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Enhanced removal of nitrate in the maize rhizosphere by plant growth-promoting *Bacillus megaterium* NCT-2, and its colonization pattern in response to nitrate

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HIGHLIGHTS

- *Bacillus megaterium* NCT-2 significantly promoted nitrate removal from the soil.
- The strain successfully colonize maize roots and the rhizosphere.
- The strain significantly improved the growth of maize seedlings.
- The NCT-2 population in the roots was significantly affected by nitrate content.
- Soil nitrate concentration had no significant effect on NCT-2 distribution.

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ABSTRACT

High soil nitrate concentrations can lead to the secondary salinization of soils. *Bacillus megaterium* NCT-2 is a wild-type strain isolated from secondary salinized soil and is very effective in reducing nitrate. Laboratory and greenhouse experiments were carried out to investigate its nitrate reduction capabilities, colonization pattern, and plant growth promotion responses to nitrate content in the soil. *B. megaterium* NCT-2 was marked with a green fluorescent protein (*gfp*) gene and was left to successfully colonize maize roots and the rhizosphere. Inoculation with *gfp*-tagged NCT-2 significantly promoted nitrate removal from the soil and improved plant growth. Confocal microscopy results revealed that NCT-2 is an endophyte that can colonize the meristematic and elongation zones of the root tip, and the middle segment of the root. Soil nitrate concentration had no significant effect on NCT-2 distribution. The *gfp*-tagged NCT-2 populations in the roots and rhizosphere soil first increased, but then decreased, and at the end of the experiment, colonization levels in the rhizosphere soil stabilized at ~5×10^4 CFU g^{-1} soil. However, the levels in the roots increased again to $1–3 \times 10^4$ CFU g^{-1} root in the different treatments. The NCT-2 population in the roots was significantly affected by nitrate content. A nitrate-nitrogen concentration of 72 mg kg^{-1} was the optimum concentration for NCT-2 colonization of maize roots. This study will improve the agricultural application of NCT-2 as a biofertilizer for nitrate removal and plant growth promotion.

1. Introduction

The intensive growth of horticultural crops has led to large chemical fertilizer consumption rates in China, which has resulted in serious environmental and food safety problems (Song et al., 2009). The replacement of chemical inputs with biotechnological products is becoming an important alternative to the use of

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chemicals (Luo et al., 2010). Extensive research has been conducted on whether plant beneficial microbes can be potentially used as bio-fertilizers to reduce the use of chemical fertilizers. Some of the strains, known as plant growth-promoting rhizobacteria (PGPR), can directly or indirectly benefit the health and yield of the plant. The *Bacillus* genus members are typical representatives of PGPR, and are often considered to be the best candidates for developing efficient bio-products because they produce a large number of bioactive compounds and can sporulate (Ongena and Jacques, 2008).

To be of benefit to plants, PGPR need to colonize the plant root system (Compant et al., 2010; Ji et al., 2008). PGPR should also be able to colonize host plant rhizospheres if they are to have agricultural application (Krzyzanowska et al., 2012). Tracking the presence of a bacterial strain in an overcrowded environment, such as the rhizosphere, requires selective detection methods (Krzyzanowska et al., 2012). Reporter gene technology is an important approach that is used to monitor microorganisms in various environmental samples (Ramos et al., 2000). Green fluorescent protein (GFP) has been widely used as a reporter for the in-situ detection and location of bacterial cells in soil and in the rhizosphere (Zhang et al., 2010), and in plants (Liu et al., 2006; Njoloma et al., 2006; Ramos et al., 2000; Timmusk et al., 2005; von der Weid et al., 2005; Zhang et al., 2011). Previous *Bacillus* colonization studies focused on their colonization distribution in various plants (Liu et al., 2006). *Bacillus mojavensis* AB1 colonized the leaves and twigs of coffee plants (Nair et al., 2002). *Bacillus pumilus* SE34 colonized tomato roots, stems, and leaves 6 weeks after inoculation (Yan et al., 2003). Colonization of *Lemna* and *Arabidopsis* root tips is strongly favored by *Bacillus amyloliquefaciens* FZB42 (Fan et al., 2012), and *Bacillus megaterium* C4 cells were mainly found in the intracellular spaces of maize and rice (Liu et al., 2006).

Plant growth conditions, including soil type and the occurrence of stress factors, play a part in determining the composition of plant microflora (Krzyzanowska et al., 2012). Although some previous studies have researched the relationships between PGPR, plants, and nitrogen (Florio et al., 2017; Mantelin et al., 2006; Mantelin and Touraine, 2004), few of them have investigated the PGPR colonization pattern in response to nitrate. A novel bacterium, *B. megaterium* NCT-2, which was isolated and identified from secondary salinization soil taken from greenhouses in China, showed a high nitrate reduction capability (Chu et al., 2017; Shi et al., 2011). In a previous study, the NCT-2 strain was shown to remove excessive nitrate in secondary salinization soil and promote plant growth (unpublished, Dan Zhang did the experiment and offered us the data). Therefore, *B. megaterium* NCT-2 could be applied as a bio-fertilizer. The commercial application of *B. megaterium* NCT-2 requires both the efficient colonization of plant tissues and long-term maintenance in the soil. The rhizosphere colonization pattern and plant growth promotion of NCT-2 in response to soil nitrate content was determined by tagging a natural isolate of NCT-2 with GFP. The localization of the bacterium population in the maize rhizosphere, and a number of maize growth parameters were investigated in plants and soils containing various nitrate concentrations. This study improves understanding of *B. megaterium* NCT-2 plant-microbe interactions in response to nitrate content, and nitrate reduction and plant promotion abilities.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacillus megaterium NCT-2 (China General Microbiological Culture Collection [CGMCC] no. 4698) was isolated and identified from secondary salinization soil in Shanghai, China. A shuttle plasmid pHIS1525, which replicates in *Escherichia coli* and *B. megaterium*, was used to introduce the *gfp* gene into *B. megaterium* NCT-2. The NCT-2 strain was sensitive to tetracycline and ampicillin, but the pHIS1525 plasmid contained tetracycline and ampicillin resistance genes.

2.2. Construction of GFP-labeled B. megaterium NCT-2

The *gfp* sequence was amplified using the *gfp*-sense/*gfp*-anti (GCAGATCTGAATGGTGAGCAAGGGCGAG/GGCGGATCCTTACTTGTACAGCTCGTCCATG) primer set and cloned into the pMD18-T vector. Then the pMD18-T-*gfp* plasmid was transformed into *E. coli* Trans109. The transformants containing the recombinant pMD18-T-*gfp* plasmid were selected and sequenced. The pHIS1525-*gfp* plasmid was constructed by ligating the gene *gfp*, digested by BglII and BamHI from pMD18-T-*gfp*, into the corresponding restriction sites of the pHIS1525 plasmid, which was then transformed into *E. coli* DH5α. The transformants containing the pHIS1525 plasmid harboring the *gfp* gene were selected. Then the pHIS1525-*gfp* plasmid was isolated and the entire encoding sequence was tested to verify the absence of undesired mutations introduced by the PCR.

The competent *B. megaterium* NCT-2 cells were prepared as follows: first a single colony of *B. megaterium* NCT-2 was incubated in 5 mL of Luria Bertani (LB) broth medium and left to grow overnight at 35 °C with shaking at 200 rpm. Second, 1 mL of the culture was incubated in 50 mL of LB medium and allowed to grow at 35 °C with shaking at 200 rpm until an OD_{600} of 1.0 was reached. Next, the cells were centrifuged for 15 min at 5000 rpm at 4 °C. Then the pellets were resuspended in 5 mL of SMMP buffer and lysozyme was added to a final concentration of 0.1 mg mL^{-1}. The mixture was then incubated at 35 °C and shaken at 200 rpm for 30 min. After this, the cells were centrifuged for 10 min at 2000 rpm and 25 °C. The pellets were resuspended in 5 mL SMMP buffer, and centrifuged for 10 min at 2000 rpm and 25 °C. Finally, the pellets were resuspended in 5 mL SMMP buffer, and a 500 mL aliquot of competent cells was placed in a sterile polypropylene tube. These competent cells were stored at −80 °C for future use. The SMMP buffer was made by mixing equal volumes of 2 × SMM (40 mM maleic acid, 80 mM NaOH, 40 mM $MgCl_2$, 1 M sucrose, pH 6.5) with 2 × AB_3 (antibiotic medium No. 3, DIFCO).

The GFP-labeled *B. megaterium* NCT-2 was constructed by transferring pHIS1525-*gfp* into competent *B. megaterium* NCT-2. The transformation process was as follows: 5 μL of pHIS1525-*gfp* plasmid was added to 20 μL of SMMP buffer and the mixture was incubated at 35 °C for 20 min. Then the mixture was added to 500 μL of competent cells and 1.5 mL PEG-P (400 g L^{-1} PEG-6000 in 1 × SMM) was gently added. The mixture was incubated at 25 °C for 2 min. Exactly 5 mL of SMMP buffer were added and mixed thoroughly and then the mixture was centrifuged at 3000 rpm at 25 °C for 10 min. The pellets were resuspended in 1 mL SMMP buffer and incubated at 35 °C for 45 min. The cells were transferred to the incubator shaker at 35 °C and were shaken at 200 rpm for 45 min. The culture was gently mixed with 2.5 mL of CR5-top agar, and then poured on selective medium (LB plate containing 10 μg mL^{-1} tetracycline). The CR5-top agar was made by mixing 1.25 mL of solution A (206 g L^{-1} sucrose, 13 g L^{-1}MOPS, 1.2 g L^{-1} NaOH, pH 7.3) with 713 μL of solution B (14.04 g L^{-1} agar, 0.7 g L^{-1} casamino acids, 35.09 g L^{-1} yeast extract), 288 μL of 8 × CR5 salts (2 g L^{-1} K_2SO_4, 80 g L^{-1} $MgCl_2$, 0.4 g L^{-1} KH_2PO_4, 17.6 g L^{-1} $CaCl_2$), 125 μL of 12% (w/v) proline, and 125 μL of 20% (w/v) glucose. The transformants that emitted green fluorescence, were screened using fluorescence microscopy (Olympus DX51, Olympus, Tokyo, Japan).

The NCT-2 and *gfp*-tagged NCT-2 growth was evaluated by taking a single colony of these two strains and culturing them