Microbiological Research **164** (2009) 461—468

Available online at www.sciencedirect.com

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Microbiological Research

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Nitrate respiration in *Pseudomonas stutzeri* A15 and its involvement in rice and wheat root colonization

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Received 6 November 2006; received in revised form 22 January 2007; accepted 5 March 2007

KEYWORDS
Pseudomonas stutzeri;
Nitrate respiration;
Rice;
Rhizosphere colonization

Summary

Unlike most bacteria, the nitrogen-fixing rice-associated *Pseudomonas stutzeri* A15 disposes of three different nitrate reductases that enable conversion of nitrate to nitrite through three physiologically distinct processes, called nitrate assimilation, nitrate respiration and nitrate dissimilation. To study the role of nitrate respiration in rhizosphere fitness, a *Pseudomonas stutzeri narG* mutant was constructed and characterized by assessing its growth characteristics and whole-cell nitrate reductase activity in different oxygen tensions. Unexpectedly, the *Pseudomonas stutzeri* A15 *narG* mutant appeared to be a better root colonizer, outcompeting the wild type strain in a wheat and rice hydroponic system.

Introduction

Nitrate is present in many environments and can be reduced by bacteria to nitrite for three different purposes: (i) utilization as a nitrogen source for growth (nitrate assimilation); (ii) generation of proton motive force to synthesize ATP by using nitrate as the terminal electron acceptor (nitrate respiration); or (iii) dissipation of excess reducing equivalents (nitrate dissimilation). These physiologically distinct processes are carried out by three different nitrate reductase systems, which differ concerning cellular location, structure, biochemical properties, regulation of expression, and gene organization (Moreno-Vivian and Ferguson, 1998; Richardson and Watmough, 1999; Richardson et al., 2001).

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0944-5013/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2007.03.003

Assimilation of nitrate starts with the active uptake of nitrate by an ABC-type transporter. The assimilatory nitrate reductase (encoded by the *nas* genes) converts nitrate to nitrite in the cytoplasm. Nitrite is subsequently reduced by the assimilatory nitrite reductase to ammonium, which is incorporated into carbon skeletons (Lin and Stewart, 1998; Reitzer, 2003). Nitrate assimilation capacity may provide a competitive advantage since it enables bacteria to use nitrate as alternative nitrogen source.

The dissimilatory nitrate reductase is found in the periplasm and is encoded by the *nap* genes. Different physiological roles have been attributed to the periplasmic nitrate reductase. Emerging evidence indicates that Nap activity is used for redox balancing by dissipating excess reductant. Maintenance of an appropriate redox balance can be necessary for optimal bacterial growth in some physiological conditions (Richardson, 2000; Potter et al., 2001). Nap activity may be particularly important when bacteria are grown on highly reduced carbon sources or during anaerobic growth of photosynthetic bacteria (Sears et al., 1997; Richardson, 2000).

The respiratory nitrate reductase is composed of three subunits, encoded by *narGHJI*. This operon is highly conserved amongst different species. The soluble $\alpha\beta$-complex (α and β subunit encoded by *narG* and *narH*, respectively), is anchored to the plasma membrane by subunit γ (encoded by *narI*). NarJ is required for maturation and assembly of the NarGHI enzyme (Philippot, 2002). Electrons are drawn from the quinol pool to the γ-subunit and subsequently transferred via NarH to the catalytic site of NarG. The oxidation of the quinol pool at the periplasmic side of the inner membrane results in the release of two protons, thereby coupling the reduction of nitrate in the cytoplasm to proton translocation, and generating the proton motive force necessary for ATP synthesis (Berks et al., 1995; Philippot and Hojberg, 1999; Richardson, 2000). This system enables bacteria to grow in anaerobic conditions using nitrate as an alternative electron acceptor in the respiratory chain.

Pseudomonas stutzeri A15 is a nitrogen-fixing bacterium that is able to colonize and infect rice roots (Vermeiren et al., 1998, 1999; Lalucat et al., 2006). Acetylene reduction assays revealed a relatively high nitrogenase specific activity (1800 nmol C_2H_4 mg prot^{-1} h^{-1}), and therefore, strain A15 may be suitable to enhance rice plant growth by biofertilization (Vermeiren et al., 1999). Because of the specific environmental conditions in the paddy rice rhizosphere, nitrate respiration by *Pseudomonas stutzeri* A15 may play a role during root colonization and this was investigated in this study using a hydroponic model system.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas stutzeri strains were grown overnight at 30 °C or 37 °C in Luria Bertani (LB) medium (Sambrook and Russell, 2001), in minimal medium M9 (containing 6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCl, 0.12 g/l MgSO4, and 2 g/l glucose) or in MMAB medium (containing 5 g/l Na-malate, 3 g/l K_2HPO_4, 1 g/l NaH_2PO_4, 1 g/l NH_4Cl, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 0.15 g KCl, 0.01 g/l of $CaCl_2 \cdot 2H_2O$, 0.0025 g/l $FeSO_4 \cdot 7H_2O$, 0.005 g/l biotin, and microelements; Vanstockem et al., 1987). *Escherichia coli* strains were grown at 37 °C in LB medium. Antibiotics were added at the following concentrations when required: 100 μg ml^{-1} rifampicin (Rif), 50 μg ml^{-1} kanamycin (Km), or 100 μg ml^{-1} ampicillin (Ap).

Recombinant DNA techniques and DNA sequence analysis

Standard techniques for subcloning, mating experiments and agarose gel electrophoresis were performed as described by Sambrook and Russell (2001). Plasmid DNA was isolated using either the GFX Micro Plasmid Prep Kit (Amersham Biosciences) or QIAprep Spin Miniprep Kit (Westburg). Genomic DNA of *Pseudomonas stutzeri* A15 was isolated with the Puregene kit (Gentra Systems).

DNA sequencing was performed with an Avant Genetic Analyzer-3100 (Applied Biosystems, USA). Homology searches were performed using the Blast programs on http://www.ncbi.nih.nlm.gov/blast.cgi and http://www.pseudomonas.com. Multiple DNA or protein sequence alignments were performed with the ClustalW 1.8 software (Thompson, et al., 1994).

Construction of a *Pseudomonas stutzeri* A15 *narG* mutant

Based on the alignment of several NarG homologues, a pair of degenerated primers, Pseu-521 (5′-GGNTGGGCSCAYTAYGTSGG-3′) and Pseu-522 (5′-TGRATNCCCCAYTTCTGRTGSGG-3′), was designed to amplify an internal fragment of *Pseudomonas stutzeri* A15 *narG*. The 1.6-kb fragment was cloned