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Synthesis and detoxification of nitric oxide in the plant beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 and its effect on biofilm formation

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ABSTRACT

Nitric oxide (NO) is an important gas signal that regulates many biological processes, and due to the high nitrogen recycling activity in the rhizosphere, NO is an important signaling molecule in this region. Thus, an understanding of the effect of NO on the rhizomicrobiome, especially on plant beneficial rhizobacteria, is important for the use of these bacteria in agriculture. In this study, the effect of exogenous NO on the beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 was investigated. The results showed that low concentrations of NO increased the ability of the strain SQR9 to form biofilms, while high concentrations of NO inhibited the growth of this bacterium. The SQR9 gene *yflM* encodes nitric oxide synthase (NOS), which is used to synthesize NO, while the gene *ykvO* encodes a sepiapterin reductase that is used to synthesize tetrahydrobiopterin, the coenzyme of NOS. Isothermal titration calorimetry and high-performance liquid chromatography analyses demonstrated an interaction between YkvO and NADPH. SQR9 has two *hmp* genes, although only one was observed to be responsible for NO detoxification through oxidization. This study revealed the effect of NO on plant beneficial rhizobacterium and assessed the ability of this strain to adapt to exogenous NO, which will help to improve the application of this strain in agricultural production.

1. Introduction

Agricultural production is encountering many challenges from the overuse of chemical fertilizers and pesticides. The application of plant growth-promoting rhizobacteria (PGPR) is an efficient way to promote the sustainable development of agriculture. However, the beneficial effects that PGPR exert on plants largely depend on their ability to colonize roots, for which biofilm formation is the most crucial step. Biofilm matrices are composed of multiple components, including proteins, exopolysaccharides and nucleic acids, which play diverse roles in biofilm formation, such as in cell attachment to surfaces and in biofilm stabilization via promoting interactions between components and the development of three-dimensional biofilm architectures. The formation of biofilm matrices is controlled by several extracellular polysaccharide and protein synthesis genes, such as *epsD* and *tasA* [1]. The rhizosphere is a crucial niche for complex interactions between plant roots, soil and microbes, and the signals regulating these interactions originate from each member of the tripartite [2]. In addition, the formation of the extracellular matrix of the biofilm is also enhanced by root-secreted signals [3].

Nitric oxide (NO) is a water-soluble free radical reactive diatomic gas produced by both plants and microbes that is an important signal in the rhizosphere. As a plant gas hormone, NO regulates plant root growth, disease resistance and abiotic stress tolerance through different signaling pathways [4–6]. NO is synthesized via the oxidation of L-arginine by the enzyme nitric oxide synthase (NOS), which is present in eukaryotes and in some bacteria [7,8]. In eukaryotes, NO binds to the H-NOX (heme-nitric/

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oxygen binding) domain, a family of hemoprotein sensors, and regulates numerous metabolic pathways [9]. Recent data indicates that the H-NOX domains, which are protoporphyrin IX hemoproteins that bind gaseous ligands at a ferrous iron center, are encoded in many bacterial genomes, and H-NOX proteins have approximately picomolar affinity for NO [10–13]. For instance, an H-NOX domain-containing protein that has both diguanylate cyclase and phosphodiesterase activities is encoded in *Shewanella woodyi* and is used to regulate biofilm formation by regulating the concentration of cyclic-di-GMP (C-di-GMP) [14]. NO has also been shown to regulate an H-NOX-associated two-component signaling pathway in the bacteria *Shewanella oneidensis* and *Pseudoalteromonas atlantica* [15–17]. In addition to regulating the activity of canonical histidine kinases, H-NOX proteins also regulate the activities of hybrid histidine kinases, including one known to participate in quorum sensing (QS) in *Vibrio harveyi* [18]. In addition to the abovementioned signaling pathways, NO has also been shown to be involved in bioluminescence, microbial symbiotic relationship and flagellar motility [7,19,20].

Multiple regulatory systems in bacteria have been reported to mediate responses to NO exposure. NO is primarily produced in agricultural soil and from biological nitrogen transformation processes, such as nitrification and denitrification, which are the primary sources of NO production [21]. In the rhizosphere, bacteria are exposed to NO from environmental sources at concentrations vary widely, from cytotoxic micromolar levels to sub-nanomolar levels [10]. Bacteria are also exposed to high concentrations of NO during denitrification, a process in which bacteria respire nitrate or nitrite under oxygen-limiting conditions [22]. To cope with the exogenous/endogenous NO stress, bacteria have evolved elaborate mechanisms to sense and detoxify NO. NO dioxygenase (NOD), which catalyzes the oxidization of NO to nitrate to detoxify NO, was identified in various bacteria, such as *Escherichia coli* and *Salmonella typhimurium* [23,24]. Flavohemoglobin (Hmp) is encoded by *hmp* and has a highly conserved active site in both its heme- and flavin-binding domains. This protein exhibits robust dioxygenase activity and is commonly present in gram-positive and gram-negative bacteria [23,25–27]. Hmp is a primary detoxification protein that has been shown to catalyze the oxidation of free NO to NO_3^- in the presence of O_2 and NADH in *E. coli* [23,25,28]. It is unclear whether the expression of *hmp*, which is directly induced by NO, contributes to NO detoxification in *Bacillus* [29]. In addition, NO reductase (NOR), which catalyzes the reduction of NO to N_2O, was shown to participate in the detoxifying process in *Pseudomonas aeruginosa* [30].

In this study, we investigated the effect of NO on biofilm formation in the plant beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9, which was isolated from the rhizosphere of a cucumber plant and showed a strong ability to promote plant growth, suppress soil-borne diseases and colonize roots [31,32]. In previous studies, we investigated the key genes for biofilm formation in SQR9 and signal transduction with cucumber plants [33]. Although NO is a gas signal synthesized by both plants and microbes, it has not yet been studied with respect to its synthesis and detoxification in plant beneficial rhizobacteria. This results of this study revealed that the low concentrations of NO induced biofilm formation of SQR9, while high concentrations inhibited its growth. SQR9 encodes nitric oxide synthase to synthesize NO using tetrahydrobiopterin (BH_4) as a coenzyme. Interestingly, although SQR9 has two *hmp* genes, only one was observed to be responsible for NO detoxification through oxidization.

2. Materials and methods

2.1. Strains and culture conditions

The strains and plasmids used in this study are described in Table 1. *E. coli* TOP10 was used as the host strain for plasmid pET28a. *B. amyloliquefaciens* SQR9 (CGMCC accession NO. 5808, China General Microbiology Culture Collection Center, NCBI accession NO. CP006890) was incubated at 37 °C in Luria-Bertani medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter). The ability of the wild-type SQR9 strain and its mutants to form biofilms was investigated in MSgg medium (5 mM potassium phosphate, 100 mM morpholinepropanesulfonic acid [MOPS], 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 mM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg/ml tryptophan, 50 μg/ml phenylalanine, and 50 μg/ml threonine, pH 7.0) [34].

2.2. Gene knockouts

To delete the *ykvO*, *yflM*, *hmp* and *hmp1* genes in SQR9, approximately 1 kb fragments upstream and downstream of each target gene were amplified. Next, the 1.1 kb chloramphenicol (Cm) and 1.7 kb erythromycin (Em) resistance genes were amplified from the plasmids pNW33n and Pax01, respectively, which overlapped the upstream and downstream fragments of each target gene. All PCR products were gel purified using an AxyPrep DNA gel purification and extraction kit (Axygen, Hangzhou, China). In the first step of the overlapping PCR, the reaction mixture contained 14.7 μl

Table 1
Bacterial strains and plasmids used in this study.

Strains and plaimids	Genotype	origin/reference
Plasmids		
pNW33 N	Cm^R, *E. coli-Bacillus* shuttle vector	[57]
Pax01	Em^R	[58]
pET-28a	Expression vector	Novagen
Strains		
B. amyloliquefaciens		
SQR9	Wild type isolate	[31]
Δ*yflM*	Complete deletion of the *yflM* gene, Cm^R	This study
Δ*ykvO*	Complete deletion of the *ykvO* gene, Em^R	This study
Δ*hmp*	Complete deletion of the *hmp* gene, Em^R	This study
Δ*hmp1*	Complete deletion of the *hmp1* gene, Cm^R	This study
Δ*hmp*Δ*hmp1*	Deletion in both *hmp* and *hmp1* gene, CmR, EmR	This study
E. coli		
BL21 (DE3)	F^- *ompT hsdSB*(rB^-mB^-) *gal dcm* λDE3 (harboring gene 1 of the RNA polymerase from the phage T7 under the *PlacUV5* promoter)	Invitrogen (Shanghai, China)
Top 10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ψ80 *lacZ* ΔM15 Δ*lacX74 nupG recA1 araD139*Δ(*ara-leu*) *7697 galE15 galK 16 rpsL* (Str^R) *end A1*$λ^-$	Invitrogen (Shanghai, China)

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