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Phenotypic variation in *Azospirillum brasilense* Sp7 does not influence plant growth promotion effects

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ABSTRACT

The *Azospirillum* genus comprises free-living, plant growth-promoting, nitrogen-fixing bacteria found in the rhizosphere of plant roots. Azospirilla are able to promote plant growth mainly through improvement of root development. Bacterial surface components, such as extracellular polysaccharides and proteins, are involved in root colonization. Phase variation – or phenotypic variation – is one of the mechanisms by which microorganisms adapt to environmental changes. This phenomenon is characterized by the presence of a sub-population of the bacteria presenting a different phenotype relative to the major population. In this study we characterized phenotypic variation of *Azospirillum brasilense* Sp7. When plated on solid media, some *A. brasilense* colonies were shown to possess a much more mucoid morphology, producing 7.5–8 times more exopolysaccharide with different monosaccharide composition than the parental strain Sp7. The rate of appearance of this kind of variant colonies was 1 in 5000, in agreement with the accepted rate for the phase/phenotypic variation phenomenon. The variants were significantly more resistant to heat and UV-exposure than the parental strain and displayed genomic changes as seen by the different band patterns following ERIC-PCR, BOX-PCR and RAPD analyses. In plant inoculation experiments under greenhouse conditions, with maize, wheat, soybean and peanuts, the EPS overproducing variants performed as similar as the parental strain. Therefore, EPS overproduction did not confer an apparent advantage to *A. brasilense* in terms of induction of plant growth promotion.

1. Introduction

Bacteria of the genus *Azospirillum*, belonging to the class alphaproteobacteria, are free-living, plant growth-promoting, nitrogen-fixing bacteria found in the rhizosphere of plant roots (Baldani et al., 2005; Wisniewski-Dyé et al., 2011). Azospirilla are able to promote plant growth through improvement of root development (Spaepen et al., 2009). Evidence suggests that production of phytohormones by the bacteria plays an important role in plant growth promotion (Spaepen et al., 2009). The beneficial effects result in increased crop yield, in many plants of agronomic importance (Bashan and de Bashan, 2010; Fibach-Paldi et al., 2012).

Bacterial surface components, such as extracellular polysaccharides and proteins, are involved in root colonization (Burdman et al., 2000a). Extracellular polysaccharides secreted by *Azospirillum* comprises lipopolysaccharides (LPS) and capsular polysaccharides (CPS), which form an adherent cohesive layer on the cell surface, and exopolysaccharides (EPS) that form an extracellular matrix that has little or no association with the cells. EPS properties contribute to cell protection against environmental stresses, attachment to surfaces, nutrient gathering and cell antigenicity. The relative monosaccharide composition and the molecular weight of the EPS and CPS in *Azospirillum* vary within species and strains, growth conditions and physiological states (Burdman et al., 2000b; Bahat-Samet et al., 2004).

Phase variation – or phenotypic variation – is one of the mechanisms by which microorganisms adapt to environmental changes. This phenomenon is characterized by the presence of a sub-population presenting a different phenotype from the major population. This sub-population appears at a relatively high ratio, more than 10^{-5} (as compared with less than 10^{-6}, as for spontaneous mutations), but during appropriate conditions can become

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dominant. Phase variation has been described for many different bacterial genera belonging to diverse taxonomic groups and displaying different ecological behaviors (*eg.* pathogens, saprophytes and symbionts). Phase variation can be associated with changes in various phenotypes, such as motility, synthesis of pili, and expression of capsule and production of antifungal metabolites. The molecular mechanisms associated with phase variation are diverse and include genetic and epigenetic changes, such as DNA inversions, duplications and deletions, transpositions, homologous recombinations, slipped-strand mispairings as well as differential methylation patterns (Wisniewski-Dyé and Vial, 2008). In phase variation, the expression of a given factor is either ON or OFF and the changes (variations) are usually reversible (i.e. ON ↔ OFF), resulting in a heterogeneous population (Henderson et al., 1999).

Several studies with some *Azospirillum* species and strains have identified phenotypic variants. First studies reported frequently occurring spontaneous mutants in *Azospirillum brasilense* Sp7 with increased resistance to stress conditions like salt stress and iron acquisition (Hartmann et al., 1992) as well as the stable variant $4V_I$ of *Azospirillum lipoferum* 4B, which lost the ability to swim and had altered carbohydrate utilization abilities (Alexandre and Bally, 1999). In another report, an *A. brasilense* WN1 stable variant was reported to be non-motile and to produce translucent colonies in contrast to the parental strain. In this study, a plasmid pattern change was observed: while the wild-type cells harbors five plasmids, the variants carried only four of them, with the disappearance of a 260-kb replicon (Vial et al., 2006). A recent study has described several phenotypic variants of *A. brasilense* Sp7 that were obtained after exposure to prolonged starvation or after re-isolation from maize roots. Some of the variants were found to produce significantly more EPS, and of different monosaccharide composition, and displayed DNA rearrangements compared to the parental strain (Lerner et al., 2010).

In the present study we further examined physiological aspects of phenotypic variation in *A. brasilense* Sp7. The most frequent variants of these strains were found to overproduce EPS. Three representative EPS variants were randomly selected for more detailed characterization. Plant growth promotion of the variants was assessed with four plant species in the greenhouse, and it was demonstrated that the variants in general do not differ in their plant growth promotion effects as compared with the parental strain.

2. Materials and methods

2.1. Media and bacterial growth conditions

A. brasilense Sp7 and variants of this strains that were obtained in this study were routinely grown at 30 °C in fructose minimal medium, with high carbon to nitrogen ratio (C:N) (Burdman et al., 1998) containing (g l^{-1}): D-fructose (6.67), $MgSO_4$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), K_2HPO_4 (6.0), KH_2PO_4 (4.0), yeast extract (Difco) (0.1), NH_4Cl (0.214) and microelements as described (Okon et al., 1977). For preparation of solid fructose minimal medium, agar (Difco) was amended at 15 g l^{-1}. Cell growth was determined spectrophotometrically at OD_{590}, by dilution plating on D-fructose minimal medium or by drying the bacteria in an oven at 80 °C for 48 h, and measuring the dry cell weight.

2.2. Assessment of phenotypic variation in A. brasilense Sp7 and characterization of variants

To assess the rate of appearance of phenotypic variants, a single colony of the parental strain was used to inoculate 5 ml of D-fructose minimal medium. After overnight incubation at 30 °C with shaking (200 rpm), a 10^{-5} dilution was plated onto fructose minimal medium plates. In all experiments the morphology of the colonies was examined after 3 days of incubation. At least 10,000 colonies were screened in each experiment. The stability of the variants was tested by transferring individual colonies to new minimal medium plates incubated at 30 °C.

2.3. Polymerase chain reaction (PCR)

PCR was performed to confirm that colonies showing varied morphology were *A. brasilense* phenotypic variants rather than contaminations. Total DNA was isolated using the Wizard Genomic DNA Purification kit (Promega). All suspected variants were tested for the *A. brasilense ipdC* gene, encoding indole-pyruvate decarboxylase, a key enzyme in indole-3-acetic acid (IAA) synthesis. This gene, and the corresponding PCR primers, has been suggested as a reliable and specific indicator for *A. brasilense* species (Shime-Hattori et al., 2011). Each 25 μl PCR reaction contained 12.5 μl of Taq Master MIX (Lambda Biotech), $MgCl_2$ (1 mM), BSA (0.04 μg μl^{-1}), and 1.6 μM of each *ipdC* primer: A32f (forward), 5′-ACCCCTCCACAATTTCCGGCGCAT-3′, and A42r (reverse), 5′-CGCCACCCCTAGAGTGGAGCTGTA-3′ (Shime-Hattori et al., 2011). PCR amplifications were performed in an automated thermal cycler (Eppendorf Mastercycler Gradient Machine) with an initial denaturation (94 °C, 2 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s) and extension (72 °C, 1 min), and a single final extension (72 °C, 5 min). PCR products were then electrophoresed directly on 1% agarose gels, visualized by ethidium bromide staining, excised from the gel with the HiYield Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience) and sequenced at Hy Labs Co.

2.4. DNA fingerprinting analyses

Phenotypic variants were compared with the parental strain by repetitive-PCR [Enterobacterial Repetitive Intergenic Consensus (ERIC)- and BOX-PCR] and random amplified polymorphic DNA (RAPD) analysis. Repetitive-PCR reactions were performed as described by Louws et al. (1994). Reaction mixtures (25 μl) contained 12.5 μl of Taq Master mix (Lambda Biotech), $MgCl_2$ (3 mM), BSA (0.02 μg ul^{-1}), 2 μM of each primer [ERICIR, 5′-GTAAGCTCCTGGGGATTCAC-3′, and ERIC2, 5′-AAGTAAGTGACTGGGGTGAGCG-3′ for ERIC-PCR; BOXA1R 5′-CTACGGCAAGGCGACGCTGACG-3′ for BOX-PCR) and 100 ng of template (genomic) DNA. PCR amplifications were performed in an automated thermal cycler with an initial denaturation (95 °C, 7 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (42 and 53 °C for ERIC- and BOX-PCR, respectively, 1 min) and extension (65 °C, 8 min) with a single final extension (65 °C, 16 min). Samples of 8 μl from each reaction were separated by gel electrophoresis on 1.5% agarose gels at 60 V for 3 h, and the gels were stained with ethidium bromide. RAPD analysis was performed as described by Vial et al. (2006). Briefly, reaction mixtures (25 μl) contained 12.5 μl Taq master mix (Lambda Biotech), primer F1253 (5′-GTTTCCGCCC-3′) (2 μM), BSA (0.02 μg ul^{-1}), $MgCl_2$ (2 mM) and bacterial DNA (~100 ng). Amplification conditions were: initial denaturation (95 °C, 5 min) followed by 35 cycles of denaturation (95 °C, 45 s), annealing (36 °C, 1 min) and extension (72 °C, 2 min) with a final extension at 72 °C for 7 min and 60 °C for 10 min. PCR products were analyzed as described for repetitive-PCR above.

2.5. Extraction of EPS and determination of EPS monosaccharide composition

EPS were extracted from D-fructose minimal medium liquid cultures, by fractionation with cold ethanol as described by del