RESEARCH LETTER

Denitrification-derived nitric oxide modulates biofilm formation in *Azospirillum brasilense*

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Abstract

Azospirillum brasilense is a rhizobacterium that provides beneficial effects on plants when they colonize roots. The formation of complex bacterial communities known as biofilms begins with the interaction of planktonic cells with surfaces in response to appropriate signals. Nitric oxide (NO) is a signaling molecule implicated in numerous processes in bacteria, including biofilm formation or dispersion, depending on genera and lifestyle. *Azospirillum brasilense* Sp245 produces NO by denitrification having a role in root growth promotion. We analyzed the role of endogenously produced NO on biofilm formation in *A. brasilense* Sp245 and in a periplasmic nitrate reductase mutant (*napA*::Tn5; Faj164) affected in NO production. Cells were statically grown in media with nitrate or ammonium as nitrogen sources and examined for biofilm formation using crystal violet and by confocal laser microscopy. Both strains formed biofilms, but the mutant produced less than half compared with the wild type in nitrate medium showing impaired nitrite production in this condition. NO measurements in biofilm confirmed lower values in the mutant strain. The addition of a NO donor showed that NO influences biofilm formation in a dose-dependent manner and reverses the mutant phenotype, indicating that Nap positively regulates the formation of biofilm in *A. brasilense* Sp245.

Introduction

The rhizosphere is a region of intense microbial activity driven by root exudation, where beneficial free-living bacteria can be found. The bacteria belonging to this group are called plant growth-promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1986). *Azospirillum* is a PGPR included in the alpha subclass of proteobacteria, which promotes growth and yield of agronomic and ecological important plant species (Okon & Labandera-Gonzalez, 1994; Bashan & de-Bashan, 2010). *Azospirillum brasilense* produces plant growth regulators mainly indole-3-acetic acid (IAA), which is associated with the beneficial effects observed after inoculation (Baca & Elmerich, 2007). *Azospirillum brasilense* Sp245 inoculation lead to an increase in the number and the length of root hairs and lateral roots (Bashan & de-Bashan, 2010). Early studies showed that *Azospirillum* cultures excrete appreciable amounts of nitrite (NO_2^-) produced by nitrate (NO_3^-) respiration (Didonet & Magalhães, 1997). Zimmer *et al.* (1984) showed that denitrification ability in *Azospirillum*, reduction of NO_3^- to molecular nitrogen (N_2) via NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O), depends on oxygen and NO_3^- concentrations. Furthermore, NO_2^- can replace IAA in several phytohormones assays (Zimmer *et al.*, 1988; Bothe *et al.*, 1992; Didonet & Magalhães, 1993). When ascorbate was added to cultures of *A. brasilense* Sp7 grown in NO_3^- as the nitrogen source, the phytohormonal effect was enhanced (Zimmer *et al.*, 1988). Additionally, the promoting effect of *Azospirillum* on the formation of root hairs and lateral roots was due not only to IAA, but also probably to NO_2^-, as was suggested by Zimmer & Bothe (1988). Later on, studies showed that NO production by *A. brasilense* Sp245 was responsible, at least in part, of the effects on root growth and proliferation (Creus *et al.*, 2005).

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NO is a small highly diffusible gas that functions as a versatile signal molecule through interactions with cellular targets (Lamattina *et al.*, 2003). The synthesis of NO in Gram negative bacteria relies mainly in denitrification pathway. This pathway is the dissimilatory reduction of NO_3^- to gaseous end products (Zumft, 1997), which occurs in four enzymatic controlled steps with NO as an obligatory intermediary (Ye *et al.*, 1994). Both nitrate and nitrite reductases are key regulatory enzymes of the pathway (Zumft, 1997). In *A. brasilense* Sp245, a periplasmic nitrate reductase (Nap) is coded by five genes and is arranged in an operon. The *napEDABC* operon was identified and characterized by Steenhoudt *et al.* (2001a). Kanamycin-resistant mutant (named Faj164, *napA*::Tn5) expresses the assimilatory nitrate reductase activity but is devoid of both Nap and membrane-bound respiratory nitrate reductase (Nar) activities, suggesting that *A. brasilense* Sp245 does not have Nar activity (Steenhoudt *et al.*, 2001a). In addition, the presence of *nirK* genes (*nirK1* and *nirK2*) encoding a NO-producing nitrite reductase copper-containing enzyme type was reported in *A. brasilense* Sp245 (Pothier *et al.*, 2008).

Azospirillum brasilense is able to produce considerable quantities of NO under aerobic conditions, and as stated before, NO production is required for *Azospirillum*-induced lateral root formation (Creus *et al.*, 2005). Interestingly, the mutant Faj164 that produces 5% of NO compared to the Sp245 wt strain in NO_3^- supplemented media was unable to induce the promoting effect on the tomato root growth system (Molina-Favero *et al.*, 2008). Consequently, NO production might be another beneficial trait for plants inoculated with *Azospirillum* (Molina-Favero *et al.*, 2008; Bashan & de-Bashan, 2010; Fibach-Paldi *et al.*, 2012).

To produce beneficial effects, *Azospirillum* has to interact with the plant surface to form complex multicellular assemblies such as aggregates and biofilms that are initiated by an attachment process (Burdman *et al.*, 2000). Biofilms are defined as surface-attached multicellular aggregates, typically encased in a self-produced extracellular polymeric matrix (Ramey *et al.*, 2004). Several factors like mechanical and nutritional stress, and inorganic and quorum-sensing molecules among others, regulate biofilms assembly and disassembly (Karatan & Watnick, 2009). In response to these factors, secondary messengers like cyclic diguanosine monophosphate (c-di-GMP) are activated (Hengge, 2009) leading to biofilm formation or modification (Karatan & Watnick, 2009). Recently, it was shown that NO stimulates biofilm formation by controlling the levels of c-di-GMP (Plate & Marletta, 2012). On the other hand, Barraud *et al.* (2006, 2009) showed that NO triggered the disassembly of *Pseudomonas aeruginosa* biofilms acting upstream of c-di-GMP signaling pathway. More evidences of this complex picture are the results reported by Schmidt *et al.* (2004) who showed that cultures of *Nitrosomonas europaea* treated with exogenous NO gas enhanced biofilm formation. Considering that *A. brasilense* produces high amounts of NO in NO_3^- supplemented medium (Molina-Favero *et al.*, 2008), it was interesting to test the effect of endogenous NO production on the ability of this beneficial bacterium to form biofilms.

Hence, we proposed that NO could be involved in the signaling process for biofilm formation in *A. brasilense*. To determine this, we tested cultures of *A. brasilense* Sp245 and its isogenic Nap mutant Faj164 under static growth conditions for their ability to form biofilm on abiotic surfaces. We also evaluated the effects of the addition of a NO donor on biofilm formation.

Materials and methods

Bacterial strains, plasmids, and constructions

Azospirillum brasilense Sp245 wt, isolated from surface-sterilized wheat roots (Baldani *et al.*, 1986), and *A. brasilense* Faj164, a knockout mutant of Sp245 with a Tn5 insertion in the *napA* gene of the operon (Steenhoudt *et al.*, 2001a), were used. Both strains produce equal negligible quantities of NO in media with NH_4Cl as N source, while in KNO_3-supplemented media, the isogenic mutant Faj164 produces only 5% of the NO in aerobic conditions (Molina-Favero *et al.*, 2008).

Enhanced green fluorescent protein (eGFP) was used for tagging *A. brasilense* strains (Wisniewski-Dyé *et al.*, 2011). To construct *egfp*-containing strains, both *A. brasilense* strains were transformed by biparental conjugation using the *Escherichia coli* S17.1 harboring the broad range plasmid pMP2444 as the donor strain (Bloemberg *et al.*, 2000). Transconjugants were isolated in Nfb with 25 μg mL^{-1} Gentamicin, and the stability of the plasmid was tested by streaking out single colonies on Luria–Bertani (LB) medium for 80 successive generations (Carreño-López *et al.*, 2000).

Static growth conditions

Bacteria were grown on Agar Congo Red (ACR) plates (Rodríguez-Cáceres, 1982) for 5 days and then isolated typical colonies were chosen and each one was transferred to 125-mL flasks containing 25 mL of LB (Difco) medium plus 5 mM $MgSO_4$ and 3.3 mM $CaCl_2$. These precultures were incubated at 30 °C with orbital agitation (100 r.p.m.) for 16 h until risen to 1.1–1.4 $OD_{540\,nm}$. Cells were harvested by centrifugation at 7500 ***g*** (Labnet Z300K) for 10 min, washed with phosphate buffer (66 mM), and resuspended to a final $OD_{540\,nm} = 2$.

Cultures were diluted 1/100 in fresh Nfb-malic medium (Dőbereiner & Day, 1976) modified to achieve a relation C : N = 2 using malic acid at 27.6 mM and supplemented with 13.8 mM NH_4Cl or 13.8 mM KNO_3 as N source. Two mL per well was transferred to sterile clear flat-bottom polystyrene 24-well plates (Costar) and incubated without agitation for 5 days at 30 °C. All media used for Faj164 strain were supplemented with Kanamycin (25 μg mL^{-1}; Sigma). For pMP2444-transformed strains, Gentamicin (25 μg mL^{-1}; Sigma) was also added.

Growth and biofilm formation quantification

At 24-h (d1), 96-h (d3), or 120-h (d5) total growth, adhered plus planktonic cells were quantified by OD_{540nm} measurements. Bacterial biofilm over walls of wells was mechanically removed and mixed with planktonic cells using sterile plastic sticks and agitation. This procedure efficiently removes biofilm and allows reading OD_{540nm} using a micro plate reader (Spectra MR; Dynex Technologies). Also, viable bacteria were enumerated by dilution plating on ACR, using drop plate method (Herigstad *et al.*, 2001). Biofilm formation was determined using crystal violet staining (O'Toole & Kolter, 1998). Briefly, each well was added with 0.5 mL of 0.5 % crystal violet. Plates were incubated for 30 min at room temperature, and then washed carefully three times with tap water. Dye attached to the wells was extracted with 2 mL of 33% acetic acid. $OD_{590\,nm}$ in each well was determined using a micro plate reader. Data were normalized by total growth estimated by $OD_{540\,nm}$.

Confocal microscopy of *A. brasilense* biofilms

Both pMP2444-transformed *A. brasilense* Sp245 and Faj164 strains grew for d1, d3, and d5 under static growth conditions as indicated above. The biofilms formed were three times smoothly washed with PBS, and observed directly over the plates with a confocal laser scanning microscope (CLSM) at emission wavelength of 488 nm (Argon laser) and excitation wavelength of 505 nm (Carl Zeiss, LSM 5 Pascal, Axioskop 2 Mot). Exciting laser intensity, background level contrast, and electronic zoom were maintained at the same level. Stained biofilms were observed and imaged using the Neofluar 10×/1.65 objective. Each experiment was carried out twice.

NO_2^- and NO production in statically grown cultures

NO_2^- concentration, an indirect estimator of NO production (Mur *et al.*, 2011), was determined in free cell supernatants using the inNO-T-II system (Innovative Instruments, Inc) following the manufacturer instructions. Real-time bacterial NO production was determined by amperometric method with a NO-specific amiNO-2000 microelectrode, using the inNO-T-II system. Microelectrode was previously stabilized by 15-min running in PBS buffer pH 7.2, followed by 15-min running in fresh Nfb-malic medium. Microelectrode was inserted 3–4 mm in static bacterial cultures. Recording time of NO production was 40 min per well, and the conversion of picoamperes to μM of NO was carried out according to manufacturer instruction.

Active reduction of NO_2^- to NO in Faj164 mutant was determined fluorometrically, according to Molina-Favero *et al.* (2008). Fluorescence intensity was measured with a Fluoroskan Ascent microplate reader (Labsystems, 480-nm excitation, 525-nm emission) every 4 min for 2 h with 10 μM of the NO-specific fluorescent probe 4,5-diamino-fluorescein diacetate in presence of 0.1 mM $NaNO_2$.

Exogenous NO donor treatments of *A. brasilense* static cultures

To determine the effect of exogenous NO treatment, the NO donor S-nitrosoglutathione (GSNO) was used. GSNO was prepared freshly every day according to Hart (1985), and from the beginning of the experiment, the corresponding wells were added with 1, 25, 50, 100 μM, or 10 mM GSNO every 24 h up to d3. Biofilm formation was evaluated using crystal violet staining as described above. The effect of GSNO treatment on cell viability was evaluated by dilution plating on ACR.

Experimental design and data analysis

All experiments, except amperometric determinations of NO that was determined twice, were performed in three complete independent assays each one with four replicas and repeated at least two times. Media ± SE are presented for each variable determined.

Results

Growth *of Azospirillum* under static condition

Azospirillum brasilense Sp245 and Faj164 isogenic *napA*:: Tn5 mutant were grown in NH_4Cl- or KNO_3-supplemented minimal Nfb liquid medium in cell culture plates without agitation for d1, d3, or d5. In NH_4Cl, both strains grew gradually and to the same extent for the whole period assayed (Fig. 1). The similar growth kinetic showed by both strains indicates that, as was expected, the Nap activity is not required for growth in NH_4Cl-supplemented medium. On

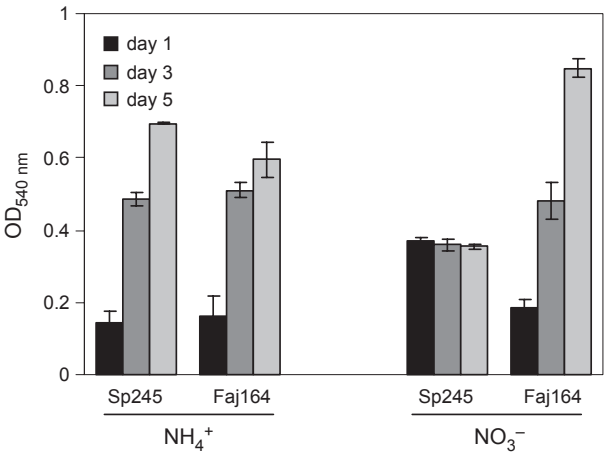

Fig. 1. Static growth of *Azospirillum brasilense* Sp245 and isogenic *napA*::Tn5 mutant Faj164. *Azospirillum brasilense* Sp245 and Faj164 strains were cultivated for 5 days in 24-well plastic plates without agitation in Nfb broth supplemented with 13.8 mM NH_4Cl or 13.8 mM KNO_3 as N source. At 24 h (d1), 72 h (day 3), and 120 h (day 5), total growth (planktonic plus attached cells after disaggregation) was determined by OD_{540nm}. Values are means ± SE of three independent cultures with four replicas each.

the other hand, in KNO_3 Nfb medium, remarkable differences were observed between both strains. The Sp245 wt strain grew fast the first day and then stopped growing (Fig. 1). However, Faj164 strain grew slowly on d1 and gradually increased its growth surpassing wt strain in d5 (Fig. 1). A remarkable observation was that Faj164 strain showed similar growth kinetics both in KNO_3- and NH_4Cl-supplemented media. Moreover, it resembled the wt growth pattern in NH_4Cl-supplemented medium (Fig. 1).

Biofilm formation on abiotic surface

Azospirillum brasilense Sp245 wt and Faj164 mutant strains were assayed for their ability to produce biofilm in two N sources, as indicated earlier. Biofilm formation was quantified with crystal violet. Moreover, attached cells in the biofilm were observed by CLSM. The amount of biofilm produced in each media was significantly different. In NH_4Cl-supplemented medium, biofilm formation was similar for both strains (Fig. 2a). In this medium, biofilms formed at d1 and d3 showed loosely attachment to the well in comparison with d5 where adherence was tighter (Fig. 2b). Significantly, higher biofilm formation occurred in KNO_3 Nfb, showing the wt strain a 10-fold increase in attached cell on d3 compared to NH_4Cl Nfb and fourfold increase on d5 (Fig. 2a). Besides, the wt strain showed a twofold increase of attached cells on d3 compared to Faj164 (Fig. 2a and b). The fact that both strains grew similarly at d3 (Fig. 1) but the wt strain formed a greater biofilm (Fig. 2a) indicated a defect on biofilm formation caused by the deficiency of Nap activity. Nevertheless, the difference observed between both strains at d5 was less pronounced (Fig. 2).

NO_2^- and NO production in statically grown cultures

The NO_2^- concentration was determined in the supernatants of biofilms in each N source (Fig. 3a). No detectable NO_2^- production occurred in medium supplemented with NH_4Cl in both strains during the assay (Fig. 3a). However, remarkable differences were observed when the strains were grown with KNO_3 (Fig. 3a). Whereas the

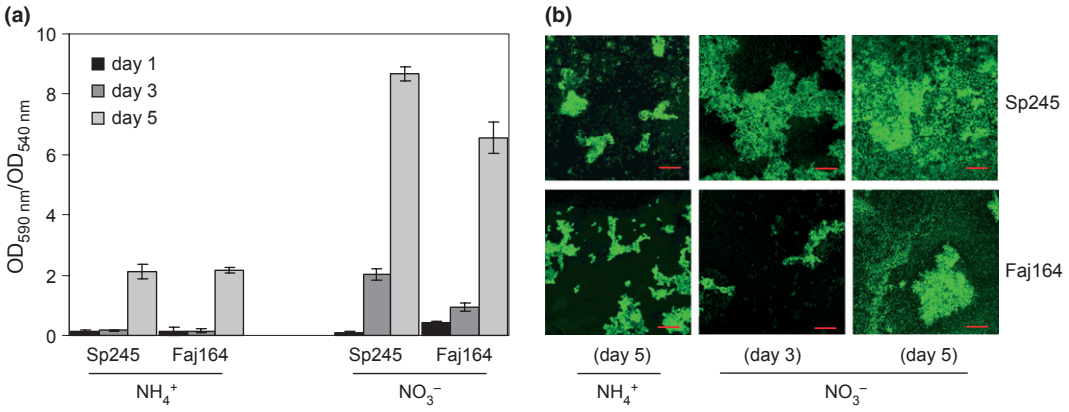

Fig. 2. Biofilm formation under static growth in *Azospirillum brasilense* Sp245 and isogenic *napA*::Tn5 mutant Faj164. (a) *Azospirillum brasilense* Sp245 and Faj164 strains were cultivated for 5 days in 24-well plastic plates without agitation in Nfb broth supplemented with 13.8 mM NH_4Cl or 13.8 mM KNO_3 as N source. At 24 h (d1), 72 h (day 3), and 120 h (day 5), crystal violet staining of the biofilm formed (OD_{590nm}) was determined and normalized by the total cell growth (OD_{540nm}). Values are means ± SE of three independent cultures with four replicas each. (b) Confocal laser microphotographs of representative day 3 and day 5 biofilms of *A. brasilense* Sp245 and Faj164 harboring pMP2444 (*egfp*) plasmid. Magnification is 10×, and red bars represent 25 nm.

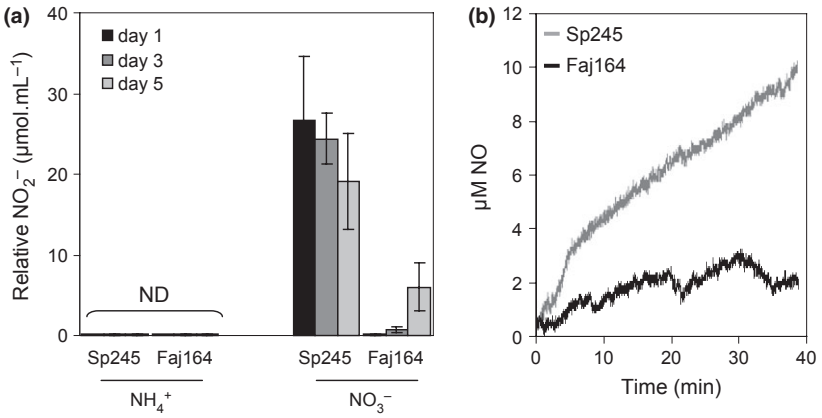

Fig. 3. NO_2^- and NO production in biofilm of *Azospirillum brasilense* formed in static growth. (a) NO_2^- quantification was carried out on supernatants of *A. brasilense* Sp245 wt or Faj164 strains at 24 h (day 1), 72 h (day 3), and 120 h (day 5) of static growth using a microelectrode (in NO-T-II system) operating to determine of NO_2^- ion. NH_4Cl or KNO_3 was provided as N source to Nfb media. Each NO_2^- determined value was normalized by the total growth of the corresponding culture at OD_{540nm}. Values are means ± SE of three independent cultures with four replicas each. (b) The real-time NO production (µM) *in vivo* by the biofilm of wt and Faj164 strains after 3 days of static growing in KNO_3 Nfb medium was estimated by measuring the change in electric potential in steady state during 40 min with the inNO-T-II system provided with a microelectrode. The figure is representative of results obtained after the measurement of two replicas for each strain in two independent assays. nd: not detected.

Sp245 strain was able to produce measurable concentrations of NO_2^- after 24 h in the supernatant of biofilm (*ca.* 30 µmol mL^{-1}), the Faj164 mutant did not produce detectable amounts of NO_2^-. While wt strain slightly decreased the NO_2^- production (arriving to *ca.* 20 µmol mL^{-1} on d5), no NO_2^- concentration was found neither on d1 nor on d3 in mutant biofilm supernatant. Nevertheless, NO_2^- in Faj164 biofilm supernatant was detected at d5 (*ca.* 5 µmol mL^{-1}) (Fig. 3a).

Amperometric determination of NO production derived from NO_2^- was measured in wt and Faj164 static growing cultures. *In situ* production of NO was determined at d3 (Fig. 3b), and data from both strains confirmed the preceding results on NO_2^- production (Fig. 3a). While wt strain produced *ca.*10 µM of NO in 40 min of measurement, the production of NO by mutant strain was < 2 µM (Fig. 3b). Amperometric measurements of NO were determined only in biofilms of d3 to compare similar grown cultures in both strains, evaluated by OD_{540nm} (Fig. 1) and CFU mL^{-1} (data not shown).

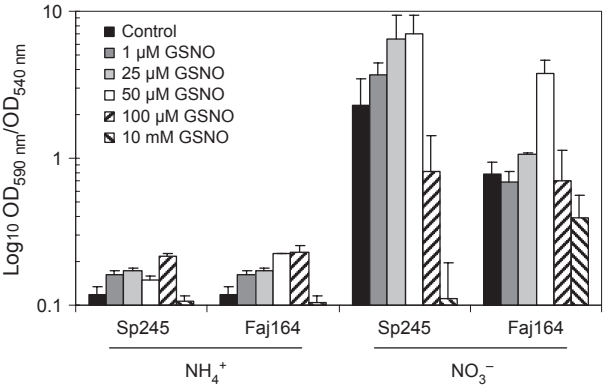

Fig. 4. Exogenously applied NO donor GSNO effects on biofilm formation in *A. brasilense*. Biofilms of Sp245 and Faj164 mutant were allowed to form in 24-well plastic plates with NFb supplemented with NH_4Cl or KNO_3 as N source. Different GSNO concentrations were applied to each well from the start of the culture and added every 24 h. Biofilm formation was quantified with crystal violet 72 h after culture, and data normalized by the total growth of the corresponding culture at $OD_{540\ nm}$. Values are means ± SE of three independent cultures with four replicas each for each GSNO concentration tested. The *Y*-axis is shown as a logarithmic scale.

GSNO effects on biofilms

To assess the role of NO as a signal molecule inducing biofilm formation in *A. brasilense*, different concentrations of GSNO (NO donor) were added to the plates from culture initiation and every 24 h. The addition of GSNO to both media increased biofilm formation in both strains (Fig. 4). In NH_4Cl-supplemented media, a dose response to GSNO up to 100 µM on biofilm formation was produced and no differences were observed between wt and Faj164 strains (Fig. 4). In KNO_3-supplemented media, the wt strain showed gradual increase of biofilm up to 50 µM GSNO (Fig. 4). The addition of 50 µM GSNO to the Nap mutant restored the biofilm formation ability (Fig. 4). These data indicate the role of NO as an early

signal to induce formation of biofilm in *A. brasilense*. Neither lesser than 50 μM nor higher concentrations of GSNO restored the biofilm forming phenotype in the mutant strain, indicating that minor exogenous concentrations could be insufficient to trigger biofilm formation, and higher ones could be cytotoxic. The latter was corroborated by the diminished CFU mL^{-1} counts, where GSNO affected cell viability at 100 μM in KNO_3-containing medium (data not shown). On the other hand, in NH_4Cl-containing medium, GSNO affects cell viability only at 10 mM (data not shown).

Discussion

In natural environments, bacteria are often challenged by changing conditions, including different classes of nutrients availability, and various oxygen tensions (Danhorn & Fuqua, 2007). Some bacteria sense signals and environmental changes, and adjust their lifestyle from planktonic to sessile modes, triggering the formation of biofilms (Karatan & Watnick, 2009). Apart from providing different metabolic pathways, different N sources, NH_4Cl or KNO_3, generate different quantities of endogenous NO in *A. brasilense* Sp245 aerobic cultures (Molina-Favero *et al.*, 2008). Therefore, we tested these two sources of N in the growing media in static conditions and concluded that there was a direct correlation between the presence of NO_3^- as a nitrogen source, and the quantity of biofilm formed (Fig. 2a and b).

NO is a widespread intracellular and intercellular signaling molecule that regulates several functions that promote beneficial effects during the bacteria–plant interaction (Creus *et al.*, 2005; Molina-Favero *et al.*, 2008; Cohen *et al.*, 2010). There are diverse reports on the function of NO in biofilm formation. Schmidt *et al.* (2004) showed that treating *N. europaea* cultures with gaseous NO induced changes in growth characteristics, turning cells into nonmotile forms that produced biofilm on the reactor walls. Nevertheless, *P. aeruginosa* growing in aerobic conditions showed that a rise in the NO content in the preformed biofilm induced its dispersion and stimulated swarming motility (Barraud *et al.*, 2006). This process occurred when the dominating conditions became anaerobic in the biofilm, inducing respiratory Nir activity. In addition, *P. aeruginosa* Δ*nirS* mutants, which produce less NO, showed a high degree of biofilm formation, while Δ*NorCB* mutants, which accumulate NO, showed an increased dispersion of the biofilm formed (Barraud *et al.*, 2006). These results point to a different regulatory mechanism for biofilm formation or dispersion in ammonium-oxidizing bacteria and denitrifiers or pathogenic bacteria.

Data presented in this paper could shed light on previous results obtained by Siuti *et al.* (2011) who showed that biofilm formation in *A. brasilense* Sp7 was greater in media containing $NaNO_3$ compared to NH_4Cl or N-lacking media. These results could be explained given that NO is produced in huge amounts in NO_3^- containing medium compared to NH_4^+ supplemented ones. Moreover, the fact that exogenous NO donor not only increased biofilm formation in the wt strain but also reversed the phenotype of biofilm formation in the *napA*::Tn5 mutant further supports the hypothesis that NO is a signal for biofilm formation in *A. brasilense* (Fig. 4). Interestingly, the response to exogenous NO supply was not only limited to NO-producing conditions (e.g. KNO_3-containing media; Fig. 3a). In NH_4Cl-containing media, both strains also showed an increase in biofilm formation but in much less size than the biofilms produced in KNO_3-supplemented medium (note the log *y*-axis scale, Fig 4b). This result indicates that the mechanism involved in NO responses in *A. brasilense* could be functional in both N sources.

Rhizobacteria can encounter both forms of N in the soil, NH_4^+ and NO_3^-. In fact, the spatial and temporal availability of NH_4^+ and NO_3^- in soils is highly heterogeneous, within centimeters from the roots and changing over the course of a day (Bloom *et al.*, 2003). In this context, biofilm formation by *Azospirillum* could be strongly influenced by the availability of N forms in the microsites of the soil. Our results are in agreement with this hypothesis and point to strengthen the critical role played by NO. As plant roots are common sites for biofilm formation (Danhorn & Fuqua, 2007), the importance of NO as a regulator of the process in PGPR and the mechanisms involved are worthy areas of research. It was described that in *N. europaea*, *Nitrosolobus multiformis*, and *Nitrospira briensis*, NO activate gene transcription required for attachment and initial formation of biofilm (Schmidt *et al.*, 2004). The switch into biofilm growing mode was dependent on NO concentration in the medium. At high NO concentrations, cells produced biofilm for long periods, while the gradual depletion of NO correlated with an increase of motility. Nitrite in supernatants of static cultures of Sp245 wt strain was detected in higher quantities from d1 to d5 (Fig. 3a) while biofilm formation was only observed until d3 and it was notably higher on d5 (Fig. 2). Taking into account that static growth of this strain was constant along the full assay (*ca.* 0.4 OD_{540nm}, Fig. 1), this could indicate that the presence of NO signal on d1 is not sufficient to trigger biofilm formation until d3 (Figs 2 and 3a). A possible shift between NO synthesis (d1) and well-developed biofilm (d3) could be happening. The change from planktonic mode of life to biofilm form includes several physiological switches and the *novo* synthesis of bacterial cell wall components as well as extracellular matrix compounds (Hengge, 2009; Karatan & Watnick, 2009). Our results

indicate that NO acts positively and is an early signal in biofilm formation in *A. brasilense*, as was previously reported by Schmidt *et al.* (2004) and Plate & Marletta (2012) in *N. europaea* and *Shewanella oneidensis*, respectively.

In contrast, in *P. aeruginosa* and *Staphylococcus aureus*, which are opportunistic pathogens, NO mediates the dispersion of biofilms within a nontoxic nM range of concentrations (Barraud *et al.*, 2006; Schlag *et al.*, 2007). In these bacteria, a completely different function for NO was described. The NO signal is mainly produced by catabolic reactions from eukaryotic host cells attacked by pathogens, using NO as a protection in the immune system. Therefore, *S. aureus* has evolved a nitrosative stress response, required for its resistance to innate immunity of the host (Richardson *et al.*, 2006). Moreover, NO acts as a signal enhancing biofilm formation in *Neisseria gonorrhoeae*. The genes coding for nitrate and nitrite reductases, as well as genes involved in oxidative stress tolerance, are up-regulated by NO (Falsetta *et al.*, 2011). This suggests that the effect of NO on biofilm dispersal is a species-specific phenomenon with different bacteria using NO for opposing dispersal strategies.

Contrary to d3, at d5, Faj164 produced significant quantities of biofilm (Fig. 2a and b) in KNO_3-containing medium, which correlated with the presence of NO_2^- in the growth medium (Fig. 3). As cellular lysis is a common process in matured biofilms (Webb, 2006), we speculate that some lysis could by the source of NO_2^- released to growth medium in Faj164 strain. The presence of *nirK* genes (*nirK1* and *nirK2*) encoding a NO-producing nitrite reductase was reported in *A. brasilense* Sp245 (Pothier *et al.*, 2008), and NO_2^- reduction step is functional in Faj164 mutant (data not shown). This NO production could trigger biofilm formation as occur in Sp245 wt strain leading to restore the ability to form biofilms.

In *A. brasilense* Sp245, the Nap is required to synthesize NO (Molina-Favero *et al.*, 2008), but additional physiological roles have been ascribed to this enzyme (Steenhoudt *et al.*, 2001a). It might provide a pathway for dissipation of excess reducing equivalents when cells are grown on highly reduced C substrates as is reported for other bacteria (Richardson & Ferguson, 1992; Sears *et al.*, 1993, 1997). In this way, a spontaneous chlorate-resistant mutant of *A. brasilense* Sp245, named Sp245chl1, defective in both cytosolic assimilatory and periplasmic dissimilatory nitrate reductase activity, was found to be significantly affected in its ability to colonize roots of wheat and rice seedlings (Steenhoudt *et al.*, 2001b). These data further support the Nap activity as an important component in PGPR for root colonization ability. The effect of dissipation of redox equivalents excess should not be ruled out in biofilm development, and it deserves more investigation in the future.

Although the exact nature of gene regulation during initial stage of biofilm formation in *A. brasilense* is still not understood, evidence from others' bacterial models could be valuable. A link between NO and c-di-GMP as was reported in *P. aeruginosa* (Barraud *et al.*, 2009) and *S. oneidensis* (Plate & Marletta, 2012) could not be ruled out in *A. brasilense* Sp245. The genetic approach to unravel these important mechanisms in *A. brasilense* will shed light on the biofilm and root colonization development.

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Authors' contribution

A. Arruebarrena Di Palma and C.M. Pereyra are joint first authors and contributed equally to this work.

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