Applied Microbiology and Biotechnology (2022) 106:7891–7903
https://doi.org/10.1007/s00253-022-12250-0

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Engineering D-glucose utilization in *Azospirillum brasilense* Sp7 promotes rice root colonization

Vijay Shankar Singh[1] · Basant Kumar Dubey[2] · Sushant Rai[1] · Surendra Pratap Singh[3] · Anil Kumar Tripathi[1]

Received: 11 August 2022 / Revised: 7 October 2022 / Accepted: 12 October 2022 / Published online: 5 November 2022

Abstract

Bacteria of the genus *Azospirillum* include several plant associated bacteria which often promote the growth of their host plants. Although the host range of *Azospirillum brasilense* Sp7 is much wider than its close relative *Azospirillum lipoferum* 4B, it lacks the ability to efficiently utilize D-glucose for its growth. By comparing the genomes of both the species, the genes of *A. lipoferum* 4B responsible for conferring D-glucose utilization ability in *A. brasilese* Sp7 were identified by cloning individual or a combination of genes in a broad host range expression vector, mobilizing them in *A. brasilense* Sp7 and examining the ability of exconjugants to use D-glucose as sole carbon source for growth. These genes also included the homologs of genes involved in *N*-acetyl glucosamine utilization in *Pseudomonas aeruginosa* PAO1. A transcriptional fusion of the 5 genes encoding glucose-6-phosphate dehydrogenase and 4 components of glucose phosphotransferase system were able to improve D-glucose utilization ability in *A. brasilense* Sp7. The *A. brasilense* Sp7 strain engineered with D-glucose utilization ability showed significantly improved root colonization of rice seedling. The improvement in the ability of *A. brasilense* Sp7 to colonize rice roots is expected to bring benefits to rice by promoting its growth.

Key points

- *Genes required for glucose utilization in Azospirillum lipoferum were identified.*
- *A gene cassette encoding glucose utilization was constructed.*
- *Transfer of gene cassette in A. brasilense improves glucose utilization and rice root colonization..*

Keywords *Azospirillum* · D-glucose utilization · Phosphotransferase system · Rice root colonization

Introduction

Plant growth-promoting rhizobacteria (PGPR) inhabit rhizosphere, root surface, and root interior, and stimulate plant growth and development (Kloepper and Schroth 1981; Santoyo et al. 2016) through a number of direct and indirect mechanisms which include production of phytohormones and siderophores, biological nitrogen fixation, nutrient solubilization, and induction of systemic resistance (Barriuso et al. 2008). PGPR such as *Herbaspirillum seropedicae* or *Gluconacetobacter diazotrophicus* colonize plant roots endophytically by occupying intercellular spaces, and *Pseudomonas fluorescens* or *Azospirillum brasilense* are present on root surfaces and their proximity in the rhizosphere (Santoyo et al. 2016; Steenhoudt and Vanderleyden 2000). Application of PGPR as inoculants has been shown to increase the productivity of a variety of crops (e.g., maize, tomato, lettuce, wheat, soybean, rice, and apples) under normal as well as stressful conditions (Deshmukh and KhareP 2016; Etesami and Alikhani 2016; Bhattacharyya and Jha 2012; Dobbelaere et al. 2001; Okon and Labandera-Gonzalez 1994; Cassán et al. 2020).

The rhizosphere is a complex microbial habitat comprising of an integrated network of plant roots, soil and a diversity of microbes. It is strongly influenced by plant metabolism through secretion of an array of root exudates,

✉ Anil Kumar Tripathi
tripathianil@rediffmail.com

[1] School of Biotechnology, Institute of Science, Banaras Hindu University, Varanasi 221005, India

[2] Plant Biotechnology Division, Council of Scientific and Industrial Research-Central Institute of Medicinal and Aromatic Plants, Lucknow, India

[3] Department of Botany, Dayanand Anglo-Vedic (PG) College (Affiliated to CSJM University, Kanpur) Civil Lines, Kanpur 208001, India

which mediate rhizosphere interactions by serving as energy sources for microorganisms and acting as chemical attractants or repellents (Bais et al. 2001; Estabrook and Yoder 1998). The capacity of root exudates to attract bacteria is attributed to some of their individual components. Studies on the attraction and migration of beneficial rhizosphere bacteria towards root exudates provide important information about ecological traits for root colonization. Microbes that display chemotaxis towards root exudates of the host plant show strong attachment to the host plant which allows stronger association and better beneficial effects to the plant (Yang et al. 2009; Bashan et al. 2014). Understanding, predicting and controlling the structure and function of the rhizosphere can be a key to harnessing plant–microbe interactions and other rhizosphere activities.

Bacteria of the genus *Azospirillum* are very popular PGPR and consist of more than 20 species (Fukami et al. 2018). *Azospirillum lipoferum* and *Azospirillum brasilense* were first described in 1978 (Tarrand et al. 1978). One of the several phenotypic characteristics which differentiate *A. brasilense* from *A. lipoferum* (Baldani et al. 1986) is its poorability to take up D-glucose accounting for its poor ability to grow on D-glucose as sole source of carbon (Westby et al. 1983; Goebel and Krieg 1984; Martinez-Drets et al. 1984; Singh et al. 2021). In bacteria, the transport of D-glucose or D-fructose is mediated via phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase systems (PTS), which consist of three main components: enzyme I (EI), histidine-phosphorylatable phosphoryl carrier protein (HPr), and enzyme 2 (EII). The EII often consists of three domains (EIIA, EIIB, and EIIC), which may be fused in a single polypeptide chain or may exist as two or three interactive chains. In the case of glucose-PTS, a phosphoryl group is first transferred from PEP to EI, which then transfers the phosphoryl group to HPr (Deutscher et al. 2014). The phosphoryl group is then sequentially transferred from phospho-HPr to EIIA, EIIB, and then finally to D-glucose to form glucose-6-phosphate, which is then transported inside the cell and acted upon by glucose-6-phosphate dehydrogenase to start the pentose phosphate pathway. Thus, to uptake and metabolize D-glucose, bacterial cells require a D-glucose PTS and glucose-6 phosphate dehydrogenase.

A. brasilense is a very efficient colonizer of the rhizosphere of a large number of legume and non-legume plants. It exhibits a positive chemotaxis towards a large number of organic compounds such as amino acids, saccharides, and organic acids, typical for plant roots exudates (Reinhold et al. 1985). Malate, succinate, and D-fructose are the most effective attractants for *A. brasilense* (Zhulin et al. 1988; Singh et al. 2019). It can survive, grow, and compete with other microbes of the rhizosphere, attach to the host root surfaces, promote plant growth via phytohormone production, and can cope with several abiotic stresses encountered in the rhizosphere (Dobbelaere et al. 2003). Due to its strong chemotaxis towards and proficiency in the utilization of dicarboxylates, *A. brasilense* is a vigorous colonizer of C4 plants (Zhulin et al. 1988; Reinhold et al. 1985; Singh et al. 2019).

Rice is the most widely consumed staple food for a large part of the global population. D-glucose is the most prominent component of the root exudates of rice seedlings constituting over 90% of the total carbohydrates (Bacilio-Jiménez et al. 2003). But, *A. brasilense* does not show any notable chemotaxis towards rice root exudates (Bacilio-Jiménez et al. 2001) due to its poor ability to utilize D-glucose, and hence it faces a limitation to effectively colonize rice roots (Calvo et al. 2014; Yang et al. 2009). The endophytic bacteria of rice, *Corynebacterium flavescens* and *Bacillus pumilus*, however, show significant chemotaxis towards rice root exudates (Bacilio-Jiménez et al. 2001). These differential responses can be due to their ability to utilize D-glucose. The composition and concentration of sugars and amino acids from rice root exudates provides a clear advantage to the bacteria that utilize D-glucose over *A. brasilense* allowing them to compete better than *A. brasilense* (Bacilio-Jiménez et al. 2001; Hozore and Alexander 1991). These studies suggest that *A. brasilense* could compete and colonize well in the rice rhizosphere if it acquires the ability to utilize D-glucose. In this study, we identified *A. lipoferum* genes that are involved in the uptake and utilization of D-glucose, transferred them into *A. brasilense*, evaluated the ability of the engineered strain to utilize D-glucose and colonize rice roots, and eventually showed that the engineered strain showed improved utilization of D-glucose and enhanced colonization of the roots of rice seedlings.

Materials and methods

Bacterial strains, plasmids, and chemicals

Bacterial strains and plasmids used in this study are described in Table 1. *A. brasilense* Sp7 was grown and maintained in minimal medium (MM) supplemented with malate (40 mM) as sole carbon source (Vanstockem et al. 1987) while *A. lipoferum* 4B was grown in biotin supplemented low phosphate minimal medium (Goebel and Krieg 1984) and maintained in low salt Luria–Bertani (LB) agar plates which contained only 5 g/L NaCl at 30 °C. For experiments, late log phase cultures of *A. brasilense* Sp7 and *A. lipoferum* 4B, grown overnight in low salt LB broth, were pelleted by centrifugation at 5000 rpm for 5 min, washed with 0.85% saline. The ability to utilize D-glucose was monitored by measuring growth in secondary cultures of different strains inoculated in 30 ml MM supplemented with filter-sterilized

Table 1 Glucose transport and catabolism associated genes in *Azospirillum* spp. and *Pseudomonas aeruginosa*

A. lipoferum 4B Gene ID	Enzyme/protein encoded	*A. brasilense* Sp7 homolog gene ID (% identity/% similarity)	*Pseudomonas aeruginosa* PAO1 homolog gene ID (% identity/% similarity)
AZOLI_p20578	PEP-dependent phosphotransferase system, EI/HPr/EIIA components (EIIA)	AMK58_28220 (73.4/81)	PA3760 (42/56)
AZOLI_p20577	Fused glucose-specific PTS enzymes: IIB and IIC component (EIICB-Glc)	AMK58_28215 (81/87)	PA3761 (41/60)
AZOLI_p20575	*N*-acetylglucosamine-6-phosphate deacetylase (NagA)	AMK58_10545 (53.4/64)	PA3758 (41/55)
AZOLI_p20574	Phosphatase	Absent	Absent
AZOLI_p20572	*N*-acetyl glucosamine utilization regulator, GntR family (NagR)	AMK58_16695 (35/50)	PA3757 (49/63)
AZOLI_p20571	*N*-acetylmuramic acid 6-phosphate etherase (MurQ)	Absent	Absent
AZOLI_p20570	*N*-acetylglucosamine PTS system EIICBA (NagE)	Absent	PA3761(54/70)
AZOLI_p30596	Glucose-6-phosphate 1-dehydrogenase (G6PD)	Absent	PA3183 (53/68)

D-glucose (40 mM) with an initial OD_{600} value of 0.05. Different *E. coli* strains like DH5α and S17.1 were grown in LB medium at 37 °C. All chemicals used in media preparation and for the experiment were purchased by Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Corporation (St. Louis, USA) or Hi-media (Mumbai, India). Restriction enzymes, DNase, RNases, were purchased from New England Biolabs (NEB) (Ipswich, USA) while Taq DNA polymerase, phusion high fidelity DNA polymerase, real-time (RT)-PCR kit, and T4-DNA ligase were purchased from Thermo Fisher Scientific (Waltham, USA). As per requirements, ampicillin (50 µg/ml), tetracycline (10 µg/ml), and chloramphenicol (20 µg/ml) were added in culture media for the growth of *A. brasilense* Sp7 and its derivatives.

Bioinformatic analysis

Amino acid sequences of the genes associated with D-glucose, D-fructose, and *N*-acetylglucosamine (NAG) transport and their catabolism were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG) database for gene analysis and sequence comparison. Level of sequence identity and similarity were calculated with the help of Basic Local Alignment Search Tool (BlastP) of National Centre for Biotechnology Information (NCBI) and Bioedit software and Transporter Classification Data Base (TCDB). Sequence analysis and construction of genetic maps were performed with the help of vector NTI software (Thermo Fisher Scientific, Waltham, USA) or manually.

Construction of recombinant plasmids for complementation

To enhance the D-glucose utilization ability of *A. brasilense* Sp7, a gene encoding glucose-6-phosphate-1-dehydrogenase (G6PD) (of 1521 bp), and two downstream genes (of 2207 bp) encoding glucosamine-fructose-6-phosphate amino transferase (GFPAT) and *N*-acetylglucosamine-6-phosphate deacetylase (NagA) present in the genome of *A. lipoferum* were amplified by polymerase chain reaction (PCR) with two sets of primers (DF/DR and PDF/PDR, Table 3) and cloned into a broad host range modified pBBR1MCS-2 vector under the control of a constitutive Km promoter (Singh et al. 2019). The G6PD gene was also PCR amplified using another set of primers (DF1/DR1, Table 3) and cloned in another isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible broad host range vector, pMMB206 (Morales et al. 1991; Singh et al. 2017). The resulting recombinant plasmids (pVS1, pVS3, and pVS2) were electroporated or conjugatively mobilized in *A. brasilense* Sp7 and exconjugants selected on minimal agar medium supplemented with D-glucose (40 mM) as sole carbon source. Similarly, three other PCR were carried out. In the first one, the divergently oriented genes encoding EIIA (EI/HPr/EIIA) and EIICB_Glc components (4756 bp) of the D-glucose phosphotransferase system of *A. lipoferum* with their native promoters was amplified using primer pair PG2/PGR. In the second one, the genes encoding EIIA and EIIBC were amplified along with the gene encoding GFPAT (5940 bp) using primer pair PG3/PGR, and in the third, the genes encoding EIIA and EIIBC were amplified along with the genes encoding GFPAT and NagA (7120 bp) using primer pair PG3/PGR and NagA along with EIIA and

EIIBC components (7120 bp) using the primer pair PG1/PGR. The amplicons were cloned in the broad host range vector, pBBR1MCS-3 (Kovach et al. 1995) and the resulting plasmids (pVS5, pVS6 and pVS4) were conjugatively mobilized in *A. brasilense* Sp7 to evaluate their D-glucose utilization ability.

For further enhancement of the D-glucose utilization ability of *A. brasilense* Sp7, open reading frames (ORFs) of GFPAT, NagA, EIICB_Glc, EIIA and G6PD gene along with their Shine Dalgarno (SD) sequences, but lacking transcriptional terminator sequence were amplified with different sets of primers (Table 3) to create recognition sites for the two different restriction endonucleases, and the amplicons were cloned in a modified pBBR1MCS-2 vector. The resulting plasmid (pVS7) was conjugatively mobilized in *A. brasilense* Sp7 and evaluated for D-glucose utilization ability.

Measurement of D-glucose consumption during bacterial growth

D-glucose consumption during bacterial growth was measured using a colorimetric method (DuBois et al. 1956) for measuring pentose and hexose sugars present in culture media with a few modifications. For this, overnight cultures of *A. brasilense* Sp7, *A. brasilense* Sp7 (pVS7), and *A. lipoferum* grown in low salt LB broth were pelleted by centrifugation, and after washing with 0.85% saline, reinoculated at initial 0.05 OD_{600} for growth in D-glucose minimal medium (Vanstockem et al. 1987) as well as in minimal medium containing biotin, low phosphate, and D-glucose (Goebel and Krieg 1984). Bacterial growth was monitored by collecting samples from 12 to 96 h in triplicates for D-glucose estimation. For this, 200 µl D-glucose supplemented medium was mixed with 5 µl phenol (80%) and 500 µl concentrated sulfuric acid in microtubes. Samples were kept in a water bath at 30 °C for 20 min, after which color development was recorded at OD_{490} using a spectrophotometer. For estimation of unknown concentrations of D-glucose, a standard curve was plotted using1, 2, 4, 16, and 32 µg of D-glucose and the slope of the curve was derived.

RT-PCR and analysis of co-transcription

For cotranscription analysis of the EIIBC_Glc gene with GFPT and NagA genes, total RNA was isolated using the TRIzol method from cells of late log phase culture (after 120 h) of *A. lipoferum* 4B grown in minimal D-glucose medium. RNA isolation and cDNA synthesis were performed as described earlier (Singh et al. 2019). After treatment with Turbo DNase (Thermo Fisher Scientific, Waltham, USA) for 1 h at 37 °C, reverse transcriptase PCR was carried out with 200 ng of RNA and EIIBC_Glc specific primer (GS1, Table 2) using a one-step RT-PCR kit (Thermo Fisher Scientific, Waltham, USA). The cycle condition used was 42 °C for 60 min followed by heat inactivation at 72 °C for 10 min. Positive and negative controls were included in RT-PCR for checking primer functionality and DNA contamination. Cotranscription analysis was carried out by specific PCR amplification from cDNA using different sets of primers (Table 3).

Rice root colonization

A. brasilense Sp7 and its derivative *dctP::km* mutant harboring plasmid, pVS7, were genetically tagged with GFP via conjugative mobilization of plasmid pVS8 (Singh et al, 2019). Dehulled seeds of rice (*Oryza sativa* variety TN1) were surface sterilized as described earlier (Singh et al, 2019) with minor modifications. The dehulled seeds were allowed to germinate for 4 days on Whatman filter soaked with Hoagland's medium (Hoagland and Arnon 1950) before shifting them for hydroponic growth in the same medium. Cultures of *gfp*-tagged *A. brasilense* Sp7, *dctP::km* mutant, and its D-glucose utilizing recombinant strains grown in LB broth and containing a total of 10^5 cells per ml were pelleted, washed, and inoculated in each tube 4 days after seedling growth in the Hoagland's medium in the presence of IPTG (0.4 mM). One set of the seedlings was further grown for 10 more days in the controlled plant tissue culture chamber at 24 °C with a 15-h light and 9-h dark regimen without any bacterial inoculation. After 10 days, rice roots inoculated with *gfp*-tagged *A. brasilense* and its D-glucose utilizing recombinant were washed and used for observing the extent of root colonization under a confocal microscope as described earlier (Singh et al. 2019).

To determine the number of colony-forming units (CFU) of the inoculated strains, pieces of root tips from three different hydroponically grown seedlings were cut and their weight determined. The root tips were crushed and homogenized in 300 µl LB broth, serially diluted in the same medium, and spread on LB agar plates supplemented with chloramphenicol (20 µg/ml). After 48 h of incubation at 30 °C in an incubator, the number of colonies on each plate was counted. The experiment was done in triplicate.

Statistical analysis

Experiments on measurement of growth and D-glucose consumption were performed in triplicates in three independent experiments. The mean significant values were determined by using SPSS 17 package software (SSP Inc., Chicago,

Table 2 Bacterial strains and plasmids

Strains or plasmids	Relevant properties	References/Sources
Bacterial strains		
Azospirillum brasilense Sp7	Wild-type strain (ATCC 29145)	(Nur et al. 1981)
Azospirillum lipoferum 4B	Wild-type strain (ATCC 29707)	(Wisniewski-Dyé et al. 2011)
E. coli DH5α	Δ*lacU*169 *hsdR*17 *recA1 endA*1 *gyrA*96 *thiL relA1*	Gibco/BRL
E. coli S.17–1	Smr *recA thi pro hsdR* RP4-2 (Tc::Mu; Km::Tn*7*)	(Simon et al. 1983)
Plasmids		
pMMB206	Broad host range, low copy number, expression vector, Cmr	(Morales et al. 1991)
pBBR1MCS-2	Broad host range, medium copy number, *lacZ* containing promoterless vector, Kmr	(Kovach et al. 1995)
pBBR1MCS-3	Broad host range, medium copy number, *lacZ* containing promoterless vector, Tetr	(Kovach et al. 1995)
pVS1 (**Ga**)	Constitutively expressing G6PD gene of *A. lipoferum* under the control of Km promoter in pBBR1MCS-2	This work
pVS2 (**G**)	G6PD gene of *A. lipoferum* cloned at the *Bam*HI and *Hin*dIII restriction sites of the pMMB206 vector	This work
pVS3 (**GI**)	Constitutively expressed NagA and GFPAT genes of *A. lipoferum* under the control of Km promoter in pBBR1MCS-2	This work
pVS4 (**GII**)	Divergently organized genes encoding EIIA and EIIBC of *A. lipoferum* along with their native promoters cloned at the *Xba*I site of the pBBR1MCS-3 vector	This work
pVS5 (**GIII**)	Divergently organized genes encoding EIIA and EIIBC with downstream gene encoding GFPAT, of *A. lipoferum* cloned at the *Xba*I site of the pBBR1MCS-3 vector	This work
pVS6 (**GIV**)	Divergently organized genes encoding EIIA and EIIBC with two downstream genes encoding NagA and GFPT of *A. lipoferum* cloned at the *Xba*I site of the pBBR1MCS-3 vector	This work
pVS7 (**GV**)	Modified pBBR1MCS-2 vector constitutively expressed transcriptionally fused NagA, GFPAT, Glc-PTS and G6PD genes of *A. lipoferum* under the control of Km promoter	This work
pVS8	*gfp* gene cloned at the *Bam*HI and *Hin*dIII restriction sites of the pMMB206 vector	This work

IL, USA). Analysis of the variance followed by Duncan's multiple comparison test was used to analyze significance between more than two treatments. Differences were considered significant at $P < 0.05$.

Results

Comparison of the genes involved in the uptake of D-glucose in A. brasilense Sp7 and A. lipoferum

Since *A. brasilense* Sp7 shows very poor growth on D-glucose as sole carbon source but *A. lipoferum* 4B grows well on D-glucose, we examined their genomes for the presence of genes encoding enzymes for the uptake and metabolism D-glucose on KEGG. The gene encoding G6PD was present in the genome of *A. lipoferum* (AZOLI_p30596) but absent in *A. brasilense* Sp7 (Table 1). The D-glucose PTS in *A. lipoferum* 4B consists of two divergently organized ORFs (AZOLI_p20577 and AZOLI_p20578) (Fig. 1a), in which AZOLI_p20578 codes for the EI/HPr/EIIA component of the PEP-dependent D-glucose PTS, and AZOLI_p20577 codes for fused glucose-specific PTS enzymes: IIB and IIC components (EIICB-Glc). In *A. brasilense* Sp7 also, two divergently organized ORFs AMK58_28220 and AMK58_28215 encode EIIA and EIICB-Glc components, respectively (Fig. 1a). The EIIA and EIICB proteins of *A. brasilense* Sp7 and *A. lipoferum* 4B showed 73.4% and 81% identity, respectively (Table 1). The EIIBC proteins from both the species were of almost identical size (594 and 595 aa) showing no gaps or deletions. In case of EIIA, however, the *A. lipoferum* 4B protein was of 856 aa while that of *A. brasilense* Sp7 was only 836 aa. Alignment of the deduced amino acid sequence of the two EIIA proteins showed an absence of a stretch of 14 aa at the N-terminus and a deletion of 4 aa stretch in the middle of the EIIA protein of *A. brasilense* Sp7 (Supplemental Fig. S1). In the case of *A. lipoferum* 4B, two other genes (AZOLI_p20575 and AZOLI_p20574) encoding GFPAT and NagA are organized in the same orientation as AZOLI_p20577 (Fig. 1a). Another two related genes further downstream included *murQ* (AZOLI_p20571) and *nagE* (AZOLI_p20570) which

Table 3 Primers used in this study

Primers	Sequence 5' to 3' direction
(a) Primers used for cloning in pBBR1MCS-3 and pMMB206	
PG1	CTAGTCTAGACGAAACTCCCTCACACTAGCTC
PG2	CTAGTCTAGAATGATGGAACCAGTAGAATACCAG
PG3	GCTCTAGATCAGACGGTCTTCGTCACTTTG
PGR	CTAGTCTAGAATTTGTGCGCCTCTGCTCTGC
DF1	CGCGGATCCAGAGGTCCGAAGATGCAACCC
DR1	CCCAAGCTTGAACTTTTTTCACCACCATGACG
GF	CGCGGATCCATGGTGAGCAAGGGCGAG
GR	CCCAAGCTTTTACTTGTACAGCTCGTCCA
(b) Primers used for cloning in modified pBBR1MCS-2 vector for constitutive overexpression	
DF	CCGCTCGAGCATGCAACCCCGCTCCACCG
DR	TCCCCCCGGGCATGACGATTTTTTGAGCGACC
PDF	CCGCTCGAGGATGACAGAGCAGAAGCCGCC
PDR	TCCCCCCGGGGAAACTCCCTCACACTAGCTCG
PAF	CCGCTCGAGCATGACAGAGCAGAAGCCGC
PAR	CCCCCCGGGGGGCCCAAGCTTGGGCACACTAGCTCGCCCGCCAC
PBF	CCCAAGCTTGGGGGACAAATTGCGGGAGAGCC
PBR	CCCCCCGGGGGGGCTCTAGAGCGATGGGACGCGCGCTATTCG
PCF	GCTCTAGAGCAGAATGAGGACCAACCAAACATG
PCR	CCCCCCGGGGGGGAAGATCTTGTCAGATCGCCGGCCACGC
PDF1	GAAGATCTTCTGCCACCGACGCGAGAGGTC
PDR1	CCCCCCGGGGGGGAACTTTTTTCACCACCATGACG
(c) Primers used for co-transcription analysis	
GS1	CAGTTCGGACAGAGG
GS2	CCTCGCGTAGCAGC
BF	CTTCGCCACCACCCGTCTGC
BR	TCGATCAGGTATTTGCCGTAGG
GFF	GTCTATGAACTGGCACTGGC
GFR	CGAAATGGATGCCCAAGACC
CNT	GAAACCGTGGTGTTCGAGGAC

encoded an *N*-acetylmuramic acid 6-phosphate acetylase and an *N*-acetylglucosamine PTS system EIICBA or EIICB component, respectively (Fig. 1a).

A search in the TCDB showed that *A. lipoferum* 4B ORFs AZOLI_p20572, AZOLI_p20574, AZOLI_p20575, AZOLI_p20577, and AZOLI_p20578 were the homologs to PA3757 (NagR), PA3758 (NagA), PA3759 (NagS), PA3760 (NagF), and PA3761 (NagE), respectively, of *Pseudomonas aeruginosa* PAO1 (Table 1 and Fig. 1a) which are involved in the utilization of NAG (Korgaonkar and Whiteley 2011). Although ORFs AZOLI_p20577 and AMK58_28215 were identified as fused glucose-specific PTS enzymes consisting of the components IIB and IIC (EIICB-Glc), AZOLI_p20578 and AMK58_28220, they were identified as NAG phosphotransferase transporters of the Glc family which are known to transport D-glucose, glucosamine, NAG, and a large variety of α- and β-glucosides. Several of these PTS transporters lack their own IIA domains and use D-glucose IIA protein (Saier 2015). Most of these transporters have the B and C domains linked together in a single polypeptide chain. We did not find the homologs of the remaining three genes of NAG utilization gene cluster in the vicinity of AMK58_28215 and AMK58_28220 in *A. brasilense* Sp7. A homolog of NagA, showing 53.4% identity with the NagA of *A. lipoferum* 4B, was found at a different location in the genome of *A. brasilense* Sp7 as AMK58_10545.

Co-transcription analysis of gene encoding EIICB-Glc, GFPAT and NagA

Since the ORFs AZOLI_p20574 (NagA), AZOLI_p20575 (GFPAT), and AZOLI_p20577 (EIICB-Glc) were organized together in the direction opposite to that of AZOLI_p20578 (EIIA) (Fig. 1b). The first nucleotide of the start codon (ATG) of AZOLI_p20574 overlaps with the last

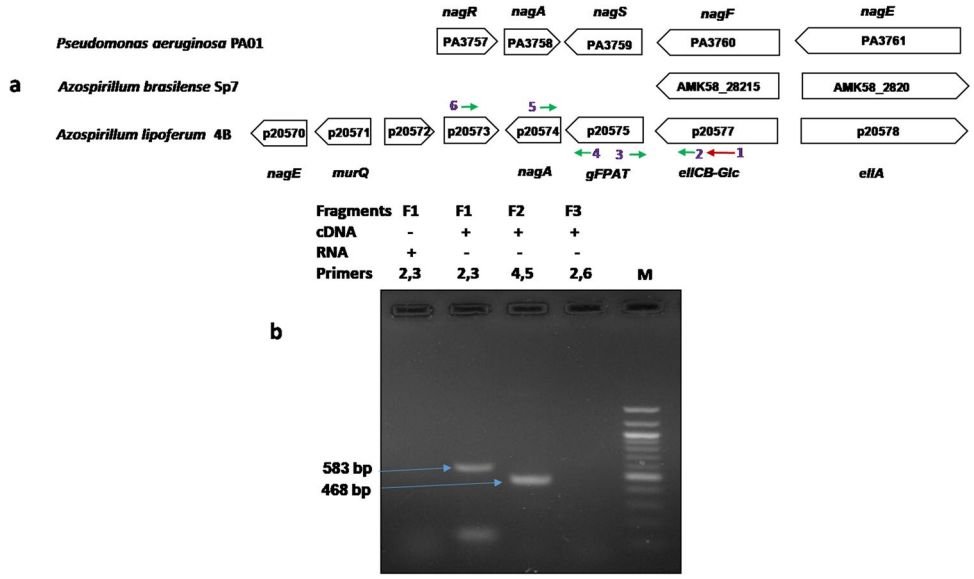

Fig. 1 **a** Organization of the genes encoding EIIA and EIIBC of the glucose phosphotransferase system (PTS) in *Azospirillum lipoferum* 4B, *Azospirillum brasilense* Sp7, and *Pseudomonas aeruginosa* PAO1. Identities of the genes of each bacterium are shown within the arrows depicting orientation of each gene. Identities, similarities, and annotation of the proteins encoded by the genes of *A. lipoferum* 4B with their orthologs in *A. brasilense* Sp7 and *P. aeruginosa* PAO1 are shown in Table 1. **b** Co-transcription analysis of the genes encoding glucose PTS in *A. lipoferum* 4B. The agarose gel shows the result of PCR amplifications. Lane 1: using primer 2 and 3 from purified DNase-treated RNA; Lane II and Lane III with primer pairs 2, 3 and 4, 5 using cDNA as template. Lane IV using primers 2 and 6 with cDNA as template. Primers 1, 2, 3, 4, 5, and 6 are primers GS1, BF, BR, GFF, GFR, and CNT, respectively as listed in Table 3. Lane V shows 100 bp molecular weight marker (NEB). Location of the primers is shown in Fig. 1b above and below the genes of *A. lipoferum* 4B

nucleotide of the stop codon (TGA) of AZOLI_p20575 at the junction of the two genes suggesting co-transcription and even coupled translation of the two genes. The gap of 178 bp between AZOLI_p20577 and AZOLI_p20575, however, raised a doubt whether the genes encoding EIICB-Glc was also co-transcribed with the genes encoding GFPAT and NagA. RT-PCR analysis of the cDNA synthesized by the gene-specific primer GS1 revealed that EIICB-Glc- and GFPAT-specific primers (BCF/BCR) produced an amplicon of 583 bp while GFPAT- and NagA-specific primers (GFPTF/GFPTR) produced as amplicon of 468 bp (Fig. 1a, b). This analysis of the transcripts of EIICB-Glc, GFPAT, and NagA genes of *A. lipoferum* 4B indicated that AZOLI_p20574 (NagA), AZOLI_p20575 (GFPAT), and AZOLI_p20577 (EIICB-Glc) were transcribed together as a single transcriptional unit.

Role of A. lipoferum 4B genes in conferring D-glucose utilization ability to A. brasilense Sp7

Although the genes encoding EIIA and EIIBC of the D-glucose PTS were present in the genomes of both *A. brasilense* Sp7 and *A. lipoferum* 4B, it is only the latter which utilizes efficiently both D-glucose and NAG for its growth. Since G6PD was absent in *A. brasilense* Sp7, we cloned the gene encoding G6PD from *A. lipoferum* 4B in pMMB206 (as G) and in pBBR1MCS-2 (as Ga) (Fig. 2a), expressed it in *A. brasilense* Sp7, but did not observe any improvement in the growth of *A. brasilense* Sp7 on MM plate containing D-glucose as sole carbon source. Since EIIA and EIIBC encoding genes were present in *A. brasilense* Sp7, we decided to examine if the expression of neighboring genes GFPAT and NagA (GI, Fig. 2a) without or with G6PD (G) would improve D-glucose utilization. But, this also failed to improve D-glucose utilization in *A. brasilense* Sp7 (Fig. 2b, c). We then cloned the two genes encoding EIIA and EIICB from *A. lipoferum* 4B (GII, Fig. 2a) and expressed it without and with G6PD (G) in *A. brasilense* Sp7, but still failed to see any improvement in the growth of *A. brasilense* Sp7 on D-glucose (Fig. 2b, c). Next, we cloned two amplicons of DNA regions of *A. lipoferum* 4B, one containing the genes encoding GFPAT, EIIA, and EIICB (GIII) and the other containing the genes encoding NagA, GFPAT, EIIA, and EIICB (GIV)(Fig. 2a), and expressed them without or with G6PD (G). In the case of GIII without or with G, we did not find any improvement in D-glucose utilization (Fig. 2b, c). However, in the case of GIV, we could see a conspicuous improvement in the growth of *A. brasilense* Sp7 on D-glucose as a sole source of carbon (Fig. 2b), which showed further enhancement when G was also expressed along with GIV (Fig. 2b, c). These observations indicated that G6PD,

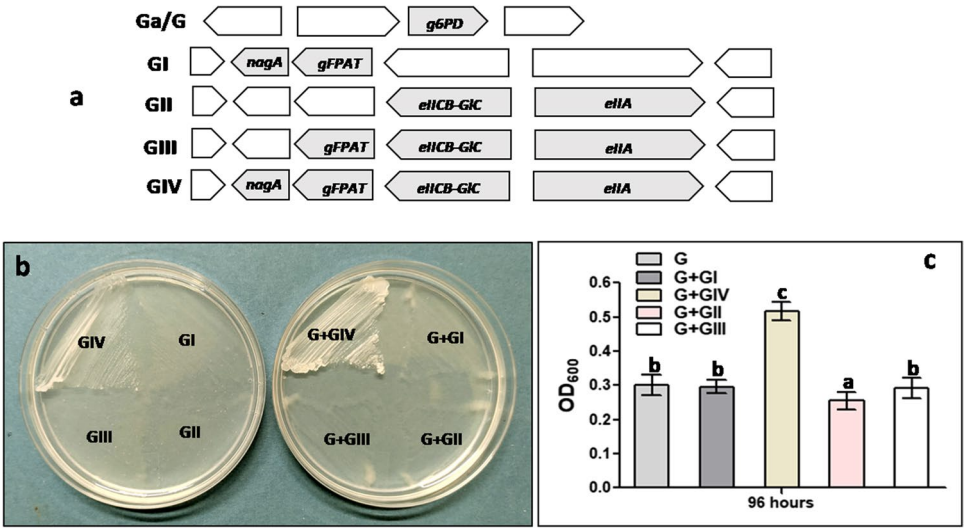

Fig. 2 **a** Representation of the set of genes of *A. lipoferum* 4B that were cloned in different recombinant plasmids constructed to test the genes required for glucose utilization in *A. brasilense* Sp7. Recombinant plasmids containing G6PD in pMMB206 and pBBR1MCS-2 were designated as G (pVS1) and G1 (pVS2), respectively. Recombinant plasmids harboring genes encoding NagA + GFPAT, EIIA + EIIBC, GFPAT + EIIBC + EIIA, and NagA + GFPAT + EIIBC + EIIA were designated as GI (pVS3), GII (pVS4), GIII (pVS5), and GIV (pVS6), respectively. The genes cloned in each recombinant plasmid are highlighted in light grey. **b** Comparison of the growth of *A. brasilense* Sp7 harboring GI, GII, GIII, and GIII without (plate on left) or with G (plate on right) on minimal medium agar plates containing glucose as sole carbon source. **c** Growth measured as optical density of the cultures of *A. brasilense* Sp7 harboring G + GI, G + GII, G + GIII, and G + GIII without (plate on left) or with G (plate on right) in minimal medium containing glucose as sole carbon source after 96 h

EIIA, EIICB, GFPAT, and NagA were required for conferring D-glucose utilization ability to *A. brasilense* Sp7.

After finding that the genes encoding G6PD, EIIA, EIICB, GFPAT, and NagA enhanced the ability of *A. brasilense* Sp7 to utilize D-glucose when they were expressed with their native promoters, we asked whether these genes will further enhance D-glucose utilization ability if all the 5 genes were put together as a transcriptional unit under a constitutively expressed promoter. For this, we constructed a D-glucose utilization cassette (GV) by cloning all the five genes (in the sequence NagA, GFPAT, EIIA, EIICB, and G6PD) downstream of a constitutive promoter of the kanamycin (Km) resistance gene (Fig. 3a), and found that it further improved the D-glucose utilization ability of *A. brasilense* Sp7.

When we compared the growth of *A. brasilense* Sp7 (GV) strain with its parent in the minimal medium having high phosphate and D-glucose (as sole carbon source) but lacking biotin, the growth of *A. brasilense* Sp7 (GV) was considerably better than that of *A. brasilense* Sp7 (Fig. 3b, c). In this medium, *A. brasilense* Sp7 (GV) grew considerably faster than *A. lipoferum* 4B (Fig. 3b, c), and consumed significantly more glucose than *A. lipoferum* 4B (Fig. 3d). A comparison of the growth of *A. brasilense* Sp7 (G + GIV) with that of *A. brasilense* Sp7 (GV) showed that the growth of *A. brasilense* Sp7 (GV) was twice that of *A. brasilense* Sp7 (G + GIV) (Figs. 2 and 3). This difference is expected to be due to the superiority of the constitutive expression of the 5 genes under the kanamycin promoter over the expression of GIV genes with their native promoters.

Since *A. lipoferum* 4B requires low phosphate and biotin to grow optimally on glucose as carbon source, we compared the growth of *A. brasilense* Sp7 (GV) with its parent and with *A. lipoferum* 4B in the low phosphate medium supplemented with biotin. In this medium, *A. lipoferum* 4B grew better than *A. brasilense* Sp7 (GV) (Fig. 3e, f). Analysis of the D-glucose in the spent medium after 96 h showed that *A. lipoferum* 4B consumed more glucose than *A. brasilense* Sp7 (GV) (Fig. 3g).

Evaluation of the ability of engineered A. brasilense Sp7 to colonize rice roots

After ascertaining that *A. brasilense* Sp7 (GV) grows well on D-glucose as sole carbon source, we investigated its ability to colonize rice roots (Fig. 4). For this, we genetically tagged *A. brasilense* Sp7, a *dctP::km* mutant of *A. brasilense* Sp7, and *A. brasilense* Sp7 harboring GV with green fluorescent protein (GFP) (Singh et al. 2019). The *dctP::km* mutant of *A. brasilense* Sp7 is compromised in the uptake of dicarboxylates and hence colonizes the roots of a C4 plant poorly (Singh et al. 2019). We compared colonization of the roots of the rice seedlings by different

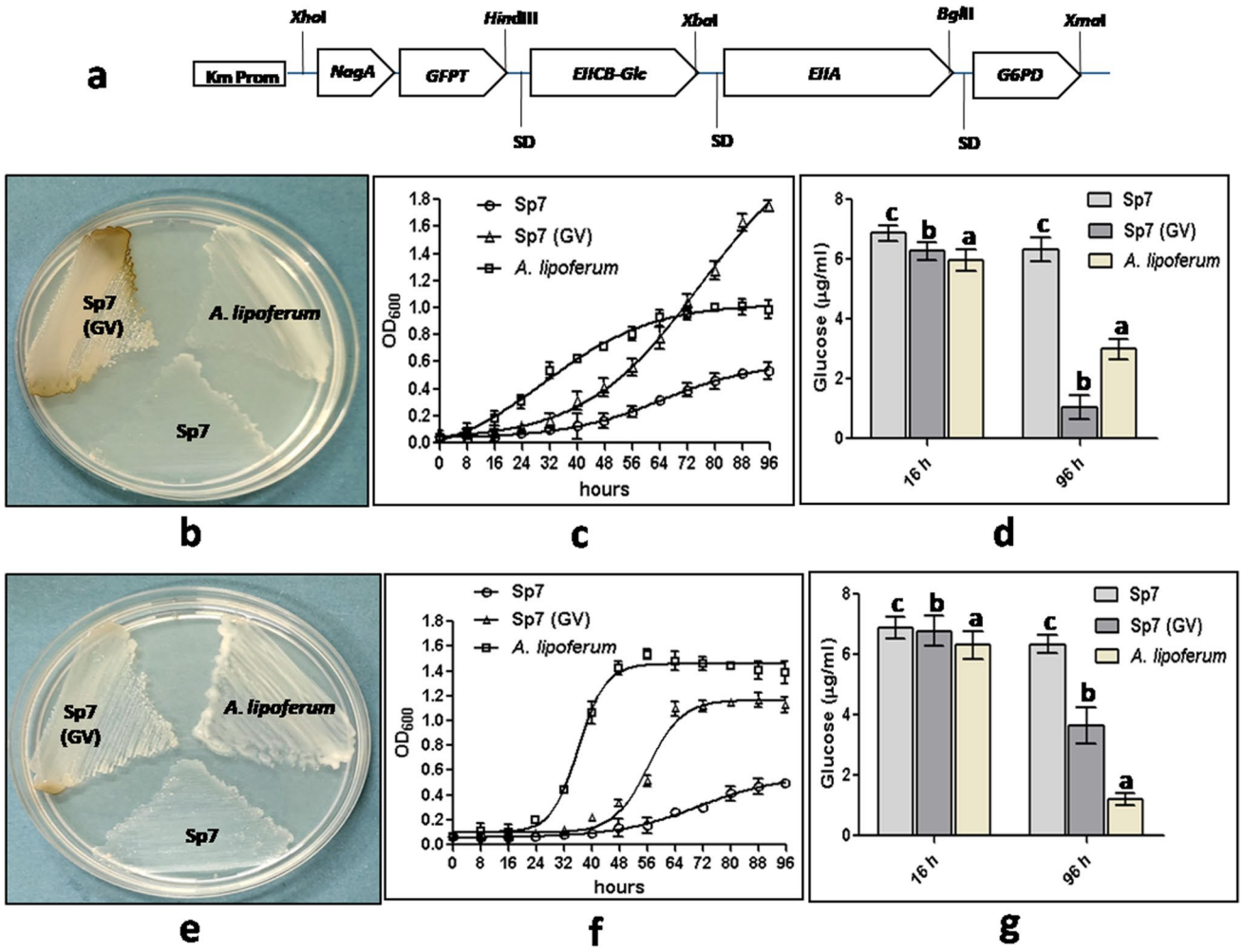

Fig. 3 **a** Organization of the genes encoding NagA, GFPAT, EIIBC, EIIA, and G6PD on GV (pVS7). Comparison of the growth of *A. lipoferum* 4B, *A. brasilense* Sp7, and *A. brasilense* Sp7 (GV) on agar plate (**b**, **c**) and liquid medium (**d**, **e**) and glucose utilization (**d**, **g**) in minimal medium containing glucose as sole carbon source with high phosphate lacking biotin (**b**, **c**, **d**) and low phosphate plus biotin (**e**, **f**, **g**)

GFP-tagged strains of *A. brasilense* Sp7 by confocal laser scanning microscopy 12 days after inoculation (Fig. 4). *A. brasilense* Sp7 showed some colonization due to its ability to utilize the limited amount of dicarboxylates present in the root exudates of rice seedlings, but the *dctP::km* mutant *A. brasilense* Sp7 showed very poor colonization. The *A. brasilense* Sp7 (GV), however, showed profuse intercellular colonization due probably to its ability to utilize the abundant amount of D-glucose exuded by the roots of rice seedlings. A large number of intense green fluorescing cell aggregates or clumps scattered on the subapical region of the roots inoculated with GFP-tagged *A. brasilense* Sp7 (GV) suggested that the engineered strain with its D-glucose utilizing ability colonized the roots of rice seedlings much better than its parent.

A quantitative assessment of the rice root colonization (Fig. 5) showed that the CFU isolated from the rice roots inoculated with *A. brasilense* Sp7 (GV) showed about tenfold increase in comparison to those inoculated with *A. brasilense* Sp7. This difference in CFU was more pronounced in case of *dctP::km* mutant. While the CFU from the roots inoculated with of *dctP::km* mutant declined from 10^5 to 10^2, the CFU from the roots inoculated with *dctP::km* (GV) were at par with those inoculated with *A. brasilense* Sp7 (GV).

Discussion

In this study, we have shown that the genes involved in D-glucose utilization in *A. lipoferum* 4B can improve the ability of *A. brasilense* Sp7 to utilize D-glucose and to colonize the roots of rice seedlings better. Earlier studies on the comparison of *A. brasilense* Sp7 and *A. lipoferum* 4B on their ability to utilize carbon compounds for growth showed

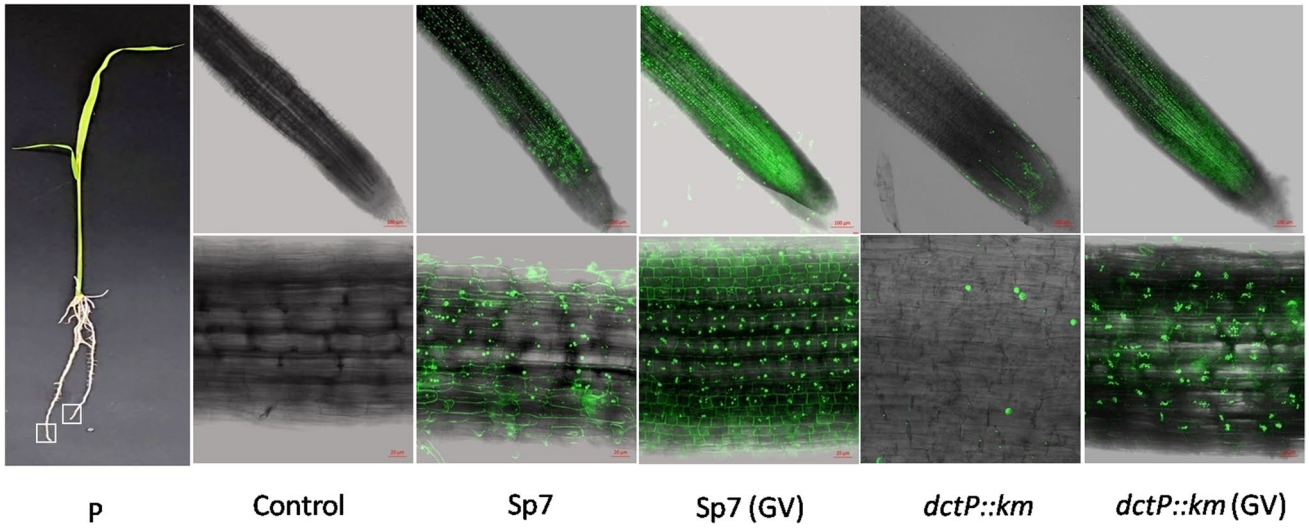

Fig. 4 Confocal laser scanning microscopic images (10× and 40×) of surface sterilized apical and sub-apical regions (shown in boxes, Fig. P) of the roots of *Oryza sativa* 10 days after inoculation with *gfp*-tagged *A. brasilense* Sp7, *dctP::km* mutant of *A. brasilense* Sp7, *dctP::km* (GV), and *A. brasilense* Sp7 (GV). Roots showing green fluorescence show roots colonization by the inoculated strains. However, the absence of green fluorescence on uninoculated control roots shows absence of background green fluorescence in the roots

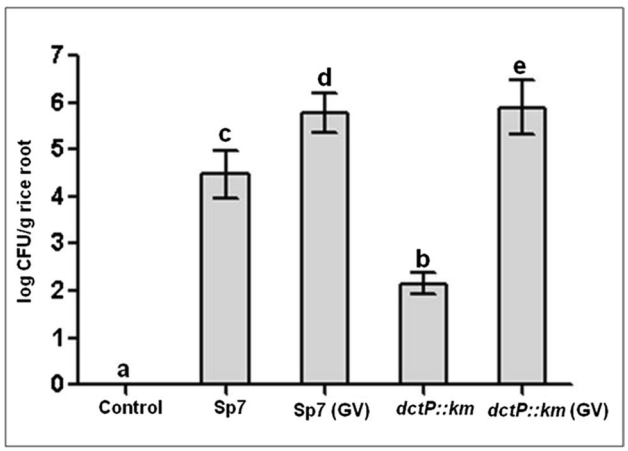

Fig. 5 Quantification of colonization of *A. brasilense* Sp7, *dctP::km*, *dctP::km* (GV), and *A. brasilense* Sp7 (GV) as colony-forming units (CFU) obtained from the roots 10 days after inoculation which were inoculated with $10.^5$ cells 4 days after seedling growth in liquid medium. Mean ± standard deviation of triplicates from three experiments are indicated, and differences between the 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)

that *A. lipoferum* 4B utilizes both D-glucose and D-glucosamine well for its growth but *A. brasilense* Sp7 fails to do so (Goebel and Krieg 1984; Loh et al. 1984; Martinez-Drets et al. 1984). This inference was based on the comparison of the growth on different carbon sources including malate or succinate on which *A. brasilense* Sp7 grows very rapidly. In these studies, which involved prolonged incubation of bacterial strains on minimal medium agar plates, the authors showed that *A. brasilense* Sp7 grows very poorly on D-glucose as carbon source. The previously observed inability of *A. brasilense* Sp7 to use D-glucose as a sole carbon source, and the absence of hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphofructokinase had indicated towards an absence of the catabolic Embden-Meyerhof-Parnas pathway and a hexose monophosphate pathway in *A. brasilense* Sp7 (Westby et al. 1983; Goebel and Krieg 1984; Martinez-Drets et al. 1984). But, some strains of *A. brasilense* were reported to grow very slowly on D-glucose (Tarrand et al. 1978). *A. brasilense* Sp7 was shown to utilize about 5% of the available D-glucose, and its rate of oxygen consumption on D-glucose was about one-quarter of that on succinate as carbon source (Goebel and Krieg 1984; Loh et al. 1984). These reports suggest that D-glucose utilization ability may not be entirely absent in *A. brasilense* but very inefficient in comparison to other carbon sources such as dicarboxylates, D-fructose, glycerol etc.

D-glucose represents over 90% of the carbohydrates in the root exudates of rice seedlings, indicating that this sugar is the main source of carbon in the rhizosphere of young rice plants (Bacilio-Jiménez et al. 2003). In view of the abundance of D-glucose in rice root exudates, and the poor ability of *A. brasilense* Sp7 to utilize D-glucose, we set out to engineer the D-glucose utilization ability in *A. brasilense* Sp7 to improve its ability to colonize rice rhizosphere. The prima facie analysis of the genome of *A. brasilense* Sp7 indicated that it lacks only G6PD. Hence, we hypothesized that cloning and expression of the *A. lipoferum* 4B gene encoding G6PD on a broad host range expression vector should be

sufficient to confer D-glucose utilization ability to *A. brasilense* Sp7. But, transfer of G6PD was not enough for improving its D-glucose utilization ability. Although the deduced amino acid sequence of the EIIBC proteins in both the species was highly similar, EIIA protein showed a truncation of 14 aa at the N-terminus and a deletion of 4 aa stretch in case of *A. brasilense* Sp7 (Supplemental Fig S1) that the EIIA protein of *A. brasilense* Sp7 may not be functional. This may be one of the reasons why the transfer of EIIA and EIIBC together with G6PD from *A. lipoferum* 4B did not confer D-glucose utilization ability to *A. brasilense* Sp7.

Further, the genes encoding the EIIA and EIIBC components of the PTS appear to be the part of the gene cluster involved in the utilization of NAG in *A. lipoferum* 4B as found in *P. aeruginosa* PAO1 (Fig. 1). A similar PTS is likely to be involved in the utilization of D-glucose in *A. lipoferum* 4B as we did not find any other PTS dedicated for D-glucose transport in its genome. Usually, NAG is transported via a NagE transporter, phosphorylated, and then deacetylated by *N*-acetylglucosamine-6-phosphate deacetylase (NagA) to produce glucosamine-6-phosphate, which is then converted into fructose-6-phosphate by glucosamine-6-phosphate deaminase (NagB) or glucosamine-fructose-6-phosphate aminotransferase (GFPT) (Moye et al. 2014; Boulanger et al. 2010; Gaugué et al. 2013; Korgaonkar and Whiteley 2011). The close vicinity of the genes encoding proteins responsible for the transport and catabolism of NAG to the genes encoding components of the D-glucose PTS, and co-transcription of the genes encoding EIICB-Glc, GFPAT and NagA suggests some unknown connection between D-glucose and NAG utilization in *A. lipoferum* 4B. The essentiality of the genes involved in NAG (or GlcNAc) utilization for the utilization of D-glucose suggests that D-glucose transport in *A. lipoferum* 4B may be mediated via a PTS that is used for the transport of NAG.

After ensuring that the engineered strain of *A. brasilense* Sp7 grows well on D-glucose as sole carbon source, we evaluated the ability of the engineered strain to colonize rice roots. Although some dicarboxylates might be available in the rice rhizosphere to sustain the survival of *A. brasilense* Sp7, it may not be enough to compete with other rhizosphere bacteria having the ability to efficiently utilize D-glucose for their growth (Bacilio-Jiménez et al. 2003). When colonization of the roots of the rice seedlings by the engineered strain of *A. brasilense* Sp7 was compared with its parent, both the strains showed colonization. But, the engineered strain showed clumps or aggregates of cells at many places indicating that the engineered strain colonized the roots of rice seedlings much better than its parent. To minimize root colonization due to the ability of *A. brasilense* Sp7 to utilize dicarboxylates, we compared root colonization by a *dctP::km* mutant of *A. brasilense* Sp7 (Singh et al. 2019), which was defective in the major transporter of dicarboxylates. The engineered derivative of the *dctP::km* mutant of *A. brasilense* Sp7 showed considerably higher root colonization than the *dctP::km* mutant. Recovery of 10-–15-fold higher CFU of the engineered derivative of *A. brasilense* Sp7 over its parental strains *A. brasilense* Sp7, and about 10^3–10^4 fold higher CFU of *dctP::km* mutant (GV) over *dctP::km* mutant clearly showed that strains carrying D-glucose utilization cassette showed improved root colonization than their parents. The ability of the engineered strain of *A. brasilense* Sp7 to utilize D-glucose in low as well as high phosphate medium with or without biotin indicated that it can survive and multiply in the rhizospheres that might be low in biotin and high in phosphate.

The ability of bacteria to utilize the ingredients of root exudates plays a critical role in root colonization (Bacilio-Jiménez et al. 2003; Cambell and Greves 1990; Futamata et al. 1998; Lynch and Whipps 1990). The association between plants and microbial communities living in the rhizosphere can be improved by manipulating plants to release certain chemicals or nutrients in their root exudates, and by enhancing the ability of rhizobacteria to utilize them. The establishment of a trophic link between PGPR and the plant is considered a sound approach to favor the maintenance of a sufficient microbial inoculum in the plant root system. Although PGPRs are frequently used as bioinoculants, there are only a few reports on their engineering to make them more effective root colonizers. Earlier attempts in this direction have shown that the expression of a heterologous gene encoding a siderophore receptor improved the competitiveness of *Pseudomonas fluorescens* in soil (Raaijmakers et al. 1995), and a heterologous gene encoding chitinase into the *Burkholderia vietnamiensis* led to a significantly enhanced suppression of diseases such as wheat sheath blight, cotton *Fusarium* wilt, and tomato grey mold (Zhang et al. 2012). Similarly, the expression of genes encoding proline dehydrogenase (Van Dillewijn et al. 2001), or 1-aminocyclopropane-1-carboxylate deaminase (Ma et al. 2004) increased the ability of *Sinorhizobium meliloti* to nodulate legumes; and a gene encoding trehalose-6-phosphate synthase improved the ability of *Rhizobium etli* to nodulate *Phaseolus vulgaris* (Suárez et al. 2008). Since improved root colonization is often a prerequisite to improve plant growth by any PGPR, the enhanced ability of *A. brasilense* Sp7 to colonize rice roots due to its improved ability to utilized D-glucose may lead to an improved plant growth in case of rice as well as other plants which exude relatively higher amounts of D-glucose in their root exudates.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00253-022-12250-0.

Author contribution AKT conceived the study and wrote the paper. VSS, BKD, SR and SPS performed research and analyzed the data.

Funding This work was supported by a grant from Indian Council of Agricultural Research (ICAR), New Delhi and J C Bose National Fellowship (SERB) to AKT. VSS acknowledges support of Senior Research Associateship (SRA) from the Council of Scientific and Industrial Research (CSIR), New Delhi. We thank CSIR-CIMAP for confocal microscopy facility.

Data availability The datasets used and/or analyzed during the current study are available from corresponding author on reasonable request.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

References

Bacilio-Jiménez M, Aguilar-Flores S, del Valle MV, Pérez A, Zepeda A, Zenteno E (2001) Endophytic bacteria in rice seeds inhibit early colonization of roots by *Azospirillum brasilense*. Soil Biol Biochem 33:167–172. https://doi.org/10.1016/s0038-0717(00)00126-7

Bacilio-Jiménez M, Aguilar-Flores S, Ventura-Zapata E, Pérez-Campos E, Bouquelet S, Zenteno E (2003) Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. Plant Soil 249:271–277. https://doi.org/10.1023/a:1022888900465

Bais HP, Loyola-Vargas VM, Flores HE, Vivanco JM (2001) Root-specific metabolism: the biology and biochemistry of underground organs. In Vitro Cell Dev Biol – Plant lant 37:730–741. https://doi.org/10.1007/s11627-001-0122-y

Baldani JI, Baldani VL, Seldin L, Döbereiner J (1986) Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. Int J Syst Evol Microbiol 36:86–93. https://doi.org/10.1099/00207713-36-1-86

Barriuso J, Ramos Solano B, Fray RG, Cámara M, Hartmann A, Gutiérrez Mañero FJ (2008) Transgenic tomato plants alter quorum sensing in plant growth-promoting rhizobacteria. Plant Biotechnol J 6:442–452. https://doi.org/10.1111/j.1467-7652.2008.00331.x

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez JP (2014) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant Soil 378:1–33. https://doi.org/10.1007/s11104-013-1956-x

Bhattacharyya PN, Jha DK (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350. https://doi.org/10.1007/s11274-011-0979-9.

Boulanger A, Déjean G, Lautier M, Glories M, Zischek C, Arlat M, Lauber E (2010) Identification and regulation of the *N*-acetyl glucosamine utilization pathway of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*. J Bacteriol 192:1487–1497. https://doi.org/10.1128/jb.01418-09

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant Soil 383:3–41. https://doi.org/10.1007/s11104-014-2131-8

Cambell R, Greves M (1990) Anatomy and communitystructure of the rhizosphere. In: Lynch JM (ed) The rhizosphere. Wiley & Sons, Chichester, pp 11–34

Cassán F, Coniglio A, López G, Molina R, Nievas S, de Carlan C, Donadio F, Torres D, Rosas S, OliveraPedrosa F, de Souza E, DíazZorita M, de-Bashan L, Mora V (2020) Everything you must know about *Azospirillum* and its impact on agriculture and beyond. Biol Fertil Soils 56:461–479. https://doi.org/10.1007/s00374-020-01463-y

Deshmukh Y, KhareP PD (2016) Rhizobacteria elevate principal basmati aroma compound accumulation in rice variety. Rhizosphere 1:53–57. https://doi.org/10.1016/j.rhisph.2016.07.001

Deutscher J, Aké FM, Derkaoui M, Zébré AC, Cao TN, Bouraoui H, Kentache T, Mokhtari A, Milohanic E, Joyet P (2014) The bacterial phosphoenolpyruvate: carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. Microbiol Mol Biol Rev 78:231–256. https://doi.org/10.1128/mmbr.00001-14

Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Vanderleyden J, Dutto P, Okon Y (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Funct Plant Biol 28:871–879. https://doi.org/10.1071/pp01074

Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit Rev Plant Sci 22:107–149. https://doi.org/10.1080/713610853

DuBois M, Gilles KA, Hamilton JK, Rebers PA, Fred S (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356. https://doi.org/10.1021/ac60111a017

Estabrook EM, Yoder JI (1998) Plant-plant communications: rhizosphere signaling between parasitic angiosperms and their hosts. Plant Physiol 116:1–7. https://doi.org/10.1104/pp.116.1.1

Etesami H, Alikhani HA (2016) Co-inoculation with endophytic and rhizosphere bacteria allows reduced application rates of N-fertilizer for rice plant. Rhizosphere 2:5–12. https://doi.org/10.1016/j.rhisph.2016.09.003

Fukami J, Cerezini P, Hungria M (2018) *Azospirillum*: benefits that go far beyond biological nitrogen fixation. AMB Express 8:73. https://doi.org/10.1186/s13568-018-0608-1

Futamata H, Sakai M, Ozawa H, Urashima Y, Sueguchi T, Matsuguchi T (1998) Chemotactic response to amino acids of fluorescent pseudomonads isolated from spinach roots grown in soils with different salinity levels. Soil Sci Plant Nutr 44:1–7. https://doi.org/10.1080/00380768.1998.10414421

Gaugué I, Oberto J, Putzer H, Plumbridge J (2013) The use of amino sugars by *Bacillus subtilis*: presence of a unique operon for the catabolism of glucosamine. PLoS ONE 8(5):e63025. https://doi.org/10.1371/journal.pone.0063025

Goebel EM, Krieg NR (1984) D-Fructose catabolism in *Azospirillum brasilense* and *Azospirillum lipoferum*. J Bacteriol 159:86–92. https://doi.org/10.1128/jb.159.1.86-92.1984

Hoagland DR, Arnon DI (1950) The water-culture methodfor growing plants without soil. Circ. No. 347, CaliforniaAgricultural Experiment Station

Hozore E, Alexander M (1991) Bacterial characteristics important to rhizosphere competence. Soil Biol Biochem 23:717–723. https://doi.org/10.1016/0038-0717(91)90140-f

Kloepper JW, Schroth MN (1981) Plant growth-promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathology 71:642–644. https://doi.org/10.1094/phyto-71-642

Korgaonkar AK, Whiteley M (2011) *Pseudomonas aeruginosa* enhances production of an antimicrobial in response to *N*-acetylglucosamine and peptidoglycan. J Bacteriol 193:909–917. https://doi.org/10.1128/jb.01175-10

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM II, Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175–176. https://doi.org/10.1016/0378-1119(95)00584-1

Loh WH, Randles CI, Sharp WR, Miller RH (1984) Intermediary carbon metabolism of *Azospirillum brasilense*. J Bacteriol 158:264–268. https://doi.org/10.1128/jb.158.1.264-268.1984

Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. Plant Soil 129:1–10. https://doi.org/10.1007/bf00011685

Ma W, Charles TC, Glick BR (2004) Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Sinorhizobium meliloti* increases its ability to nodulate alfalfa. Appl Environ Microbiol 70:5891–5897. https://doi.org/10.1128/aem.70.10.5891-5897.2004

Martinez-Drets G, Del Gallo M, Burpee C, Burris RH (1984) Catabolism of carbohydrates and organic acids and expression of nitrogenase by azospirilla. J Bacteriol 159:80–85. https://doi.org/10.1128/jb.159.1.80-85.1984

Morales VM, Bäckman A, Bagdasarian M (1991) A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 97:39–47. https://doi.org/10.1016/0378-1119(91)90007-x

Moye ZD, Zeng L, Burne RA (2014) Fueling the caries process: carbohydrate metabolism and gene regulation by *Streptococcus mutans*. J Oral Microbiol 6:24878. https://doi.org/10.3402/jom.v6.24878

Nur I, Steinitz YL, Okon Y, Henis Y (1981) Carotenoid composition and function in nitrogen-fixing bacteria of the genus *Azospirillum*. J Gen Microbiol 122:27–32. https://doi.org/10.1099/00221287-122-1-27

Okon Y, Labandera-Gonzalez CA (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol Biochem 26:1591–1601. https://doi.org/10.1016/0038-0717(94)90311-5

Raaijmakers JM, Sluis LVD, Bakker PA, Schippers B, Koster M, Weisbeek PJ (1995) Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp. Can J Microbiol 41:126–135. https://doi.org/10.1139/m95-017

Reinhold B, Hurek T, Fendrik I (1985) Strain-specific chemotaxis of *Azospirillum* spp. J Bacteriol 162:190–195. https://doi.org/10.1128/jb.162.1.190-195.1985

Saier MHJ (2015) The bacterial phosphotransferase system: new frontiers 50 years after its discovery. J Mol Microbiol Biotechnol 25:73–78. https://doi.org/10.1159/000381215

Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda MC, Glick BR (2016) Plant growth-promoting bacterial endophytes. Microbiol Res 183:92–99. https://doi.org/10.1016/j.micres.2015.11.008

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nat Biotechnol 1:784–791. https://doi.org/10.1038/nbt1183-784

Singh VS, Dubey AP, Gupta A, Singh S, Singh BN, Tripathi AK (2017) Regulation of a glycerol-induced quinoprotein alcohol dehydrogenase by σ54 and a LuxR-type regulator in *Azospirillum brasilense* Sp7. J Bacteriol 13(199):e00035-e117. https://doi.org/10.1128/jb.00035-17

Singh VS, Tripathi P, Pandey P, Singh DN, Dubey BK, Singh C, Singh SP, Pandey R, Tripathi AK (2019) Dicarboxylate transporters of *Azospirillum brasilense* Sp7 play an important role in the colonization of finger millet (*Eleusine coracana*) roots. Mol Plant Microbe Interact 32:828–840. https://doi.org/10.1094/mpmi-12-18-0344-r

Singh VS, Dubey BK, Pandey P, Rai S, Tripathi AK (2021) Cometabolism of ethanol in *Azospirillum brasilense* Sp7 is mediated by fructose and glycerol and regulated negatively by an alternative sigma factor RpoH2. J Bacteriol 27(203):e00269-e321. https://doi.org/10.1128/jb.00269-21

Steenhoudt O, Vanderleyden J (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24:487–506. https://doi.org/10.1111/j.1574-6976.2000.tb00552.x

Suárez R, Wong A, Ramírez M, Barraza A, Orozco MDC, Cevallos MA, Lara M, Hernández G, Iturriaga G (2008) Improvement of drought tolerance and grain yield in common bean by overexpressing trehalose-6-phosphate synthase in rhizobia. Mol Plant Microbe Interact 21:958–966. https://doi.org/10.1094/mpmi-21-7-0958

Tarrand JJ, Krieg NR, Döbereiner J (1978) A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can J Microbiol 24:967–980. https://doi.org/10.1139/m78-160

Van Dillewijn P, Soto MJ, Villadas PJ, Toro N (2001) Construction and environmental release of a *Sinorhizobium meliloti* strain genetically modified to be more competitive for Alfalfa nodulation. Appl Environ Microbiol 67:3860–3865. https://doi.org/10.1128/aem.67.9.3860-3865.2001

Vanstockem M, Michiels K, VanderleydenJ VAP (1987) Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn*5* and Tn*5*-mob insertion mutants. Appl Environ Microbiol 53:410–415. https://doi.org/10.1128/aem.53.2.410-415.1987

Westby CA, Cutshall DS, Vigil GV (1983) Metabolism of various carbon sources by *Azospirillum brasilense*. J Bacteriol 156:1369–1372. https://doi.org/10.1128/jb.156.3.1369-1372.1983

Wisniewski-Dyé F, Borziak K, Khalsa-Moyers G, Alexandre G, Sukharnikov LO, Wuichet K, Hurst GB, McDonald WH, Robertson JS, Barbe V, Calteau A (2011) *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. PLoS Genet 7:e1002430. https://doi.org/10.1371/journal.pgen.1002430

Yang J, Kloepper JW, Ryu CM (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Sci 14:1–4. https://doi.org/10.1016/j.tplants.2008.10.004

Zhang X, Huang Y, Harvey PR, Ren Y, Zhang G, Zhou H, Yang H (2012) Enhancing plant disease suppression by *Burkholderia vietnamiensis* through chromosomal integration of *Bacillus subtilis* chitinase gene *chi113*. Biotechnol Lett 34:287–293. https://doi.org/10.1007/s10529-011-0760-z

Zhulin IB, Tretyakova SE, Ignatov VV (1988) Chemotaxis of *Azospirillum brasilense* towards compounds typical of plant root exudates. Folia Microbiol 33:277–280. https://doi.org/10.1007/bf02925621