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For publication

Carotenoid production and phenotypic variation in *Azospirillum brasilense*

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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% to 0.026%). Strain Cd showed a lower frequency of alteration in pigmentation (0 to 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].

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, *A. 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 more clearly distinguished repetitive-PCR profile than 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

Azopirillum 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 ºC 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 ºC. 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 ºC on NA) was used to inoculate 4 mL of LB medium in glass tubes. After growth for 48 h at 35 ºC 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 ºC. This experiment was repeated three times for each strain, and for each strain/experiment, about 3 X 10^4 to 3 X 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 decarboxylase, a key enzyme in indole-3-acetic acid (IAA) synthesis. Shime-Hattori et al. [40] proposed this PCR assay as a reliable indicator of *A. brasilense* species. Primer sequences and reaction conditions (cycles and temperatures) were as described [48], with the difference being that, in this study, colony-PCR was used (instead of PCR of purified DNA as template). A sterilized toothpick was used to transfer bacteria from a single colony to the PCR reaction tube that contained a final volume of 25 μL. Primers were purchased from Hy Labs (Rehovot, Israel) and RedTaq ReadyMix PCR reaction mix (Sigma-Aldrich, St. Louis, MO) was used. Reactions were done using a Mastercycler gradient apparatus (Eppendorf, Hamburg, Germany). PCR products were electrophoresed in 1% agarose gels, visualized by ethidium bromide staining, and excised from the gel and purified with the HiYield Gel/PCR DNA fragment extraction kit (RBC Bioscience, New Taipei City, Taiwan). Purified PCR products were sequenced by Hy Labs to verify that the amplified sequence corresponds to the *ipdC* fragment.

2.4. Amplification and sequencing of rpoE1 *and* chrR1 *open reading frames*

To assess whether alterations in colony pigmentation in PVs of strains Sp7 and Cd are associated with mutations in *rpoE1* and/or *chrR1* genes, the coding DNA sequence (CDS) of these genes (576 and 687 bp, respectively) was PCR-amplified and the PCR products were sequenced. Primer sets rpoE:F1/rpoE:R1 and chrR:F1/chrR:R1 were used for amplification of *rpoE1* and *chrR1* CDS, respectively. Primer sequences and reaction conditions were as described [25]. PCR reagents, extraction of PCR products from gels and sequencing were as described above for the *ipdC* gene.

3. Results

3.1. Assessment of phenotypic variation in A. brasilense *strains upon growth in rich medium*

A. brasilense strains Sp7, Cd, Sp245, Az39 and phv2 were grown in liquid LB medium at 35 ºC for 48 h. After this incubation period, the cultures were serially diluted and plated onto LA medium. After incubation at 35 ºC for 78 h, 3 X 10^4 to 3 X 10^5 colonies of each strain were screened for visible altered phenotypes in terms of EPS production or pigmentation. The experiment was carried out three times and results are summarized in Table 1. In all experiments, phenotypic variants (PVs) were confirmed by colony-PCR using *A. brasilense* specific *ipdC* primers.

The most frequently observed alteration was a change in pigmentation, from white to pink, in strain Sp7. The rate of appearance of pink colonies in strain Sp7 varied from 0.009 to 0.026% in the different experiments (Table 1). The pink colonies of the variants of this strain (Figs. 1A and B) were similar to those of strain Cd, and generally, the pink colonies developed more slowly than the parental type ones. These

PVs remained stable after several transfers. We were also able to detect PVs of strain Cd with the opposite alteration of pigmentation, e.g., from pink to white colonies (Figs. 1C and D). The rate of appearance of white Cd colonies was lower than the rate of appearance of pink Sp7 colonies, and ranged from 0 to 0.008% in the three experiments (Table 1). Also, the white Cd variants remained stable after several transfers.

In this study, no EPS overproducing colonies were detected for strain Sp7 nor for strains Cd, Sp245 and Az39. The only exception was strain phv2, for which two variants that were more mucoid than the parental strain (Fig. 2) were detected, corresponding to a frequency rate ranging from 0 to 0.003% (Table 1). The phenotype of these two variants was stable after several transfers.

3.2. Association of alteration in pigmentation in Sp7 variants with mutations in the chrR1 *anti-sigma factor gene*

Thirunavukkarasu et al. [44] reported that transposon mutants of *A. brasilense* Sp7 impaired in the *chrR1* anti-sigma factor gene produce pink colonies. They also found that the carotenoid-producing strain of *A. brasilense*, Cd, possesses a single nucleotide deletion relative to strain Sp7 in the *chrR1* gene. The deletion is at nucleotide position 466 of the *chrR1* CDS, causing a frame shift and generation of an early stop codon. Impairment of *chrR1* in strain Cd as well as in Sp7 mutants causes constitutive activation of the concomitant sigma factor RpoE1, resulting in high expression of carotenoid synthesis genes and consequent pink pigmentation of the colonies. These and additional evidence from that study demonstrated a tight

212 regulation of carotenoid synthesis in *A. brasilense* by the ChrR1-RpoE1 anti-
213 sigma/sigma factors [44].

214 Based on this background, we hypothesized that pink carotenoid-producing
215 colonies of Sp7 variants isolated in our study could carry mutations in the *chrR1*
216 gene. To test this hypothesis, the *chrR1* CDS of 28 pink PVs of Sp7 obtained in the
217 three experiments were PCR-amplified and the products were sequenced from both
218 directions. All tested PVs carried mutations in the *chrR1* gene. Moreover, among the
219 28 PVs, seven different mutations, named chrR1_1 to chrR1_7, were detected that
220 were different from the mutation that characterizes strain Cd (Table 2). The mutations
221 in the different PVs were spread in the *chrR1* CDS and included three non-silent
222 nucleotide substitutions, three single nucleotide insertions and one single nucleotide
223 deletion. Multiple alignments of the relevant regions of the Sp7 *chrR1* gene and the
224 mutated versions of this gene in the pink variants of Sp7 and in strain Cd are shown in
225 Supplemental Fig. S1. All single nucleotide insertions or deletions occurred in regions
226 with 4-5 sequential C or G nucleotides.

227 Fig. 3 shows the impact of the seven mutations on the amino acid sequence of
228 the *chrR1* product. The most common mutation (chrR1_2) involved replacement of a
229 cysteine residue by arginine at amino acid position 36 of the ChrR1 product (Fig. 3).
230 This mutation was detected in 15 out of the 28 tested PVs (Table 2), and occurred in
231 the three independent experiments (Table 1).

232 In this study we also detected 13 Cd variants that produced white colonies
233 (Table 1). We hypothesized that these variants could be mutated in the *rpoE1* sigma
234 factor gene. However, sequencing of the *rpoE1* gene of some of these variants revealed
235 that they were identical to Sp7 *rpoE1*. These results were in agreement with the report
236 of Thirunavukkarasu et al. [44] that showed that the *rpoE1* gene of strains Sp7 and Cd

are identical. Alternatively, the white Cd variants could be revertants that recovered the functional sequence of *chrR1*. Sequencing of the *chrR1* gene of some of the white Cd variants, however, revealed that these sequences were identical to that of strain Cd, thus indicating that this phenotype is associated with genetic alterations other than in the *rpoE1* and *chrR1* genes.

4. Discussion

Phenotypic/phase variation is a widespread phenomenon in bacteria that often contributes to adaptation to environmental changes [17,45,47,51]. As described in the Introduction, this phenomenon was shown to occur in the plant growth-promoting rhizobacterium *A. brasilense* [19, 34] We previously reported two major phenotypic changes in *A. brasilense* Sp7 following starvation [23] or growth in fructose minimal medium [48]: overproduction of EPS and altered colony pigmentation, from white to pink, due to synthesis of carotenoids [30].

EPS contributes to the structure of the cell envelope, making bacteria more resistant to osmotic stress, desiccation, heat, UV radiation and antibiotics [22,49]. In *A. brasilense* Sp7, EPS overproduction is often associated with genetic rearrangements [23,48]. This is, for instance, the case of phv2, one of the strains analyzed in this study, which is an EPS-overproducing PV of strain Sp7 that possesses distinguished repetitive-PCR profiles from its parental strain [48]. Carotenoids are important components protecting bacteria against photo-oxidative stress; they quench damage caused by singlet oxygen and stop chain oxidations of free radicals [20,53]. In *A. Brasilense*, carotenoids also play a role in protection of nitrogenase from oxygen damage, and they are likely important for biological nitrogen fixation under conditions of limited nitrogen [15,30].

The objectives of the present study were to assess the frequency of phenotypic variation of *A. brasilense* Sp7 during growth in rich media and to assess the frequency of this phenomenon in four additional strains under similar conditions. Our screens focused on detection of PVs that are altered in EPS production or in pigmentation. The major insights from three independent experiments were as follows: (i) The frequency of phenotypic variation in *A. brasilense* is strain-dependent, and interestingly, no PVs of any kind could be detected in this study for strains Sp245 and Az39. (ii) In contrast to previous studies that assessed phenotypic variation under stress or growth in minimal medium, EPS-overproducing variants were rarely detected after growth in rich medium. In fact, under tested conditions, among the five assessed strains, phv2 was the only one that rendered PVs that were altered in EPS production, and this was at a relatively low frequency. (iii) Alterations in colony coloration were observed only in strain Sp7 and, to a lesser extent, in strain Cd (from white to pink and vice-versa, respectively).

In *A. brasilense*, carotenoid production is regulated by two sigma factors that belong to the extracytoplasmic function (ECF, (σ^E) family, RpoE1 and RpoE2, and their cognate anti-sigma factors, ChrR1 and ChrR2, respectively [12,44]. Unlike the *rpoE1-chrR1* operon that is activated by oxidative stress, the *rpoE2-chrR2* operon seems to be expressed constitutively [12]. It was suggested that, while the former is important for long-term resistance to oxidative stress, the latter could provide a rapid response to this stress [12]. Recently, it was shown that in *A. brasilense* Sp7, response to photo-oxidative stress and carotenoid biosynthesis are regulated by a complex network of alternative sigma factors that, in addition to RpoE1 and RpoE2 ECF sigma factors, involves two heat shock sigma factor (σ^H), RpoH1 and RpoH2 [21,35]. According to the proposed model, RpoE1 and RpoE2 promote transcription of *rpoH2*

and *rpoH1*, respectively, and in turn, the *rpoH2* and *rpoH1* products activate transcription of *crtE2* [35]. CrtE2 encodes a geranyl-geranyl pyrophosphate (GGPP) synthase that is responsible for conversion of farnesyl pyrophosphate into GGPP, a key step in carotenoid synthesis in bacteria [35]. In addition, *A. brasilense* Sp7 contains a second copy of *crtE*, *crtE1*, which is also regulated indirectly by RpoE1, but by a yet unknown sigma factor [35].

RpoE has been widely investigated in the phototrophic bacterium *Rhodobacter sphaeroides*, in which it controls the response to oxidative stress caused by singlet oxygen formation, also including regulation of carotenoid synthesis [4,5]. The function of ECF sigma factors is generally limited by a specific and reversible interaction with their cognate anti-sigma factors, which in the case of RpoE is ChrR, a zinc-dependent anti-sigma factor [4]. As aforementioned, the ability to produce high levels of carotenoids in *A. brasilense* Cd is due to a single nucleotide deletion in the coding sequence (CDS) of the *chrR1* gene. Disruption of the *chrR1* CDS in this strain results in release of RpoE1 repression, resulting in high levels of carotenoids and formation of pink-colored colonies, in contrast to the white colonies of strain Sp7 that carries a functional ChrR1 [44]. Interestingly, strain Cd was isolated from roots of *Cynodon dactylon* (durva grass) that were inoculated with *A. brasilense* Sp7 [11]. This and the fact that these strains are very closely related in terms of genetic and phenotypic traits [44] suggest that Cd could itself be a PV of Sp7.

Based on the above knowledge, we hypothesized that, similarly to strain Cd, Sp7 PVs with a pink coloration could also be affected in the *chrR1* gene. Indeed, sequencing of the *chrR1* CDS of 28 pink variants of Sp7 revealed that all of them were mutated in this gene. Moreover, among the tested variants, we found seven different types of mutations in *chrR1*, all of them differing from the mutation reported in strain

Cd [44]. This finding not only strengthens the critical role of ChrR1 in regulation of carotenoid synthesis in *A. brasilense*, but also supports the notion that ChrR1 and its concomitant sigma factor RpoE1 are the key regulators of this trait (otherwise, we would expect to find some Sp7 PVs that possess intact *chrR1* but are mutated in some of the other regulatory genes). These results also suggest that the *chrR1* CDS in *A. brasilense* may have mutation hotspots [39]. Occurrence of hotspot mutations is strong evidence of positive selection, since they may provide an adaptive advantage, like carotenoid production, under particular conditions [9].

Of the seven mutations detected in the *chrR1* CDS among the 28 pink PVs of Sp7, four were single nucleotide insertions/deletions (indels) that lead to frameshifts at different positions, as similarly reported for strain Cd [44]. Also, as in Cd, these indels occurred in spots of runs of 4-5 identical base pairs (G:C), which can be susceptible to replication mistakes [24,28]. Among all detected mutations, the most frequent was a substitution of a cysteine residue by an arginine residue at amino acid position 36 of ChrR1. This was the mutation that characterized 53.6% of the PVs analyzed (15 out of 28), and importantly, variants of this type were isolated in the three independent experiments. Cysteine residues play an important role in the structure of ChrR1 and in its interaction with RpoE1. The *R. sphaeroides* ChrR1 possesses four cysteine residues located in the N-terminus at positions 35 and 38, and at the C-terminus in positions 187 and 189 [8,29]. *A. brasilense* Sp7 ChrR1 possesses eight cysteine residues, four of them located at positions similar to those mentioned above for *R. sphaeroides* ChrR1 [44]. As is the case in *R. sphaeroides* ChrR1, those located at the N-terminus (positions 36 and 39 in *A. brasilense* Sp7 ChrR1) constitute the Hx_3Cx_2C zinc-binding domain of the protein [44]. This probably explains why the substitution of cysteine by arginine at position 36 of ChrR1 compromised its function.

In contrast to strain Sp7, no pink PVs could be detected in this study for strains Az39 and Sp245. Like Sp7, strains Az39 and Sp245 are considered model strains of *A. Brasilense*, and their genome sequences are also available at GenBank (Accession numbers CP00793-CP00798 and HE577327-HE577333, respectively). The ChrR1 and RpoE1 predicted sequences of these strains are highly conserved with those of Sp7 (99% identity between Sp7 and Az39, and 96% identity between Sp7 and Sp245 for ChrR1, and 98% identity in both cases for RpoE1). While we cannot discard the possibility that some of the minor differences between these strains in ChrR1 or RpoE1 could be determinants of observed differences in the frequency of emergence of pink PVs among these strains, it is more likely that this phenotypic distinction is due to dissimilarity in other genes involved in carotenoid metabolism. It is also possible that strain Sp7 is more prone to spontaneous mutations than the other strains. Further research is needed to elucidate this question

Further investigation is also needed to identify genetic alterations that determine the occurrence of white PVs in strain Cd. We showed that the white PVs of strain Cd have a *chrR1* gene identical to strain Cd and an *rpoE1* gene identical to both Cd and Sp7. These findings support the fact that, on the one hand, the white PVs of strain Cd are not revertants for negative regulation of carotenoid synthesis, since their ChrR1 is still non-functional. On the other hand, the lack of carotenoid synthesis ability in these PVs is not due to impairment of RpoE1. Therefore, we conclude that the lack of ability to produce carotenoids in these variants is probably due to other genetic alterations, probably in a gene (or genes) encoding enzyme(s) that are involved in the carotenoid metabolic pathway.

As mentioned above, overproduction of EPS in PVs of *A. brasilense* Sp7 has been shown to be associated with DNA rearrangements. Under stress conditions like

starvation, bacteria can undergo adaptive mutations involving movement of mobile genetic elements and subsequent DNA rearrangements, thus accelerating adaptation of microbial populations to environmental stress [13,20,41]. This could explain why phenotypic variation involving EPS overproduction occurs frequently (about 0.02%) in *A. brasilense* Sp7 under starvation or growth in minimal media [23,48], but rarely (only for strain phv2 and at an average frequency of 0.001%) during growth in rich media as observed in this study.

We reported that EPS-overproducing variants of *A. brasilense* Sp7 did not differ from the parental strain in plant growth-promoting ability in the interaction with several crops [48]. On the other hand, a spontaneous mutant of strain Sp7, which produced less EPS than the parental strain and lacked flocculation ability, had a modified pattern of colonization of wheat roots [19]. With respect to production of bacterial inoculants for agriculture, the occurrence of phenotypic variation may not be desired, since it might compromise the stability and thus the reliability of the product. In our study, under tested conditions, we could not detect the occurrence of phenotypic variation in strains Az39 and Sp245. Therefore, although we cannot discard the possibility that these strains might undergo phenotypic variation under other conditions or after prolonged growth, our results suggest that the use of these apparently more stable strains for inoculant production could be more advantageous than using a less stable strain like Sp7. In this regard, Az39 is already among the *A. brasilense* strains that have been widely investigated in terms of mechanisms of plant growth promotion and formulations for field applications [10,33], and its genome sequence was recently released [37].

Overall, this study contributes to the understanding of phenotypic variation in *A. brasilense.* The knowledge emerging from this and other studies on this topic are

useful for improving production of commercial inoculants of this bacterium. More investigation is needed to elucidate the genetic determinants that mediate generation of EPS-overproducing variants in *A. brasilense* Sp7, as well as generation of *A. brasilense* Cd variants that lack the ability to produce high levels of carotenoids.

Conflict of interest

There is no conflict of interest.

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572 **Figure legends**

573

574 Fig. 1. Detection of phenotypic variants characterized by alteration in pigmentation in
575 *A. brasilense* Sp7 and Cd. A and B: White colonies of strain Sp7 grown in LA
576 medium. Arrows indicate pink phenotypic variants (PVs) of this strain. C and D: Pink
577 colonies of strain Cd grown in LA medium. Arrows indicate white phenotypic variants
578 (PVs) of this strain. Pictures were taken under a Carl Zeiss Stemi 508 stereo
579 microscope after 48 h of growth at 35 °C.

580

581 Fig. 2. One of the EPS-overproducing PVs of *A. brasilense* phv2. phV2 (also named
582 V6) is itself a PV of strain Sp7 that produce more EPS than the parental strain [45]. A)
583 Emergence of the PV from phv2. The arrow indicates the PV, named V6V1,
584 surrounded by phv2 colonies. B) Sp7 colonies. C) phv2 colonies. D) V6V1 colonies.
585 Pictures were taken under a Carl Zeiss Stemi 508 stereo microscope after 48 h of
586 growth at 35 °C.

587

588 Fig. 3. Impact of the seven mutations detected in the *chrR1* gene product in different
589 pink PVs of strain Sp7. The amino-acid sequence of the Sp7 Chr1 is indicated at the
590 top. ChrR1_1 to ChrR1_7 correspond to mutations detected in this study as described
591 in Table 2. The ChrR1 sequence of strain Cd [44] is shown at the bottom. Amino acids
592 marked with green indicate substitutions relative to ChrR1 from Sp7. Open reading
593 frame shifts are marked with yellow and appearance of an early start codon is marked
594 with red.

595

596

Supplemental material

Fig. S1. Multiple alignment of the *chrR1* CDS of *A. brasilense* Sp7, the seven types of mutated variants identified in this study (chrR1_1 to chrR1_7), and strain Cd [44]. The multiple alignment was done using Clustal Omega at http://www.ebi.ac.uk/Tools/msa/clustalo/.

Tables

Table 1. Frequency of phenotypic variation involving change in pigmentation or overproduction of exopolysaccharides (EPS) in *A. brasilense* strains[a].

Strain[b]	Exp	Total colonies screened	Change in pigmentation			EPS overproduction		
			No. of colonies	Frequency (%)	Frequency average (%)	No. of colonies	Frequency (%)	Frequency average (%)
Sp7	1	7×10^4	6	0.009	0.014	0	0	0
	2	9×10^4	23	0.026		0	0	
	3	2×10^5	18	0.009		0	0	
Cd	1	4.5×10^4	0	0	0.003	0	0	0
	2	1.5×10^5	1	0.001		0	0	
	3	1.5×10^5	12	0.008		0	0	
phv2	1	3×10^4	0	0	0	1	0.003	0.001
	2	1×10^5	0	0		0	0	
	3	9.5×10^4	0	0		1	0.001	

[a] Results of three independent experiments (Exp 1 to 3) for each strain.

[b] Phenotypic variation in strains Sp245 and Az39 was also assessed in three experiments (total colonies screened in each experiment were 3.5×10^4, 2×10^5 and 9×10^4 for Sp245, and 3×10^4, 1×10^5 and 3×10^5 for Az39). No phenotypic variants were detected for these strains in any experiment.

615 Table 2. Summary of mutations in the *chrR1* coding sequence (CDS) of *A. brasilense*
616 Sp7 that were identified in this study following sequencing of this gene in 28 pink
617 phenotypic variants (PVs) of this strain.

Mutation no.	Nucleotide position in CDS	Change in the CDS	Change in the translated protein	No. of PVs carrying this mutation	Source (experiment no.)[a]
chrR1_1	80	Substitution: T → C	Substitution: Leu → Pro	1	Ex. 3 (1)
chrR1_2	106	Substitution: T → C	Substitution: Cys → Arg	15	Ex. 1 (1); Ex. 2 (12); Ex. 3 (2)
chrR1_3	108-111[b]	Insertion: C	ORF shift	6	Ex. 2 (6)
chrR1_4	235-238[b]	Insertion: C	ORF shift	1	Ex. 3 (1)
chrR1_5	250-253[b]	Insertion: C	ORF shift	1	Ex. 3 (1)
chrR1_6	345	Substitution: G → A	Substitution: Trp → stop	1	Ex. 2 (1)
chrR1_7	479-483[c]	Deletion: G	ORF shift	3	Ex. 3 (3)
chrR1_Cd	463-466[d]	Deletion: C	ORF shift	-	-

618

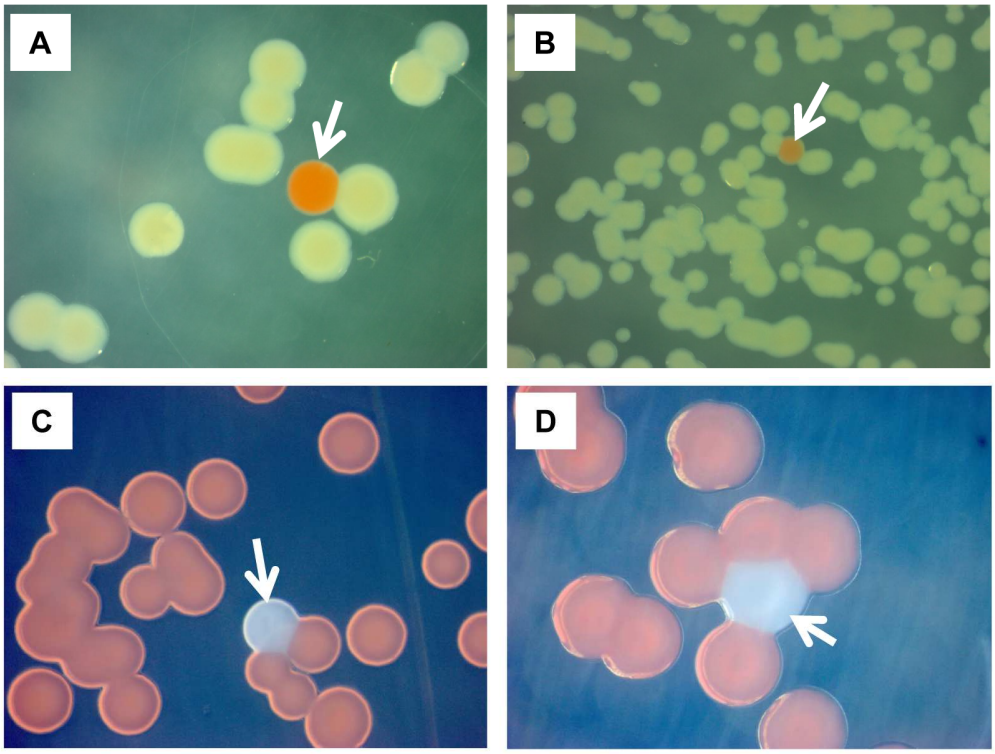
619 The mutation in this gene in *A. brasilense* Cd [44] is included at the bottom of the table.

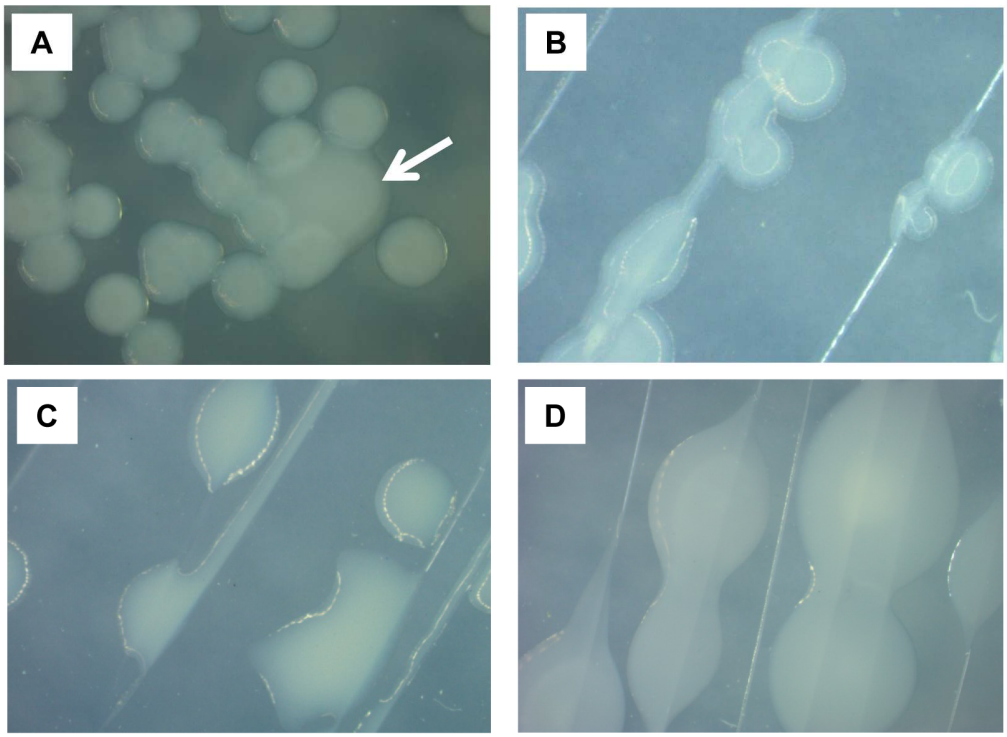
620 [a] Ex.: experiment number out of three from which PVs carrying the mutation were detected. Number in parentheses
621 indicates number of PVs with this mutation in each experiment.

622 [b] In these mutations, an additional cytosine was inserted in a run of four cytosines.

623 [c] In this mutation, a guanine was deleted from a run of five guanines.

624 [d] In the Cd *chrR1*, a cytosine was deleted from a run of four cytosines.





>Sp7_ChrR1
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPRPKLVCQPAEVPLLPEPLRRYVGSDLSRLPWKRLMRGMDCYDIPLGSGSGYDSERRGKARLMRLASGVGPPHHT
HRGIELTLVLDGGFTDDLGQFARGDLSVADDSVRHRPVADEEGCLCLAVTDAPLHFTGALGLILNPFVKF*

>Sp7_ChrR1_1
MTVPTHHPGDTLLIDYAGGALGEAASPIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPRPKLVCQPAEVPLLPEPLRRYVGSDLSRLPWKRLMRGMDCYDIPLGSGSGYDSERRGKARLMRLASGVGPPHHT
HRGIELTLVLDGGFTDDLGQFARGDLSVADDSVRHRPVADEEGCLCLAVTDAPLHFTGALGLILNPFVKF*

>Sp7_ChrR1_2
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALRPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPRPKLVCQPAEVPLLPEPLRRYVGSDLSRLPWKRLMRGMDCYDIPLGSGSGYDSERRGKARLMRLASGVGPPHHT
HRGIELTLVLDGGFTDDLGQFARGDLSVADDSVRHRPVADEEGCLCLAVTDAPLHFTGALGLILNPFVKF*

>Sp7_ChrR1_3
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPLLPPERRRDGGGRRRPAGEHRAGGGRSRLPGDGAGPAGRPAA
PAPRTPAEAGLPAGRGAAAARTAAPLRRQRPVAPALEAADARHGLLRHPSGVRLRLRFRTPRQGPADAAGQRRRTAPPH
PPGD*

>Sp7_ChrR1_4
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
PAPRTPAEAGLPAGRGAAAARTAAPLRRQRPVAPALEAADARHGLLRHPSGVRLRLRFRTPRQGPADAAGQRRRTAPPH
PPGD*

>Sp7_ChrR1_5
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPPAEAGLPAGRGAAAARTAAPLRRQRPVAPALEAADARHGLLRHPSGVRLRLRFRTPRQGPADAAGQRRRTAPPH
PPGD*

>Sp7_ChrR1_6
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPRPKLVCQPAEVPLLPEPLRRYVGSDLSRLP*

>Sp7_ChrR1_7
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPRPKLVCQPAEVPLLPEPLRRYVGSDLSRLPWKRLMRGMDCYDIPLGSGSGYDSERRGKARLMRLASGVGPPHHT
HRGLS*

>Cd_ChrR1
MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPEEVDPACLETVLARLDDLPP
LPRAPRPKLVCQPAEVPLLPEPLRRYVGSDLSRLPWKRLMRGMDCYDIPLGSGSGYDSERRGKARLMRLASGVGPPTTP
TGGLS*