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Original article

Nitric oxide metabolism and indole acetic acid biosynthesis cross-talk in *Azospirillum brasilense* SM

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Abstract

Production of nitric oxide (NO) and the presence of NO metabolism genes, nitrous oxide reductase (*nosZ*), nitrous oxide reductase regulator (*nosR*) and nitric oxide reductase (*norB*) were identified in the plant-associated bacterium (PAB) *Azospirillum brasilense* SM. NO presence was confirmed in all overexpressing strains, while improvement in the plant growth response of these strains was mediated by increased NO and indole-3-acetic acid (IAA) levels in the strains. Electron microscopy showed random distribution to biofilm, with surface colonization of pleiomorphic Azospirilla. Quantitative IAA estimation highlighted a crucial role of *nosR* and *norBC* in regulating IAA biosynthesis. The NO quencher and donor reduced/blocked IAA biosynthesis by all strains, indicating their common regulatory role in IAA biosynthesis. Tryptophan (Trp) and L-Arginine (Arg) showed higher expression of NO genes tested, while in the case of *ipdC*, only Trp and IAA increased expression, while Arg had no significant effect. The highest *nosR* expression in SMnosR in the presence of IAA and Trp, along with its 2-fold IAA level, confirmed the relationship of *nosR* overexpression with Trp in increasing IAA. These results indicate a strong correlation between IAA and NO in *A. brasilense* SM and suggest the existence of cross-talk or shared signaling mechanisms in these two growth regulators.

Keywords: *Azospirillum brasilense*; *nosR*; *nosZ*; *norBC*; *ipdC*; Scanning electron microscopy

1. Introduction

Auxins are one of the most essential plant growth regulators, involved in functions ranging from apical dominance to root growth and development [1,2]. Different *Azospirillum brasilense* strains may be endophytic [3] or colonize the plant surface [4–6] for evading competition from the surrounding rhizosphere. In plants, various studies have elucidated the role of NO in growth and development, seed dormancy/germination [7], flowering [8], photosynthesis [9], stomatal movement [10,11] and programmed cell death [12], among many others. Nitrification-denitrification by Gram-negative bacteria has been established as an enzymatic process, with NO as an obligatory intermediate [13–15]. The genes involved in these processes are shown in Fig. 1 [16].

Nitrous oxide reductase, encoded by *nosZ*, is a copper-containing enzyme and a part of the gene cluster *nosRZDFYL*. In *Pseudomonas stutzeri*, transcription of *nosZ* is dependent on a membrane-bound iron–sulfur flavoprotein regulatory component, NosR [17,18]. Nitric oxide reductase (Nor) is a membrane-bound enzyme of the heme-copper oxidase superfamily and catalyzes the reduction of NO to nitrous oxide (N_2O), an obligatory step in bacterial denitrification [19,20]. Hino and colleagues established it to be an integral membrane protein consisting of small (NorC) and large

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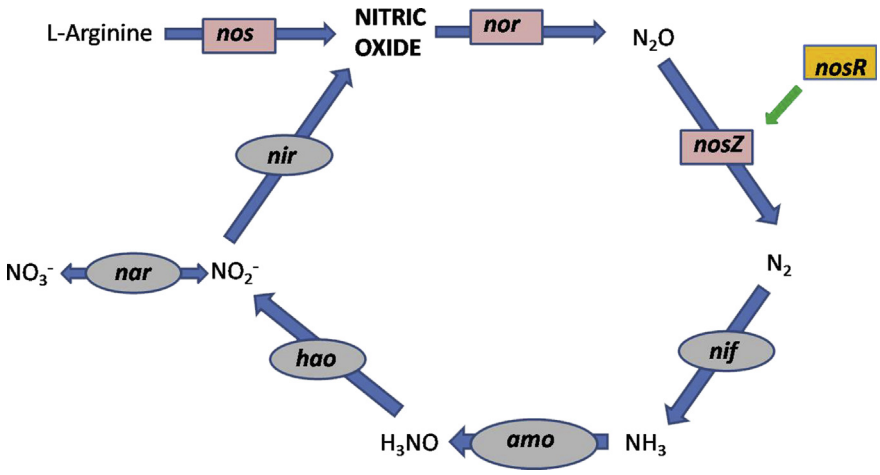

Fig. 1. NO metabolism in plant-associated bacteria. Pathway showing NO metabolism (biosynthesis and catabolism) in bacteria along with critical genes involved. *nos*, *nor*, *nosZ*, *nosR*, *nif*, *amo*, *hao* and *nar*, *nir* represent nitric oxide synthase, nitric oxide reductase, nitrous oxide reductase, transcriptional regulator of nitrous oxide reductase, nitrogenase, ammonia monooxygenase, hydroxylamine dehydrogenase, nitrate reductase and nitrite reductase, respectively.

(NorB) subunits [21]. For a long time, nitrification-denitrification was considered the sole mechanism of biosynthesis of NO in microbes. However, Chen and Rosazza reported the presence of nitric oxide synthase (Nos) in *Nocardia* (1994), which converts L-arginine (Arg) to L-citrulline in the presence of oxygen, thereby releasing NO [22]. Sequence analysis of bacterial Nos has shown that it has high homology with the mammalian oxygenase domain, but lacks the reductase domain [14,23,24]. Creus et al. [13] established that Nos-like activity could be operative in *Azospirillum*, as the addition of Arg to growth media enhances NO production, though the *nos* gene (encoding a nitric oxide synthase) is absent in the *A. brasilense* Sp245 sequenced genome.

Previous studies have shown that NO mediates IAA-induced adventitious rooting in cucumber, as well as root hair formation and lateral branching in tomato [25–29]. Some intermediates of the signaling pathway play a pivotal role in the IAA–NO interaction, leading to improved root development in plants. Studies carried out by Pagnussat et al. [25–27] conclusively demonstrated cGMP-dependent and -independent pathways for IAA–NO–triggered rooting. NO could increase the levels of cGMP via guanylate cyclase and this mediates auxin-induced adventitious rooting in plants. Also, it was established that the mitogen-activated protein (MAP) kinase cascade is activated during IAA-dependant adventitious rooting [27].

Some of our earlier work demonstrated the functional and genetic characterization of IAA biosynthesis in *A. brasilense* SM mediated by means of the indole-3-pyruvate pathway involving indole-3-pyruvate decarboxylase, IpdC [30,31]. The absence of the strain Sp245 IAA regulator, *iaaC*, in strain SM was shown to be an important factor resulting in variations in IAA patterns between different strains [30]. In the present study, we reveal production of NO by strain SM and expression of NO metabolism genes, and we investigate the existence of a correlation/cross-talk between IAA and NO produced by this plant-associated bacterium (PAB).

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. brasilense SM (MTCC 4037, India) and its derivatives were used in this study. The bacterial cultures were maintained on Luria–Bertani agar with antibiotics as per the requirement of the strain. Purity of the culture was checked on nitrogen-free basal (Nfb) medium as described [32]. For all experiments, an initial OD_{560} of 0.1 was obtained by diluting overnight-grown cultures of *A. brasilense* cultured in 20 ml of buffered standard succinate medium (SSM). Cells were grown at 30 °C, 160 rpm for all experiments. All chemicals used were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). For details of bacterial strains used in this study, refer to Table 1.

2.2. DNA manipulations and in silico analysis

A. brasilense SM genomic DNA was isolated according to standard protocols [36]. Plasmids were transferred into *A. brasilense* SM by electroporation with the Gene-pulser (Bio-Rad Labs, Hercules, CA, USA) as mentioned earlier [32]. Plasmid isolation, restriction digestion, ligation and transformation of *Escherichia coli* strains were performed by standard techniques [33]. Restriction and other enzymes were purchased from New England Biolabs. DNA was extracted from agarose gel and eluted by the GenElute Gel Extraction kit (Sigma–Aldrich).

In order to confirm the presence of NO metabolism genes in strain SM (depicted in Fig. 1), evidence was sought using PCR with primers designed from the draft genome sequence of *A. brasilense* Sp245 available at http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=2511231222 [37]. The primers were thus designed to include the entire coding region, and were able to amplify *nosZ*, *nosR* and *norBC* from strain SM genomic DNA (for