Dicarboxylate Transporters of *Azospirillum brasilense* Sp7 Play an Important Role in the Colonization of Finger Millet (*Eleusine coracana*) Roots

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Azospirillum brasilense is a plant growth-promoting bacterium that colonizes the roots of a large number of plants, including C3 and C4 grasses. Malate has been used as a preferred source of carbon for the enrichment and isolation *Azospirillum* spp., but the genes involved in their transport and utilization are not yet characterized. In this study, we investigated the role of the two types of dicarboxylate transporters (DctP and DctA) of *A. brasilense* in their ability to colonize and promote growth of the roots of a C4 grass. We found that DctP protein was distinctly upregulated in *A. brasilense* grown with malate as sole carbon source. Inactivation of *dctP* in *A. brasilense* led to a drastic reduction in its ability to grow on dicarboxylates and form cell aggregates. Inactivation of *dctA*, however, showed a marginal reduction in growth and flocculation. The growth and nitrogen fixation of a *dctP* and *dctA* double mutant of *A. brasilense* were severely compromised. We have shown here that DctPQM and DctA transporters play a major and a minor role in the transport of C₄-dicarboxylates in *A. brasilense*, respectively. Studies on inoculation of the seedlings of a C4 grass, *Eleusine coracana*, with *A. brasilense* and its dicarboxylate transport mutants revealed that dicarboxylate transporters are required by *A. brasilense* for an efficient colonization of plant roots and their growth.

Keywords: C₄-dicarboxylate, cell aggregation, DctP, DctA, nitrogen fixation, TRAP

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Bacteria of the genus *Azospirillum* are nitrogen-fixing and plant growth promoting rhizobacteria, which colonize the roots of a variety of plants including nonleguminous grasses and cereals (Okon et al. 1976, 1980; Pereg et al. 2016). The plant growth-promoting effect of *Azospirillum* spp. is observed due to its ability to produce phytohormones, siderophores, and to fix atmospheric nitrogen (Steenhoudt and Vanderleyden 2000). *Azospirillum* efficiently colonize and proliferate in the rhizosphere due to their preference for dicarboxylates as carbon source (Alexandre et al. 2000; Christiansen-Weniger 1992; Steenhoudt and Vanderleyden 2000). Under conditions of high carbon and low nitrogen, they form cysts embedded in a fibrillar matrix to form flocs or aggregates that help them in coping with stress conditions and root colonization (Burdman et al. 1998; Pereg Gerk et al. 1998; Sadasivan and Neyra 1985). They also show maximum rates of nitrogen fixation with dicarboxylates such as malic acid and succinic acid as carbon source (Martinez-Drets et al. 1984). This is why malic acid is routinely incorporated as a carbon source in semisolid media used for the enrichment and culture of *Azospirillum* spp. (Albrecht and Okon 1980; Neyra and Döbereiner 1977; Vanstockem et al. 1987). Since *A. brasilense* utilizes C₄-dicarboxylates in preference to carbohydrates (Goebel and Krieg 1984), this phenomenon was described as ‘reverse diauxie’ or ‘reverse catabolite repression’ (Mukherjee and Ghosh 1987).

Transport of C₄-dicarboxylates, such as malate, succinate, and fumarate (tricarboxylic acid cycle intermediates) in bacteria occurs via different transporters (Janausch et al. 2002). While the Dcu-type transporter family facilitates the uptake of C₄-dicarboxylates under anaerobic conditions, the most common transporters of C₄-dicarboxylates under aerobic conditions include members of DASS (divalent anion sodium symporter), DAACS (dicarboxylate amino acid-cation symporter), TRAP (tripartite ATP-independent periplasmic), and CitMHS transporter families (Youn et al. 2009). DctA represents the DAACS family of transporters, which is involved in the uptake of C₄-dicarboxylates in bacteria including *Escherichia coli*, *Bacillus subtilis*, or *Rhizobium leguminosarum* (Asai et al. 2000; Davies et al. 1999; Reid and Poole 1998). The DctABD system consists of three clustered genes, *dctA* encoding a C₄-dicarboxylates transporter protein and two divergently oriented genes, *dctB* and *dctD*, encode a two-component regulatory system involved in the sensing of C₄-dicarboxylates and regulating the expression of *dctA* (Engelke et al. 1989; Ronson et al. 1984; Yurgel and Kahn

2004). Mutation in any of the three *dct* genes causes loss of the ability to transport C₄-dicarboxylates (Engelke et al. 1987; Finan et al. 1988) in free-living rhizobia. The *dctA* mutant of *Rhizobium* also fails to fix nitrogen under symbiotic condition on plants (Reid and Poole 1998).

TRAP transporter was first identified and characterized as a C₄-dicarboxylate transporter in the purple photosynthetic bacterium *Rhodobacter capsulatus* (Forward et al. 1997; Shaw and Kelley 1991). It is encoded by a tricistronic operon (*dctPQM*), in which *dctP* encodes a high-affinity C₄-dicarboxylate binding periplasmic protein and *dctQ* and *dctM* encode integral membrane proteins (Forward et al. 1997). A *dctSR* operon, organized divergently to the *dctPQM* operon, encodes a two-component system that regulates the expression of the *dctPQM* operon (Hamblin et al. 1993).

In spite of several lines of evidences suggesting the importance of C₄-dicarboxylates for the growth, nitrogen-fixation, and rhizosphere colonization by *Azospirillum* spp., the genes involved in C₄-dicarboxylate utilization were not characterized. Although the role of dicarboxylate transport in *Rhizobium*-legume symbiosis is well-established (Ronson et al. 1981), the role of dicarboxylates and their transporters in the colonization and proliferation of plant roots by *Azospirillum* spp. has not yet been reported. In this study, we have investigated the role of two C₄-dicarboxylate transporters (DctP and DctA) of *A. brasilense* Sp7 in its ability to grow and fix nitrogen on dicarboxylates as sole carbon source. We have also shown here that the dicarboxylate transporters of *A. brasilense* Sp7 play important role in the colonization and proliferation of the roots of finger millet (*Eleusine coracana*), a C₄ grass.

RESULTS

Identification of a C₄-dicarboxylate-induced protein in *A. brasilense* Sp7.

A comparison of growth of *A. brasilense* Sp7 in minimal medium (MM) with malate, succinate, fumarate or fructose as sole source of carbon indicated that malate, succinate and fumarate were utilized much more efficiently than fructose (Fig. 1A). The growth curves of *A. brasilense* Sp7 obtained with 20-mM concentrations of the above carbon sources showed a similar pattern of growth, except that the growth with 40 mM reached 1.6 at an optical density at 600 nm (OD₆₀₀), while the OD₆₀₀ of cultures grown with 20 mM reached 1.2 (Supplementary Fig. S2). In the case of C₄-dicarboxylates, *A. brasilense* Sp7 grew exponentially from 4 to 16 h, after which a stationary phase started. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile of *A. brasilense* cultures, grown with malate, succinate, or fumarate as sole carbon source for 20 h, showed a protein of 37 kDa (Fig. 1B), which was strongly upregulated in malate-grown culture but was absent in fructose-grown cultures. This protein was also upregulated in succinate- or fumarate-grown cultures, but the intensity of the band was considerably weaker than that seen in malate-grown culture. This upregulated protein was eluted and trypsin-digested and was submitted for peptide mass fingerprinting by matrix-assisted laser desorption-ionization (MALDI) tandem time of flight assay. The peptide sequences obtained from the 37-kDa protein matched with the DctP (AZOBR_140140) protein of *A. brasilense* (Supplementary Fig. S1). This protein is a member of the tripartite TRAP transporter, which consists of three proteins, DctP, DctQ, and DctM. The gene encoding DctP was the first gene of the *dctPQM* operon (Fig. 2A) that was organized divergently to the genes encoding DctS (encoding a sensor) and DctR (encoding a regulator), which are known to regulate the expression of *dctPQM* operon in *R. capsulatus* (Forward et al. 1997).

Although DctP (AZOBR_140140) was the most prominently upregulated band in the total proteins of *A. brasilense* grown with malate as carbon source, proteins of the predicted molecular weights of DctQ (25 kDa) and DctM (47.52 kDa) could

not be seen by SDS-PAGE. To understand this anomaly, we carried out a reverse transcription-polymerase chain reaction (RT-PCR) from the total RNA isolated from *A. brasilense* Sp7 grown with malate as carbon source (Fig. 2B). The RT-PCR results showed that *dctPQM* was cotranscribed as a single transcript in *A. brasilense* Sp7. To see if the intergenic region between *dctP* and *dctQM* contains a potential terminator, we carried out an in silico analysis to search for a hairpin or step-loop structure. While open reading frames of DctQ and DctM overlap by 3 nucleotides (nt), the intergenic region of 122 nt between *dctP* and *dctQM* harbored a potential hairpin structure (Fig. 2A), providing an explanation for a relatively lower level of expression of *dctQM*. It is also possible that DctP might be relatively more stable than DctQ and DctM.

Phylogenetic and structural features of DctP protein from *A. brasilense* Sp7.

The DctP (AZOBR_140140) protein showed a maximum of 73% identity with the DctP (RCAP_rcc03024) of *R. capsulatus* and 45% identity with the DctP of *Pseudomonas aeruginosa* and *Shewanella oneidensis* (Fig. 3A). When we searched the genome of *A. brasilense* for DctP homologs, we found 14 paralogs of DctP, of which 12 were located on plasmids while two were located on the chromosome. Of the 14 DctP paralogs, 11 were part of complete *dctPQM* operons (Supplementary Fig. S3), while the remaining three were not organized as an operon and were randomly distributed in the genome. The genes encoding DctS and DctR regulators, however, were found in the vicinity of the gene encoding DctP (AZOBR_140140). A phylogenetic tree of the 14 DctP paralogs of *A. brasilense* along with the DctP orthologs that have been characterized for their substrates showed that only AZOBR_140140 clustered with the known dicarboxylate transporters (Fig. 3A). The other DctP paralogs of *A. brasilense* showed only 27 to 45% identities with the DctP of *R. capsulatus*. They clustered with DctP orthologs that are known to transport substrates other than dicarboxylates.

For analyzing the affinity of DctP paralogs other than AZOBR_140140 toward dicarboxylates, we attempted to identify the presence of conserved residues involved in dicarboxylate binding. The crystal structure of DctP protein of *Shewanella oneidensis* (Protein Data Bank [PDB] ID 4MX6) bound to L-malate and DctP protein of *P. aeruginosa* (PDB ID 4NF0) bound to succinate (Vetting et al. 2015) indicated that Val-36 and 37, Lys-42 and 95, Arg-171, Gly-173, Phe-195, Asn-211 and 215, and Tyr-238 were involved in the binding of L-malate and succinate in the pocket of the active site and were present at specific positions in both the structures (Fig. 3B). Alignment of DctP proteins from *R. capsulatus*, *A. brasilense*, *P. aeruginosa*, and *Shewanella oneidensis* showed that amino acid residues responsible for the binding of L-malate and succinate were also conserved and were present at the same positions in the DctP protein (AZOBR_140140) of *A. brasilense* and *R. capsulatus* (Supplementary Figs. S4 and S5). In the remaining 13 DctP paralogs of *A. brasilense*, however, the residues involved in the binding of dicarboxylates were not fully conserved. Structural superimposition of the crystal structures 4MX6 and 4NF0 with the modeled structures of DctP from *R. capsulatus* and *A. brasilense* showed structural similarity (RMSD < 0.13 nm) between each other, with identical pockets of the active site formed by the sequence motif VVX5KX59KX74/75RXQ/MX20F/YX16NX3NX22, which is responsible for interacting with C₄-dicarboxylates (Fig. 3B). Docking of malate, succinate, and fumarate on DctP (AZOBR_140140) protein showed that the free energy change with malate was slightly more negative than that for succinate and fumarate (Supplementary Fig. S6), indicating that malate fits better in the active site pocket of DctP protein than succinate and fumarate.

Effect of C₄-dicarboxylates on the expression of DctP protein.

To ascertain the inducibility of DctP expression by malate, a *dctP::lacZ* fusion was constructed. The effect of malate, succinate, and fructose on the expression of *dctP::lacZ* fusion yielded maximum β -galactosidase activity with malate and minimum activity with fructose (Fig. 1C). The *dctP* promoter activity with succinate was considerably higher than that on fructose but almost 45% of that shown by malate at 16 h.

Effect of inactivation of *dctP* gene on the growth of *A. brasilense* Sp7.

The *dctP* gene of *A. brasilense* Sp7 was inactivated in vitro by inserting a kanamycin resistance (Km^r) gene cassette in a cloned *dctP* gene (Supplementary Fig. S7A), which was placed

in the *A. brasilense* Sp7 genome by allele replacement via homologous recombination. The exconjugants of *A. brasilense* showing resistance to Km were further analyzed by PCR amplification of the Km^r gene from the genomic DNA of the mutant (designated as *dctP::km*). Since *dctP* is the first gene of the *dctPQM* operon, insertional inactivation of *dctP* is expected to have a polar effect on the expression of *dctQM*. The SDS-PAGE profile of *A. brasilense* and its *dctP::km* mutant grown with malate as sole carbon source showed the presence of the 37-kDa protein band in *A. brasilense* and its absence in the *dctP::km* mutant (Fig. 4B).

In comparison with *A. brasilense* Sp7, growth of the *dctP::km* mutant with malate as sole carbon source was drastically reduced (Fig. 4A). While *A. brasilense* entered into stationary phase at 20 h, the *dctP::km* mutant entered stationary phase at

Fig. 1. A, Growth curves of *Azospirillum brasilense* Sp7 in minimal medium (MM) containing malate, succinate, fumarate, or fructose (each 40 mM) as sole source of carbon. Each point of the curve shows the mean of three replicates obtained from three independent experiments. Error bars show standard deviation (SD) at each point. M = malate, S = succinate, F = fumarate, Fr = fructose. **B,** Electropherogram (12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of the total proteins from *A. brasilense* Sp7, grown in MM supplemented with different C₄-dicarboxylates as sole carbon source, showing distinct upregulation of the DctP (AZOBR_140140) protein in lane M. This protein is also present in lanes S and F but absent in lane Fr. Every lane of the gel was loaded with 20 μ g of total proteins. Lane L shows protein molecular weight marker (Bangalore genei). **C,** Effect of malate (M), succinate (S), fumarate (F) and fructose (Fr) on the expression of *dctP::lacZ* fusion in *A. brasilense* Sp7 grown in MM. The mean of triplicates in three independent experiments were calculated and are indicated with error bars in the form of SD. Data ($n = 3$) were analyzed using SPSS 17 software, and analysis of variance followed by Duncan's multiple comparison test was used to analyze significance between more than two treatments. Differences between means were compared and found significant at $P < 0.05$.

12 h. Complementation of the *dctP::km* mutant with a plasmid overexpressing the *dctPQM* operon (pDCTPOVSS) led to restoration of the growth of the mutant almost to that of *A. brasilense* (Fig. 4A). Our attempt to complement with *dctP* alone did not restore the growth of the mutant.

Effect of inactivation of *dctA* gene on the growth of *A. brasilense* Sp7.

Since inactivation of *dctP* in *A. brasilense* did not result in a complete loss of growth in media containing malate as carbon source, it was evident that *A. brasilense* uses more than one transporter to utilize C_4 -dicarboxylates for its growth. In light of this, we investigated the role of another dicarboxylate transporter, DctA in *A. brasilense* (Fig. 5A), which was homologous (62% identity and 79% similarity) to a well-characterized DctA (PA1183) transporter of *P. aeruginosa* PAO1(Supplementary

Fig. S8). The *dctA* (AZOBR_p1140111) gene was inactivated by inserting a gentamycin resistance (Gm^r) gene cassette and placing it back in the *A. brasilense* genome by allele replacement. A plasmid overexpressing the *dctA* gene was also constructed to learn if it could complement the ability of the *dctA::gm* mutant to grow on malate (Fig. 5B). The growth of the *dctA::gm* mutant on malate as sole carbon source was slower than that of *A. brasilense* Sp7 but was not as drastically affected as seen in the case of the *dctP::km* mutant (Fig. 5B).

Effect of inactivation of *dctP* and *dctA* on the growth of *A. brasilense* Sp7.

The growth of the *dctA::gm* and *dctP::km* mutants and the *dctA::gm* *dctA::gm* double mutant was compared with that of *A. brasilense* in MM with malate as sole carbon source. Figure 6 shows that the growth of the double mutant was

Fig. 2. A, Organization of the *dctPQM* operon and flanking genes in *Azospirillum brasilense* Sp7. Divergently oriented *dctSR* system forms a sensor-regulator pair for the regulation of *dctPQM* operon. A hairpin loop was located in the 120-bp intergenic region between *dctP* and *dctQ* gene. Large arrows indicate direction of the genes and their orientation while small arrows denote primers used for polymerase chain reaction (PCR) amplification or cDNA synthesis. Primers 1, 2, 3, 4, and 5 indicate primers RTMR1, RTMR2, QRTE, PRTE, and SGF, respectively. **B,** Electropherogram showing analysis of cotranscription of the *dctPQM* operon. Location of primers is shown along with the results of amplifications shown in the ethidium bromide-stained gel. Lane I shows 1 μ g of purified RNA isolated from the culture of *A. brasilense* Sp7 grown in minimum medium with malate as carbon source. Lane II shows results of PCR amplification using primers 2 and 3 with DNase-treated RNA as template (to demonstrate that the RNA used was free from genomic DNA contamination). Lane III shows cDNA (2 μ l) obtained after the reverse transcription-PCR reaction using primer 1. Lane IV shows the PCR amplicon produced using primer pair 2,3 with 20 ng of genomic DNA as template; Lane V and Lane VIII show molecular weight markers (Bangalore Genei). Lane VI shows PCR amplification with primer pair 2,3 using cDNA as template. Lane VII shows PCR amplification with primer pair 2,4 using 10 ng of genomic DNA as template. Lane IX shows the result of PCR amplification using primer pair 2,4 with cDNA as template, showing absence of a visible amplicon due to the presence of a hairpin between *dctP* and *dctQ*. Lane X shows absence of any PCR amplicon with primer pair 2,5 with cDNA as template (showing absence of cotranscription of *dctS* with *dctPQM*).

affected most adversely, compared with that of the *dctA::gm* or *dctP::km* mutants. This indicated that DctA and DctP are required for the optimal growth of *A. brasilense* on malate as carbon source.

Effect of inactivation of *dctA* and *dctP* on nitrogen-fixing ability of *A. brasilense* Sp7.

Since nitrogen-fixation ability of diazotrophic bacteria depends on their ability to transport dicarboxylates (Driscoll and Finan 1993; Ronson et al. 1981), we carried out an acetylene reduction assay to compare the nitrogen-fixation ability of the three mutants (*dctP::km*, *dctA::gmI*, and the double mutant) with that of *A. brasilense* Sp7 grown with malate as sole carbon source in semisolid medium. Figure 7 shows that nitrogen-fixation ability of the double mutant was most adversely affected, whereas that of the *dctP::km* and *dctA::km* mutants was relatively less affected individually.

Effect of inactivation of *dctA* and *dctP* on floc formation.

A. brasilense Sp7 does not flocculate in medium with a low C:N ratio (40 mM malate and 10 mM NH₄Cl), but it shows extensive flocculation in medium with a high C:N ratio (40 mM malate and 4 mM NH₄Cl). When floc-forming ability was compared between *A. brasilense* Sp7 and its three mutants in MM with a high C:N ratio, *A. brasilense* Sp7 and the *dctA::gm* mutant of *A. brasilense* showed very conspicuous floc formation (Fig. 8A and B). Floc formation in the *dctA::gm* was slightly less than that seen in *A. brasilense* Sp7, but the double mutant did not show any floc formation, reflecting carbon limitation. Floc formation in the *dctP::km* mutant was substantially lower

than that found in the *dctA::gm* mutant or the double mutant (Fig. 8B).

Effect of inactivation of *dctA* and *dctP* on the colonization and growth of roots in finger millet.

The ability of green fluorescent protein (*gfp*)-tagged *A. brasilense* Sp7 and its mutants to colonize the roots of finger millet was compared by confocal microscopy 10 days after inoculation (Fig. 9). While roots inoculated with untagged *A. brasilense* Sp7 did not show any green fluorescence, a large number of intense green fluorescing cell clumps or aggregates were observed scattered on the surface of young root zones inoculated with *gfp*-tagged *A. brasilense* Sp7 (Fig. 9). In the case of the *gfp*-tagged *dctA::gm* mutant, the number of aggregates and the size of each aggregate were considerably reduced. The number and size of the green fluorescing flocs was drastically reduced when *gfp*-tagged *dctP::km* mutant was used for inoculation, although some colonization could be seen in the root-cap region. In the case of the double mutant, however, green fluorescing flocs were completely absent in the cortical region. Some feeble green fluorescence was observed in the root-cap region.

The effect of inoculation of *A. brasilense* Sp7 and its three *dct* mutants on the root system of the finger millet seedlings was observed 20 days after bacterial inoculation. Figure 10A shows the difference in the root morphology of the seedlings with different bacterial treatments. There was a distinct difference in the length of the primary root between uninoculated and the seedlings inoculated with *A. brasilense* Sp7. The most visible difference was seen on the number of secondary roots

Fig. 3. A, Phylogram of multiple DctP paralogs of *Azospirillum brasilense* Sp7 and some other characterized orthologs. Amino acid sequences of DctP homologs were aligned using the ClustalW program, and a phylogenetic tree was constructed with MEGA 7 software using the maximum likelihood method based on the JTT matrix-based model, using 1,000 bootstrap replications to represent evolutionary history of the taxa analyzed. Branches with less than 50% bootstrap replicates were collapsed. **B**, Representation of active site residues of DctP protein interacting with L-malate and succinate in *Pseudomonas aeruginosa* (Protein Data Bank [PDB] ID: 4NF0) (green), *Shewanella oneidensis* (PDB ID: 4MX6) (magenta) and corresponding conserved residues in *A. brasilense* (orange) and *Rhodobacter capsulatus* (cyan) DctP homologs.

emerging from the primary roots (Fig. 10A and B). Roots of the seedlings inoculated with the *dctA::gm* mutant did not show a significant difference from the root morphology of the seedlings inoculated with *A. brasilense* Sp7. Seedlings inoculated with *dctP::km* mutant and the double mutant showed a distinct decline in the number of secondary roots in comparison with those inoculated with *A. brasilense* Sp7 or *dctA::gm* mutant (Fig. 10B).

DISCUSSION

Malate is often the most dominant organic acid found in the root exudates of C4 grasses. It was shown to be the strongest chemoattractant as well as a preferred source of carbon for the growth of *A. brasilense* (Alexandre et al. 2000). Chemotactic responses of rhizosphere bacteria toward organic acids correlate with the exudation of these acids by their host plants (Reinhold et al. 1985). Therefore, chemotaxis toward malate and the ability to efficiently utilize it provide a competitive advantage to bacteria that colonize the rhizosphere of C3 and C4 grasses, which release a considerable amount of malate in their root exudates (Rudrappa et al. 2008; Vranova et al. 2013). Exudation of organic acids like malate and citrate by plant roots also helps in enhancing the availability of phosphorus and iron and in conferring aluminum tolerance to the host plant (Mora-Macías et al. 2017).

The importance of malate for *A. brasilense* is also reflected by the occurrence of DctPQM (AZOBR_140140) and DctA dicarboxylate transporters, which were found to be important for growth on dicarboxylates. In addition, *A. brasilense* harbors several other DctPQM paralogs (other than AZOBR_140140) that might have reduced affinity to dicarboxylates and, hence, might be involved in the transport of dicarboxylates at a low

level, as they do not contain all the residues required for the binding of dicarboxylates. DctP paralogs other than AZOBR_140140 might be involved in the transport of substrates other than dicarboxylates, as they cluster with TRAP transporters involved in the transport of gluconate, 2-oxybutyrate, 2,3-diketo-L-gulonate, ectoine, 5-hydroxyectoine, *N*-acetylneuraminic acid, taurine, α -keto acids, glutamate, lactate, 4-chlorobenzoate, and protocatechuate (Mulligan et al. 2011; Okon et al. 1980). Although multiple paralogs of DctP were found in *Sinorhizobium meliloti*, *Corynebacterium glutamicum*, *Aromatoleum aromaticum*, and *P. aeruginosa*, only one in each was involved in the transport of dicarboxylates (Forward et al. 1997).

It was intriguing to see that DctP was the most prominently upregulated protein in the SDS-PAGE of the total proteins of the malate-grown cells of *A. brasilense*, but the expected protein band of the other two proteins (DctQ, 25 kDa, and DctM, 47.5 kDa) constituting the TRAP transporter were not visible. The presence of a hairpin loop immediately downstream of *dctP* in the intergenic region between *dctP* and *dctQM* might reduce the frequency of cotranscription quite substantially. In the case of *R. capsulatus* also, DctP was the most abundant protein in SDS-PAGE but DctQ and DctM were much less abundant (Shaw and Kelley 1991). Transcript analysis of the RNA isolated from malate-grown *R. capsulatus* by Northern hybridization with the *dctP* probe revealed an abundance of 1-kb transcript (from *dctP*) and a low abundance of 3-kb transcript (derived from *dctPQM*) (Shaw and Kelley 1991). Presence of a GC-rich region of dyad symmetry with potentially stable stem-loop structure between *dctP* and *dctQM* in *R. capsulatus* was thought to be responsible for creating a partial transcriptional terminator permitting very little read-through. This is why the *dctP* insertion mutant could be complemented with *dctPQM* only and not with *dctP* alone. Here also, *dctP::km*

Fig. 4. A, Growth curves of *Azospirillum brasilense* Sp7, *dctP::km* mutant, and *dctP::km* mutant strains complemented with the cloned copy of the *dctPQM* operon in minimal medium (MM) containing malate (40 mM) as carbon source. Mean $\pm$ standard deviation of three independent experiments, each performed in triplicate, are indicated. **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) showing presence of malate-inducible DctP protein in *A. brasilense* Sp7 and its absence in the *dctP::km* mutant, respectively. Every lane of the gel was loaded with 20 μ g of total protein obtained from *A. brasilense* Sp7 and its *dctP::km* mutant grown in MM containing malate (40 mM) as carbon source. Lane L shows protein molecular weight marker (Bangalore genei).

mutant could be complemented with the complete *dctPQM* operon but not *dctP* alone.

The *dctP::km* mutant of *A. brasilense* Sp7 showed the same initial speed of growth on malate as the parent but stopped growing after 8 h, eventually leading to a decline. The *dctA::gm* mutant initially grew more slowly than the parent but ultimately reached the same level of growth. This difference may be due to the difference between the affinity of DctA and DctP toward dicarboxylates (Valentini et al. 2011). These observations

Fig. 5. A, Schematic representation of the organization of genes around *dctA* of *Azospirillum brasilense* Sp7. The divergently oriented *dctBD* system forms a sensor-regulator pair of the DctA transporter. **B,** Growth curves of *A. brasilense* Sp7, the *dctA::gm* mutant, and *dctA::gm* complemented with a cloned copy of the *dctA* gene in minimum medium containing malate (40 mM) as sole carbon source. Each point of the curves represents mean of the triplicates from three independent experiments, and the error bars at each point show standard deviation.

Fig. 6. Growth curves of *Azospirillum brasilense* Sp7, *dctA::gm*, *dctP::km*, and the double mutant of *A. brasilense* Sp7 grown in minimal medium containing malate (40 mM) as carbon source. Each point of the curve shows mean of the triplicates from three independent experiments, and the error bars at each point indicate standard deviation.

suggest that the two transport systems play different roles. Significantly reduced diazotrophic growth and nitrogen-fixing ability of the *dctP::km* mutant and the double mutant of *A. brasilense* Sp7 in the medium containing malate as sole carbon source indicated the importance of dicarboxylates as a primary source of carbon for nitrogen fixation in *A. brasilense*. Cells require significant amounts of carbon to produce cell-wall polysaccharides in order to form flocs or aggregates (Burdman et al. 1998; Sadasivan and Neyra 1985). The drastically reduced ability of *dctP::km* and the double mutant to form flocs or aggregates in the culture medium with a high C:N ratio suggests that the defect in the dicarboxylate transporters might reduce the effective intracellular concentration of malate in the high C:N ratio medium. The reduced uptake of malate by the mutants may mimic the response of *A. brasilense* Sp7 in a low C:N ratio medium, which may be the cause for decline in flocculation by the mutants in spite of a high C:N ratio culture medium (Bible et al. 2012; Burdman et al. 1998). The decline of flocculation in the *dctP::km* and the double mutant observed in this study clearly follows the decline in the availability of carbon to the bacterium.

In rhizobia, dicarboxylate transport plays an important role in the operation of an effective, nitrogen-fixing symbiosis (Watson et al. 1988). DctA is the only transporter of dicarboxylates that is found to be essential for in-planta nitrogen fixation, as nodules infected with *dctA* mutants fail to fix nitrogen due to their inability to utilize dicarboxylates (Engelke et al. 1989; Jording et al. 1994; Reid and Poole 1998). Although *dctPQM* homologs are present in *Sinorhizobium meliloti*, they are not involved in the transport of dicarboxylates (Yurgel and Kahn 2004, 2005). In *R. capsulatus*, DctP along with DctQM constitutes a dicarboxylate transporter that is solely responsible for the transport of dicarboxylates (Forward et al. 1997). *Sinorhizobium meliloti* mutants having deletion of *dctABD* genes, but expressing *dctPQMSR* genes from *R. capsulatus* were able to grow on dicarboxylates (Forward et al. 1997). This further confirmed that *R. capsulatus* *dctPQMSR* genes encoded a complete dicarboxylate transport system, which is absent in *Sinorhizobium meliloti* (Forward et al. 1997). *P. aeruginosa* uses both DctA and DctPQM type of dicarboxylate transport

Fig. 7. Comparison of the acetylene reducing ability of *Azospirillum brasilense* Sp7, *dctA::gm*, *dctP::km*, and the double mutant after 24 h of growth in nitrogen-free semisolid minimum medium containing malate (40 mM) as carbon source. Mean $\pm$ standard deviation of triplicates from three experiments is indicated, and differences between means were calculated. Small letters above the bars represent a homogeneous subset of the treatments and indicate the result of Duncan's multiple range test (different letters have *P* values of <0.05).

systems for utilizing dicarboxylates (Valentini et al. 2011). This study also indicated that *A. brasilense* uses both DctPQM and DctA for its growth and nitrogen fixation on dicarboxylate. The genome of *A. aromaticum* does not harbor any gene encoding DctA. It, however, encodes six paralogs of DctP, and the paralog having maximum identity with the DctP of *R. capsulatus* was the most abundant protein in dicarboxylate grown

cultures (Trautwein et al. 2012). The genome of *Corynebacterium glutamicum* encodes three paralogs of DctA and three encoding TRAP transporters (DctPQM). Surprisingly, none of them is involved in dicarboxylate transport (Teramoto et al. 2008). DcsT protein of the DASS family, however, was found to be responsible for the aerobic growth of *Corynebacterium glutamicum* on dicarboxylates.

Azospirillum spp. have been shown to colonize plant roots, using various techniques including electron microscopy, immunofluorescence microscopy, β -glucuronidase (GUS)-tagging, and GFP-tagging coupled with confocal laser scanning microscopy (Bashan and Lavanony 1989; Okon and Kapulnik 1986; Ramos et al. 2002; Schank et al. 1979; Umali-Garcia et al. 1980; Vande Broek et al. 1993). Colonization of wheat roots by *A. brasilense*, using a GUS reporter system, had shown that root colonization by *A. brasilense* was initiated in the root-hair zones and the sites of emergence of lateral roots (Vande Broek et al. 1993). The process of attachment of *Azospirillum* spp. to the root surface starts with an initial loose binding, followed by a firm binding, and, then, entrapment of additional bacteria to form large bacterial aggregates (Michiels et al. 1991). In a study of *Azospirillum*-wheat interaction using *gfp* and *gusA* genes, cell aggregates and single bacteria were visualized on the surface of the plant root system, including root hairs, lateral roots, and points of emergence of lateral roots (Ramos et al. 2002). We also found a large number of aggregates of GFP-tagged *A. brasilense* at the root tip and in the zone of root elongation on the roots of finger millet.

We also noted a significant decline in the number of secondary roots of the seedlings inoculated with *dctP::km* and the double mutant of *A. brasilense* Sp7, vis-a-vis those inoculated with *dctA::gm* and *A. brasilense* Sp7. As expected, in comparison with the uninoculated seedlings, the roots inoculated with *A. brasilense* Sp7 showed a major increase in the number of secondary roots and, also, a notable difference in the length of the primary root. The difference in root morphology may be assigned to the ability of *A. brasilense* and its *dct* mutants to proliferate on the roots and to produce a proportionate amount of auxins to cause secondary root formation. Also, earlier, *A. brasilense* was shown to colonize the roots of finger millet and pearl millet, leading to considerable increases in yield

Fig. 8. A, Comparison of aggregation of *Azospirillum brasilense* Sp7, *dctA::gm*, *dctP::km*, and the double mutant under N-limited growth conditions. **B,** Bar diagram shows percentage of cell aggregation of *A. brasilense* Sp7, *dctA::gm*, *dctP::km*, and the double mutant grown in minimum medium supplemented with 40 mM malate and 4 mM NH_4Cl . Mean $\pm$ standard deviation of triplicates from the three experiments is indicated, and the difference between means were calculated. Small letters above the bars represent a homogeneous subset of the treatments and indicate the result of Duncan's multiple range test (different letters have *P* values of <0.05).

Fig. 9. Confocal laser scanning microscopy images (10 and 40 $\times$) of surface-sterilized root, *gfp*-tagged *Azospirillum brasilense* Sp7, *dctA::gm*, *dctP::km*, and the double mutant on the roots of *Eleusine coracana* 10 days after inoculation. Control shows roots inoculated with equal amount of untagged *A. brasilense* Sp7. Cells expressing green fluorescent protein are green and gray background corresponds to root image formed by transmitted light.

(Agarwala and Tilak 1988; Rai 1991; Subba Rao 1985; Tilak and Subba Rao 1987; Umali Garcia et al. 1980; Wani et al. 1985). There could be two reasons for the failure of the *dctP::km* mutant and the double mutant to colonize the roots of finger millet. The primary reason for the failure of the mutants to colonize plant roots is the severely compromised ability of the *dctP::km* mutant and the double mutant to utilize dicarboxylates for growth and nitrogen fixation. Another reason for their failure to colonize finger millet roots could be the lack of their ability to form flocs or cell aggregates, which are considered important in plant root colonization (Burdman et al. 1998).

Plants control the populations of associated fungi and bacteria in their rhizosphere by releasing root exudates (Bardgett et al. 2014; Coskun et al. 2017). *Azospirillum* strains have been shown to display chemotaxis toward several amino acids, sugars, organic acids (Barak et al. 1983; Okon et al. 1980; Reinhold et al. 1985), root exudates (Heinrich and Hess 1985), and root mucilage (Mandimba et al. 1986). It was hypothesized that specific compounds are secreted in the root hair zones of wheat roots to which *Azospirillum* species are attracted. Since biological nitrogen fixation is an energy-intensive process, availability of carbon sources in the rhizosphere is one of the major limiting factors for associative nitrogen fixation. In this study, we have provided evidence to corroborate a long-held belief that dicarboxylates play an important role in plant root colonization and associative nitrogen fixation by *Azospirillum* spp. The first evidence in support of this was obtained in a wheat cultivar that released higher amounts of C4 dicarboxylates in its root exudates and also showed significantly higher

nitrogen fixation by *A. brasilense* and transfer of fixed nitrogen to the host plant than the wheat cultivar releasing a low amount of dicarboxylates in its root exudates (Christiansen-Weniger 1992). Mutants of *A. brasilense* with defects in motility and chemotaxis were used earlier to establish the role of bacterial motility in *Azospirillum*-plant root association (Vande Broek et al. 1998). We have shown here that *A. brasilense* mutants having defects in their ability to utilize dicarboxylates show considerably reduced growth and nitrogen fixation on dicarboxylates and possess reduced ability to colonize the roots of finger millet. This is the first study demonstrating the importance of dicarboxylate transporters of *A. brasilense* in colonizing the roots of a C4 grass and in promoting proliferation of the root system. These observations pave the way for a relatively longer-term experiment using a ^{15}N isotope dilution technique to demonstrate the contribution of *A. brasilense* to the nitrogen needs of the nonlegume plants through dicarboxylate-dependent nitrogen-fixation by *A. brasilense* (Hardarson and Hera 1998).

MATERIALS AND METHODS

Bacterial strains, culture condition, plasmids, and chemicals.

Bacterial strains and plasmids used in study are described in Table 1. *A. brasilense* Sp7 was grown and maintained in MM containing 40 mM malate as carbon source and 18 mM NH_4Cl as nitrogen source (Albrecht and Okon 1980) at 30°C. For experiments, overnight-grown cultures of *A. brasilense* in Luria-Bertani (LB) (Miller 1972) or in MM with 40 mM fructose (instead of malate) as sole carbon source were pelleted, were washed with 0.85% saline, and were then reinoculated in MM supplemented with different C₄-dicarboxylates (each 40 mM) as the sole source of carbon. *E. coli* strains like DH5 α and S17-1 were grown in LB medium at 37°C. All chemicals and media components used for growing bacteria were purchased from Hi-Media, Merck, or Sigma Aldrich. Restriction enzymes, DNA ligase, *Taq* polymerase, Phusion high fidelity DNA polymerase, DNase, RNase, and alkaline phosphatase were purchased from New England Biolabs. As required, ampicillin (100 $\mu\text{g}/\text{ml}$), chloramphenicol (20 $\mu\text{g}/\text{ml}$), Km (30 $\mu\text{g}/\text{ml}$), Gm (10 $\mu\text{g}/\text{ml}$), and tetracycline (10 $\mu\text{g}/\text{ml}$) were added in the medium for *A. brasilense* Sp7 and its derivatives.

Analysis of bacterial growth.

The growth of *A. brasilense* Sp7 was monitored in MM supplemented with malate, succinate, fumarate, or fructose (each 40 or 20 mM) as sole carbon source at 30°C with shaking at 180 rpm. Overnight-grown primary cultures of *A. brasilense* (in LB medium) were pelleted, were washed with 0.85% saline, and were reinoculated in secondary MM supplemented with C₄-dicarboxylates and fructose (40 mM) as the sole carbon source. Growth was monitored by recording OD₆₀₀ at 4 h intervals and growth curves were plotted OD versus time. Growth curves of *A. brasilense* Sp7, its mutants (*dctP::km*, *dctA::gm* and double knockout mutant), and complemented strains (the *dctP::km* mutant harboring the pDCTPOVSS plasmid and the *dctA::gm* mutant harboring the pDCTAOVSS plasmid) were also plotted as above.

Effect of different carbon compounds on protein profile.

Overnight-grown culture of *A. brasilense* Sp7 in LB broth was pelleted, was washed, and was reinoculated in the MM supplemented with malate, succinate, fumarate, or fructose (each 40 mM) as a sole source of carbon. When cultures reached the late log phase, 8 ml of each culture was pelleted by centrifugation and pellets were resuspended in 1 ml of 1 $\times$ phosphate buffered saline containing 1 mM phenylmethylsulfonyl fluoride.

Fig. 10. Comparison of the roots of the seedlings of *Eleusine coracana* plants after 20 days of bacterial inoculation of *Azospirillum brasilense* Sp7 and its *dct* mutants **A**, Effect of inoculation of *A. brasilense* Sp7 and its *dct* mutants on root length and morphology. **B**, Measurement of primary root length and numbers of secondary roots. Mean $\pm$ standard deviation of the replicates is indicated, and differences between mean were calculated. Small letters above the bars represent a homogeneous subset of the treatments and indicate the result of Duncan's multiple range test (different letters have *P* values of <0.05).

The cell pellets were lysed by sonication for 5 min, at 9-s on/off conditions, at 4°C. The resulting lysate was centrifuged at 14,000 rpm at 4°C and supernatant was collected for protein estimation by Bradford assay (Bradford 1976). Protein lysate (20 µg of each) was mixed with equal volume of 2× protein cracking dye, was boiled at 95°C for 10 min, followed by a brief vortex and centrifugation. The protein samples (20 µg per lane) were then loaded in precast 12% SDS-PAGE and electrophoresis was performed at 150 V for 5 h. A 37-kDa protein that was most prominently upregulated in the malate-grown cells was analyzed by MALDI-tandem mass spectrometry (MS/MS) after trypsin digestion. The predicted peptide sequences were searched in the genome of *A. brasilense* Sp245.

Bioinformatic analysis.

The amino acid sequence of the induced DctP (AZOBR_140140) and DctA (AZOBR_p1140111) of *A. brasilense* Sp7 were retrieved from the Genoscope database, and similar sequences from other bacteria were retrieved from the National Center for Biotechnology Information database. Levels of sequence identity and similarity were calculated using multiple sequence alignment tools at the European Bioinformatics Institute server. A phylogenetic tree was constructed using the MEGA 7 software by the maximum likelihood method based on a JTT matrix-based model (Jones et al. 1992), using 1,000 bootstrap replications to represent evolutionary history of the taxa analyzed. Structure modeling, superposition, and docking experiments were done on the Discovery Studio 3.5 Suite. Gene analysis and construction of genetic organization were performed with the help of vector NTI (Thermo Fisher Scientific) software.

RT-PCR.

Total RNA was isolated from *A. brasilense* Sp7 cells grown in minimal malate medium using the TRIzol method. RNA isolation and cDNA synthesis were performed according to a method described elsewhere (Mishra et al. 2011). After treatment with RNase-free DNase (Thermo) for 1 h at 37°C, RT-PCR was carried out with 2 µg of RNA sample and *dctM*-specific primer RTMR1 (Table 2), using the One Step RT-PCR kit (Thermo) according to manufacturer instructions. The cycle condition used was 45°C for 60 min, followed by heat inactivation at 72°C for 10 min. Negative and positive controls were included in RT-PCR for checking DNA contamination and primer functionality.

Construction of the *dctP::lacZ* transcriptional fusion.

In order to study the inducibility of *dctP* (AZOBR_140140) promoter in response to various carbon compounds, transcriptional *lacZ* fusion with *dctP* promoter region was constructed. The upstream regions of the *dctP* (156 bp) gene from *A. brasilense* Sp7 was PCR-amplified using a set of primers (DCTPPF and DCTPPR [Table 2]) containing *Xba*I and *Hind*III sites in forward and reverse primers, respectively. The amplicon was cloned in pCZ750 vector (Dombrecht et al. 2001) digested with *Xba*I and *Hind*III. Once the recombinant plasmid was confirmed by colony PCR and restriction digestion, it was isolated and was transformed in *E. coli* S17.1, which was used as a donor for the conjugative mobilization of the recombinant plasmid (pVSSPP [Table 1]) in *A. brasilense* Sp7.

β-Galactosidase assay.

The effect of various C₄-dicarboxylates on the inducibility of *dctP* gene was monitored by assaying β-galactosidase activity of the cultures of *A. brasilense* Sp7 (pVSSPP) grown with 40-mM concentrations of each carbon source at 30°C, with shaking, at 180 rpm. After 6 h of growth, samples of 0.5 OD₆₀₀ were collected by centrifugation and a β-galactosidase assay of equal numbers of cells of each sample was performed (Miller 1972).

Table 2. Primers used in this study

Primers	Sequence 5' to 3' direction
DCTPPF	GCTCTAGAGTCATCCTCCACTTGTGTGTGG
DCTPPR	CCCAAGCTTCTGGCCGCACCGCCGGTCATGG
PAF	AACTGCAGGTTTCATCGGTCGTTCTCCTTCG
PAR	GAAGATCTCGTCCAACATGTACACCCAGAAG
PBF	GAAGATCTTGCTCCTTGTCTCTGTAGAGC
PBR	GGAATTCGGCCGGGATTCACATAGATGAT G
GSPF	CCCAAGCTTGATTGCTCCGACGAGGG
GSPR	CGGGATCCATGAAGTTCGTTTCGCTGC
AAF	CCGGAATTCTGCGCAGGTTGGATTTCGTACAG
AAR	GAAGATCTAGGAAGTCGATGGTCGTGTGG
ABF	GAAGATCTGCAAGTACGGCGTGTCTGTCG
ABR	AAAACATGCAGGGACGATGGTGTGACACAGAC
GSAF	CGCGGATCCAACATGCACGCCCAAGGGCAG
GSAR	CCCAAGCTTGTGACATGCGGGCCCCAGAG
RTMR1	ACAGCTTCAGCGCCACG
RTMR2	GTCATGGTGAAGAGGAAGGTC
QRTF	CTGCATCTACATGTTCTGTCTGC
PRTF	TCTACGAGCTGACCAAGGACG
SGF	AGAGGTGTTGGCACGGCAACC

Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant properties ^a	References/Sources
<i>Escherichia coli</i> DH5α	Δ <i>lac</i> U1 69 <i>hsdR</i> I 7 <i>recA</i> 1 <i>endA</i> 1 <i>gyrA</i> 96 <i>thi</i> L <i>relA</i> 1	Gibco BRL
<i>E. coli</i> S17-1	Sm ^r , <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> -M+RP4:2-Tc:Mu:Km Tn7, <i>λ</i> pir	Simon et al. 1983
<i>Azospirillum brasilense</i> Sp7	Wild-type strain (ATCC 29729)	Nur et al. 1981
<i>dctP::km</i>	<i>A. brasilense</i> Sp7 <i>dctP</i> (AZOBR_140140) gene disrupted by insertion of Km ^r cassette	This work
<i>dctA::gm</i>	<i>A. brasilense</i> Sp7 <i>dctA</i> (AZOBR_p1140111) gene disrupted by insertion of Gm ^r cassette	This work
<i>dctP::km, dctA::gm</i>	<i>A. brasilense</i> Sp7 <i>dctP</i> (AZOBR_140140) and <i>dctA</i> (AZOBR_p1140111) genes disrupted by	This work
(P+A double mutant)	insertion of Km ^r and Gm ^r cassettes, respectively	
pCZ750	pFAJ1700 containing the <i>Kpn</i> I- <i>Ascl</i> - <i>lacZ</i> gene from the pCZ367 plasmid; Tet ^r Amp ^r	Dombrecht et al. 2001
pVSSPP	pCZ750 derivative; <i>dctP-lacZ</i> ; Tet ^r Amp ^r	This work
pSUP202	ColE1 replicon, mobilizable plasmid, suicide vector suitable for <i>A. brasilense</i> , Amp ^r , Tet ^r , Cm ^r	Simon et al. 1983
pUC4K	Vector containing Km ^r cassette	GE Healthcare
pDCTPDVSS	<i>dctP</i> (AZOBR_140140) gene disruption plasmid	This work
pDCTADVSS	<i>dctA</i> (AZOBR_p1140111) gene disruption plasmid	This work
pMMB 206	Cm ^r , broad host range, low copy number, expression vector	Morales et al. 1991
pDCTPOVSS	<i>dctP</i> and its downstream genes, <i>dctQ</i> and <i>dctM</i> (<i>dctPQM</i> operon, 3,124 bp) from <i>A. brasilense</i> Sp7 cloned into <i>Eco</i> RI/ <i>Hind</i> III restriction site of pMMB 206	This work
pDCTAOVSS	<i>dctA</i> (1,320 bp) gene cloned into <i>Bam</i> HI/ <i>Hind</i> III restriction site of pMMB 206	This work
pBBR1MCS-3	Tet ^r , broad host range, medium copy number, <i>lacZ</i> gene containing promoter less vector	Kovach et al. 1995
pAKMPVSS	Constitutive Km promoter and <i>gfp</i> gene, cloned into <i>Xho</i> I/ <i>Xma</i> I restriction sites of modified pBBR1MCS-3 vector	This work

^a Sm^r, Km^r, Gm^r, Tet^r, Amp^r, and Cm^r = streptomycin, kanamycin, gentamycin, tetracycline, ampicillin, and chloramphenicol resistant.

Construction of single and double knockout mutant of *dctP* and *dctA* genes.

The *dctP::km* and *dctA::gm* mutants of *A. brasilense* Sp7 were constructed by inserting gene cassettes encoding resistance to Km and Gm, respectively, in *dctP* (AZOBR_140140) and *dctA* (AZOBR_p1140111) genes in vitro, resulting in *dctP*- and *dctA*-disruptive plasmids pDCTPDVSS and pDCTADVSS, respectively. The two recombinant plasmids were then transformed into *E. coli* S17-1 and were conjugatively mobilized in *A. brasilense* Sp7 (Singh et al. 2017). The exconjugants were selected on LB plates containing Km and Gm, respectively. The *dctP::km* and *dctA::gm* mutants were confirmed by observing expected increase in the size of the amplicons after PCR amplification of respective genes with gene-specific primers (GSPF/R and GSAF/R). Primers used for *dctP* and *dctA* knockout construction are listed in Table 2 and the recombinant plasmids constructed are listed in Table 1. For the construction of a *dctP::km* and *dctA::gm* double mutant, the *dctA* disruptive plasmid (pDCTADVSS) was conjugatively mobilized in the *dctP::km* mutant of *A. brasilense* Sp7 and the resulting exconjugants were selected on a LB plate containing both Km and Gm.

Cloning in broad host range expression vector for complementation.

The *dctPQM* operon (3,124 bp) and *dctA* (1,320 bp) genes were cloned individually in the pMMB206 expression vector (Morales et al. 1991). The resulting recombinant plasmids (pDCTPOVSS and pDCTAOVSS) were conjugatively mobilized in *dctP::km* and *dctA::gm*, respectively, to evaluate their ability to complement the respective mutants.

Acetylene reduction assay.

Acetylene reduction assay was performed as described earlier (Tripathi et al. 2002). Briefly, overnight-grown cultures of *A. brasilense* Sp7 and its mutants (single and double knockout mutants of *dctP* and *dctA* genes) grown in MM containing 40 mM fructose, 10 mM NH₄Cl, and appropriate antibiotics were reinoculated in the same medium for growth at 30°C with shaking at 180 rpm. Cultures of *A. brasilense* Sp7 and its mutants were harvested from their midlog phase and were washed twice with nitrogen-free medium. Equal numbers of cells of *A. brasilense* Sp7 and its mutants was resuspended in 11 ml of nitrogen-free semisolid minimal malate medium to make 0.2 OD₆₀₀ (Chowdhury et al. 2007) in 22 ml crimp cap headspace vials (Agilent), which were closed tightly (to make air-tight) with crimper. Sealed tubes were incubated for 24 h at 30°C for the development of the pellicle. After pellicle formation, 10% air (vol/vol) from tubes was replaced with acetylene gas, and tubes were further incubated at 30°C for another 24 h. Thereafter, the amount of acetylene gas reduced to ethylene was measured using a gas chromatograph (Agilent Technologies 7890 B GC system) equipped with a flame ionization detector and Agilent J&W GC column CP-AI203/KCl.

Cell aggregation studies.

Azospirillum brasilense Sp7 and its mutants were grown overnight in MM containing 10 mM NH₄Cl and equal numbers of cells were harvested by centrifugation for 10 min at 12,000 rpm. The cell pellets were washed twice with nitrogen-free MM and were reinoculated in fresh minimal malate medium amended with 4 mM NH₄Cl (Burdman et al. 1998). Cultures were grown with shaking at 150 rpm for 24 h at 30°C. Quantification of cell aggregation was done as described earlier (Madi and Henis 1989). Liquid cultures containing the aggregates were transferred to 50-ml test tubes and were allowed to stand for 30 min. Once the complete aggregate got settled and the suspension consisted of free cells, turbidity of each suspension (ODs) was measured at 540 nm (Genesys spectrophotometer).

The aggregate was then dispersed by sonication for 1 min, and total turbidity was measured at 540 nm (ODt). The percentage of cell aggregation was quantified as follows: % aggregation = ODt – ODs × 100 ÷ ODt.

GFP-tagging, inoculation, seedling growth conditions, confocal microscopy, and measurement of root growth.

A. brasilense Sp7 and its mutants were genetically tagged with the GFP reporter. The vector pBBR1MCS-3 (Kovach et al. 1995) was modified by introducing a gene encoding GFP under the control of a constitutively expressed Km promoter to obtain the recombinant plasmid pAKMPVSS (unpublished), which was conjugatively mobilized into *A. brasilense* Sp7 and its three mutants via *E. coli* S17-1 in separate matings. Exconjugants showing green fluorescence under UV illumination were selected for further study. Seeds of finger millet (*Eleusine coracana*) were surface-sterilized with 0.1% mercuric chloride (HgCl₂) for 1 min followed by washing six times with sterile distilled water. The seeds were then immersed in 2% (wt/vol) sodium hypochlorite for 30 min in an incubator shaker. Finally, the seeds were washed with sterile water (five or six times) with shaking and were incubated on Whatman filter paper wetted with Hoagland's solution at 30°C for 3 days, for germination. Seedlings were washed with sterile water and were transferred to a 75-ml cotton plug-tightened test tube containing 25 ml of Hoagland's solution (Hoagland and Arnon 1950) and paper brick. Seedling were allowed to grow further on paper brick submerged in Hoagland's solution in a plant growth chamber with a 15-h light and 9-h dark regimen for 5 days. The *gfp*-tagged *A. brasilense* Sp7, its three mutants, and an untagged *A. brasilense* were grown in MM with fructose (40 mM) as carbon source. One milliliter of bacterial cultures containing a total of 10⁵ cells were pelleted, washed, and inoculated, in each tube, in Hoagland's solution after 5 days of seedling growth. One set of the seedlings was further cultivated for 10 more days in a controlled plant growth chamber at 24°C with a 15-h light and 9-h dark regimen. After 10 days, the roots of finger millet inoculated with *gfp*-tagged *A. brasilense* and its C₄-dicarboxylate transporter mutants were gently washed with deionized water. The microscopic observation was performed under confocal microscope (LSM 880, Carl Zeiss) with 10 and 40× Plan-Apochromat lenses using λ_{exc}-488 nm and λ_{em}-590 to 553 nm. Images were collected in z-series with a step size of 1.0 μm and were processed with ZEN software (Carl Zeiss). The effect of inoculation of *A. brasilense* Sp7 and its three mutants on the root morphology of seedlings was monitored in 10 tubes, each containing three seedlings for each bacterial treatment. Seedlings were allowed to grow for 20 days post-bacterial inoculation and were taken out for photography of the root morphology and measurement of the length of the primary root and the number of secondary roots.

Statistical analysis.

Experiments on measurement of growth, β-galactosidase activity, flocculation, and acetylene reduction assay were performed in triplicates in three independent experiments. The mean significant values were determined by using SPSS 17 package software. Analysis of variance followed by Duncan's multiple comparison test was used to analyze significance between more than two treatments. Differences were considered significant at *P* < 0.05.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- European Bioinformatics Institute ClustalW multiple sequence alignment tool: <https://www.ebi.ac.uk/Tools/msa/clustalw2>
- Genoscope Acinetobacter baylyi ADP1 map: <https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php>
- National Center for Biotechnology Information website: <https://www.ncbi.nlm.nih.gov>