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Original Article

Carotenoid production and phenotypic variation in *Azospirillum brasilense*

Gal Reem Brenholtz, Dafna Tamir-Ariel, Yaacov Okon, Saul Burdman*

Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel

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Abstract

We assessed the occurrence of phenotypic variation in *Azospirillum brasilense* strains Sp7, Cd, Sp245, Az39 and phv2 during growth in rich media, screening for variants altered in colony pigmentation or extracellular polysaccharide (EPS) production. Previous studies showed that EPS-overproducing variants of Sp7 appear frequently following starvation or growth in minimal medium. In contrast, no such variants were detected during growth in rich media in the tested strains except for few variants of phv2. Regarding alteration in colony pigmentation (from pink to white in strain Cd and from white to pink in the others), strain Sp7 showed a relatively high frequency of variation (0.009–0.026%). Strain Cd showed a lower frequency of alteration in pigmentation (0–0.008%), and this type of variation was not detected in the other strains. In *A. brasilense*, carotenoid synthesis is controlled by two RpoE sigma factors and their cognate ChrR anti-sigma factors, the latter acting as negative regulators of carotenoid synthesis. Here, all tested ($n = 28$) pink variants of Sp7 carried mutations in one of the anti-sigma factor genes, *chrR1*. Our findings indicate that, in *A. brasilense*, phenotypic variation is strain- and environment-dependent and support the central role of ChrR1 in regulation of carotenoid production.

Keywords: *Azospirillum*; Phenotypic variation; Phase variation; Carotenoids; Extracellular polysaccharides; Anti-sigma factor

1. Introduction

The rhizosphere is the area of the soil affected by the presence of plant roots [16]. It is composed of microbial populations that differ and are often more abundant than the rest of the soil populations, due to the generally denominated "rhizosphere effect" [27]. Among rhizosphere bacteria, some strains are able to promote plant growth by various mechanisms. These bacteria, which can be potentially applied in sustainable agriculture to increase yield, are generally denominated plant growth-promoting rhizobacteria (PGPR) [42]. During the last four decades, *Azospirillum* species have been among the best studied and characterized PGPR [6]. In fact, azospirilla are currently used extensively as inoculants (e.g., bacteria in a suitable carrier) for promoting the crop yield of cereals such as maize, wheat and sorghum, as well as in co-inoculation with rhizobia to promote nodulation and growth of commercially important nitrogen-fixing legume crops [31].

Azospirillum spp. are able to fix nitrogen in association with grasses; however, biological nitrogen fixation by azospirilla does not contribute a significant amount of nitrogen to the plants in the field. On the other hand, inoculation with various *Azospirillum* strains often leads to marked effects on root development that are associated with enhanced mineral and water uptake by the roots, resulting in significant higher crop yield [31]. *Azospirillum* spp. produce and secrete phytohormones, mainly auxins (indole-3-acetic acid) and gibberellins, as well as nitric oxide, which together appear to contribute to plant growth promotion [3,26]. Several genomes of *Azospirillum* strains have been sequenced and annotated [32,50].

* Corresponding author.
E-mail addresses: gal4646@gmail.com (G.R. Brenholtz), dafnata@savion.huji.ac.il (D. Tamir-Ariel), yaacov.okon@mail.huji.ac.il (Y. Okon), saul.burdman@mail.huji.ac.il (S. Burdman).

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Phenotypic variation, also referred to as phase variation, is a known phenomenon in many bacterial species. It is characterized by the development of a secondary population with distinguished phenotype(s) from their parental strain, thus standing out from the rest of the population. Phenotypic variation is associated with genetic and/or epigenetic alterations, occurs more frequently than spontaneous mutations and sometimes involve changes in expression of global regulatory proteins [17,46,51]. Phenotypic changes can be at the single cell level (e.g. changes in surface proteins, flagella and toxins), or at the level of the whole colony [e.g. changes in morphology features like size, shape and color, and altered production of exopolysaccharides (EPS) or pigments] [36,46,52].

One of the most studied *Azospirillum* species in terms of basic research and agricultural application is *Azospirillum brasilense*. Hartmann et al. [14] reported the occurrence of osmotic stress-resistant spontaneous mutants of *A. brasilense* Sp7 after exposure to salt stress or iron deprivation. Spontaneous phenotypic variants (PVs) of *A. brasilense* Sp7 were later reported by Katupitiya et al. [19]. These variants were shown to possess different EPS characteristics than the parental strain, to produce smooth and shiny colonies, and to have a distinguished root colonization pattern relative to the parental strain. PVs of strain Sp7 were also reported by Petrova et al. [34], which also reported alterations in lipopolysaccharide (LPS) structure and altered plasmid composition. Changes in plasmid composition were also reported in PVs of *A. brasilense* Sp245 that produced colonies with a smooth morphology [18]. Phenotypic variation was also reported to occur in other well-studied *Azospirillum* species, *Azospirillum lipoferum* [1,2,47].

In previous works, we reported a number of PVs of *A. brasilense* Sp7 that were isolated after starvation [23] or growth in minimal medium with fructose as carbon source [48]. Two major changes were reported in these studies: increased production of EPS, as reflected by increased mucosity of the variant colonies relative to the parental strain, and altered colony pigmentation, from typical white colonies in the parental strain to pink coloration in the PV colonies [23,48].

The objective of the present study was to examine the occurrence of phenotypic variation of *A. brasilense* Sp7 grown in rich medium, as well as the occurrence of phenotypic variation under similar conditions in other *A. brasilense* strains, including wild-type strains Cd, Sp245 and Az39, and phv2, an EPS-overproducing PV of strain Sp7 [48]. Variant phv2 was shown to produce ~8-fold-more EPS than strain Sp7, and to possess a different EPS composition, with substantially lower concentrations of rhamnose and higher concentrations of glucose and galactose, relative to Sp7. In addition, phv2 showed a distinct repetitive-PCR profile than that of the parental strain, and was shown to be more resistant than the latter to heat and UV-radiation [48].

In contrast to our previous reports, almost no PVs altered in EPS production could be detected in this study under the tested conditions. On the other hand, a relatively high number of PVs with modified colony coloration were detected in the cases of strains Sp7 and Cd (from white to pink, and from pink to white, respectively). In *A. brasilense*, the RpoE1 sigma factor protein and its cognate ChrR1 anti-sigma factor protein are key regulators of carotenoid synthesis [12,44]. Here we show that all pink PVs of strain Sp7 carry mutations in the *chrR1* gene [12,44]. Under tested conditions, phenotypic variation was not detected at all in strains Sp245 and Az39. Overall, findings from this and previous studies support the fact that, in *A. brasilense*, phenotypic variation is a strain- and environment-dependent phenomenon.

2. Materials and methods

2.1. Media and bacterial growth conditions

A. brasilense strains used in this study were wild-type strains Sp7 [43], Cd [11], Sp245 [7] and Az39 [38], and strain phv2 (also named V6), an EPS-overproducing variant of Sp7 [48]. All strains produce white colonies except strain Cd, that produces colonies with a pink pigmentation due to production of high amounts of carotenoids [30]. Strains were kept at −80 °C in a solution containing 25% glycerol and 75% nutrient broth (NB; Difco Laboratories, MI, USA). For production of starter cultures, bacteria were grown for 48 h on nutrient agar (NA; Difco Laboratories) at 35 °C. Luria–Bertani broth (LB; Difco Laboratories) and LB-agar (LA; LB with 1.5% agar) were used for growth of bacterial strains and isolation of phenotypic variants (PVs) as described below.

2.2. Assessment of phenotypic variation in A. brasilense strains

To assess the frequency of appearance of PVs in *A. brasilense* strains, a single colony from the parental strain (grown for 48 h at 35 °C on NA) was used to inoculate 4 mL of LB medium in glass tubes. After growth for 48 h at 35 °C with shaking (200 rpm), 100 μL-aliquots from 10^{-5} dilutions were plated onto LA medium in 14 cm-diameter Petri dishes, which were incubated for 3 days at 35 °C. This experiment was repeated three times for each strain, and for each strain/experiment, about 3×10^4 to 3×10^5 colonies were screened for the appearance of PVs (e.g., alterations in pigmentation or EPS production). PVs were isolated in LA plates, grown as described above and photographed under a Stemi 508 stereo microscope (Carl Zeiss, Oberkochen, Germany).

2.3. Confirmation of PVs by microscope observation and polymerase chain reaction (PCR)

To verify whether colonies showing altered phenotypes relative to the corresponding parental strains represent PVs rather than contaminations, we first observed bacterial samples in a Leica (Wetzlar, Germany) DM500 light microscope. Further verification was done by colony-PCR with primers A32f and A42r [40]. These primers amplify a region of the *A. brasilense ipdC* gene, encoding indole-pyruvate