the greater concentration of nitrate over nitrite. This is consistent with nitrite formation not being the rate-limiting factor from the oxidation of nitrogen dioxide (Aoki *et al.,* 1982). There are few available studies about nitric oxides emissions in soils and they have found that the ratio between nitric and nitrous oxide production varies from 3 to 10:1 (Smith *et al.,* 1997). Thus, in further studies, we would expect lower emissions of nitrous oxide in this particular soil.

In conclusion, the presence of denitrification genes (nosD and nosF) for five strains of *L. sphaericus* could be associated with the activity of this microorganism *in vitro* as our results showed throughout the study. Although the low NOx emissions obtained need to be validated with field

Table 2: Sequences obtained in this study for five strains of *L. sphaericus*

Sequence name	GenBank Accession number	BLASTN identity (%)	BLASTN e-value	BLASTX identity (%)	BLASTX e-value
Nos D partial CDSa	KX981441	100	8e-165	100	2e-69
Nos D partial CDSb	KX981442	99	6e-161	98	6e-67
Nos D partial CDSc	KX981443	99	1e-163	100	2e-69
Nos D partial CDSd	KX981444	99	1e-163	100	2e-69
Nos D partial CDSe	KX981445	99	1e-163	100	2e-69
Nos F partial CDSa	KX981446	100	2e-130	100	9e-39
Nos F partial CDSb	KX981447	100	4e-158	100	7e-53
Nos F partial CDSc	KX981448	100	8e-129	10	5e-38
Nos F partial CDSd	KX981449	100	4e-158	100	7e-53
Nos F partial CDSe	KX981450	100	1e-127	100	5e-38

* a: CBAM5, b; OT4b.25, c: OT4b.31,d: III (3)7 and e:OT4b.49.

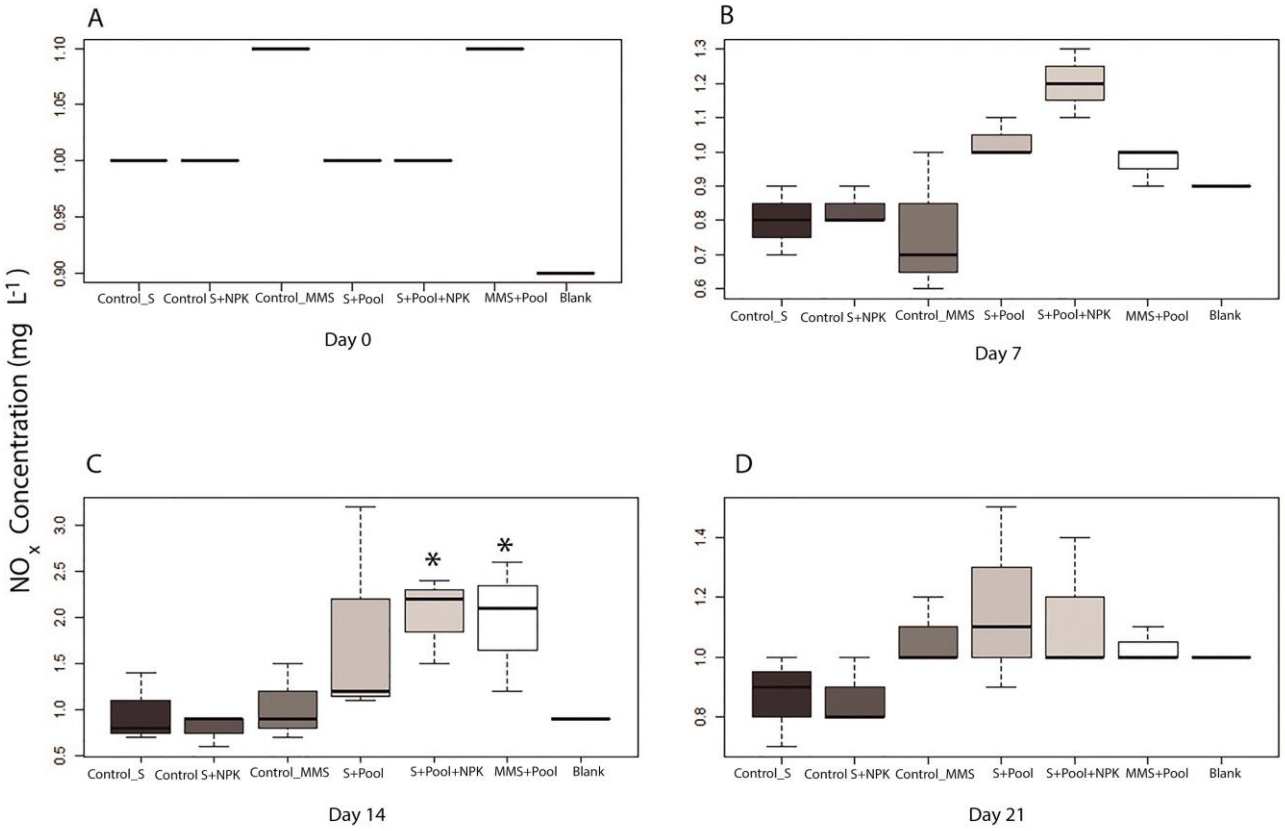

Figure 2: Variation of nitrate concentration for days 0,7,14 and 21 in close microcosms T0, T7, T14 and T21 refer to the days of sampling, pool refers to the bacterial mix (L. sphaericus III (3)7 and OT4b.49) and NPK is the fertilizer used for this assay. S refers to the soil and MMS to the minimal salts medium supplemented with ammonia. Statistical significance between treatments is indicated by *p value minor to 0.05 (Kruskal-Wallis test)

trials, they provide important evidence related to the ability of *L. sphaericus* to perform nitrification and denitrification processes. Thus, this microorganism could be considered as a promising candidate for biofertilization.

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