Modulation of PQQ-dependent glucose dehydrogenase (mGDH and sGDH) activity by succinate in phosphate solubilizing plant growth promoting *Acinetobacter* sp. SK2

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Abstract

Prospective plant growth promoting rhizobacteria isolated from the rhizosphere of *Vigna radiata* was identified as *Acinetobacter* sp. SK2 that solubilized 682 $\mu\text{g ml}^{-1}$ of tricalcium phosphate (TCP) and 86 $\mu\text{g ml}^{-1}$ of rock phosphate (RP) with concomitant decrease in pH up to 4 due to the production of gluconate. The biochemical basis of the P solubilization suggested that the gluconate production was mediated by mGDH and sGDH enzymes. Our results illustrate the role of succinate in repression of P solubilization via suppression of mGDH and sGDH activity which correlated with repression of expression of respective genes, *gdhA* and *gdhB*. SK2 also produced IAA (117 $\mu\text{g ml}^{-1}$), siderophore (87% units), HCN, ammonia and solubilized minerals of Zn and K. Our findings imply that it is important to understand the cause of failure of several phosphate solubilizing bacteria in field conditions where catabolite repression may control the expression of several genes and pathways including that of mineral phosphate solubilization. Furthermore, *Acinetobacter* sp. SK2 bearing two glucose dehydrogenase (*gdhA* and *gdhB*) genes was recognized as promising strain for P biofortification and enhanced plant growth promotion.

Keywords Mineral phosphate solubilization · mGDH · sGDH · Gluconate · Succinate-mediated catabolite repression

Introduction

Soluble phosphate (P) deficiency is pragmatic, though there is often copious amount of P in agricultural soil; it is largely present in insoluble forms that cannot be directly utilized by plants (Rodriguez and Fraga 1999). Current agriculture systems apply P fertilizers to the soil to improve crop yield. However, much of these fertilizers rapidly get immobilized into insoluble forms with only up to 20% of the applied P ultimately remaining bioavailable to the plants. P uptake by plant can be enhanced by two ways, either by increasing P solubility in soil or by decreasing P fixation in soil. The unavailable P compounds can be made available for the plant

by phosphate solubilizing bacteria (PSBs) (Richardson et al. 2009). Rhizospheric soil behaves as a rich niche for several groups of rhizobacteria among which members of the genera *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Rhizobium*, *Mesorhizobium*, *Klebsiella* and *Sinorhizobium* are known as potent PSBs and are regarded as plant growth promoting rhizobacteria (PGPR). The use of rhizobacteria as PSBs is a potential component for modern, eco-friendly agricultural practices that can reduce the high expense of chemical P fertilization, maintaining crop yields and reducing soil pollution (Miller et al. 2010; Khan et al. 2014).

Goldstein (1995) proposed that the most common mechanism for P solubilization by Gram-negative bacteria is through release of organic acids. Organic acids chelate divalent cations (such as Ca^{2+}) from complex mineral P such as hydroxyapatite or tricalcium phosphate and release free P which can be taken up by plant. Many Gram-negative bacteria employ periplasmic glucose oxidation through pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) enzyme to produce gluconate. Gluconate thus produced can be imported inside the cell through transporters where it is metabolised or can be secreted out to the

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extracellular environment where it is known to play numerous roles such as solubilization of mineral P, reduction in grazing by protists and act as an antifungal agent (Ram-machandran et al. 2006).

So far two types of PQQ-dependent GDH enzymes have been identified: membrane-bound GDH (mGDH) and soluble GDH (sGDH). The membrane-bound GDH has been reported in many Gram-negative bacteria like *Gluconobacter*, *Pseudomonas* and *Acinetobacter* species (An and Moe 2016) but sGDH is less common. sGDH has been reported in *Acinetobacter calcoaceticus*; however, its role in gluconate production is not defined (Matsushita et al. 1989). The mGDH catalyzes oxidation of glucose to gluconate in the periplasm (Goldstein 1995). *Pseudomonas fluorescens* F113 mutants for mGDH-encoding gene (*gdhA*) or PQQ biosynthesis pathway genes show impaired mineral phosphate solubilization (MPS) activity (Miller et al. 2010). Several reports cite the role of mGDH in gluconate production; however, the specific role of sGDH and the regulation mechanism is yet to be investigated. *Acinetobacter* sp. exists in natural environments including soil, fresh water, oceans, sediments, polar region and also in hydrocarbon-contaminated sites. *Acinetobacter* sp. has versatile metabolic capabilities enabling degradation of various hydrocarbon compounds (Mahjoubi et al. 2013). The metabolic pathways and regulatory mechanisms in *Acinetobacter* sp. have received extensive attention, and most of the research data have been obtained using *Acinetobacter baylyi* ADP1 (Jung and Park 2015) which is a very good bioremediation agent. *Acinetobacter* sp. has also been reported for plant growth promotion traits such as P solubilization (Gulati et al. 2010), IAA production, zinc oxide solubilization, siderophore production (Rokhbakhsh-Zamin et al. 2011) and increasing chlorophyll content in plants (Suzuki et al. 2014).

PSBs formulated as biofertilizers are applied in the rhizosphere of the respective crop plants where they are believed to solubilize P and make it available to plants. However, in rhizosphere there are several factors that affect the efficacy of the inoculated PSBs, such as soil pH, moisture, temperature, competition with existing microbiota and most importantly the type and availability of nutrients. The presence of various organic acids and sugars in rhizosphere may lead to preferential utilization of carbon sources that allows the bacteria to compete and survive among existing microbial communities. Organic acids secreted by plant roots are expected to be present in root exudates (Long et al. 2008). The survival and competence, of pseudomonads, are said to be influenced by various organic acids found in root exudates (Lareen et al. 2016). The sugar and organic acid composition of root exudates of tomato plants help in growth promoting and antifungal activity of *Pseudomonas* strains (Kravchenko et al. 2003). When the phenanthrene-degrading *P. putida* ATCC 17484 was incubated with root exudates of oat and

hybrid poplar, phenanthrene-degrading activity of this strain was repressed (Rentz et al. 2004). These results put forward the indication that the composition of root exudates may influence the performance of rhizobacteria or PSB applied as biofertilizers. Succinate-mediated repression of MPS in *Acinetobacter* is most likely to operate in field conditions as succinate is one of the important components of root exudates of many plants. Succinate-mediated repression of glucose metabolism enzymes including GDH is well reported and is termed as succinate-mediated catabolite repression (SMCR) (Mandal and Chakrabartty 1993). In *P. aeruginosa* (Patel et al. 2011) and *K. pneumonia* (Rajput et al. 2013), organic acid-mediated MPS phenotype was repressed in the presence of succinate. Similar catabolite repression like phenomenon was observed in *Rhizobium* sp. RM and RS, where in the presence of succinate, glucose and arabinose-mediated MPS was repressed (Joshi et al. 2019). Although studies on P solubilization in many more gammaproteobacteria are available, attempts to untie the biochemical and molecular basis of P solubilization and its repression by succinate in bacteria other than members of the *Pseudomonas* group are scantily reported. Here, we present the investigation of the MPS mediated by mGDH and probably by sGDH and their succinate-mediated catabolite repression (SMCR) in *Acinetobacter* sp. SK2. We found that modulation in mGDH and sGDH activity in the *Acinetobacter* sp. SK2 affected the glucose oxidation which showed profound impact on MPS phenotype. We report a possible role of sGDH of *Acinetobacter* sp. SK2 in oxidative glucose metabolism and its SMCR.

Materials and methods

Bacterial strain used and growth conditions

The TCP solubilizing isolate of *Vigna radiata* rhizospheric soil was maintained routinely on Luria–Bertani agar (HiMedia, India) and preserved at -20°C as glycerol stocks. The culture was grown at 37°C for all the experiments. For measurements of growth, GDH activity and RNA extraction, 1X M9 minimal medium was used. 5X M9 minimal medium consisted of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 34 g l^{-1} ; KH_2PO_4 , 15 g l^{-1} ; NH_4Cl , 5 g l^{-1} ; NaCl , 2.5 g l^{-1} ; 2 mmol l^{-1} , MgSO_4 and 0.1 mmol l^{-1} , CaCl_2 . Pikovskaya's (PVK) medium (with TCP) and Tris–Cl (100 mmol l^{-1} ; pH 8) buffered Rock Phosphate (TRP) medium (with RP) were used for P solubilization studies (Gyaneshwar et al. 1999). Glucose and sodium succinate (SRL chemicals, India) were added as carbon source to M9 minimal medium, PVK and TRP medium in concentrations such that the final concentration of carbon source remained constant in a given experimental setup.

Identification and characterization of isolate using 16S rRNA gene sequence

For characterization and identification, the bacterial isolate was subjected to a series of standard morphological and biochemical tests as per the Bergey's Manual of Systemic Bacteriology (Bergey et al. 1984).

The amplification of the 16S rRNA gene was carried out using F27/R1492 primer pair (Table 1). Amplified PCR product of ~1.5 kb length was separated on 1% agarose gel in 1X TAE buffer containing ethidium bromide and was commercially sequenced by Chromous Biotech Pvt. Ltd. (Bengaluru, India). The sequence alignment was carried out with sequence data available in the GenBank using Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1997) and was submitted to National Center for Biotechnology Information (NCBI). The pure culture of isolate has been submitted to the National Centre for Microbial Resource (NCMR, Pune, Maharashtra).

MPS phenotype and its repression by succinate

The MPS phenotype of the isolate was determined by spot inoculation on PVK agar that contained 100 mmol l⁻¹ glucose. The TRP agar having 100 mmol l⁻¹ glucose, 100 mmol l⁻¹ Tris-Cl and pH indicator for visualization of acidification of media (Gyaneshwar et al. 1999) was used for establishing strong MPS phenotype. Succinate-mediated repression of TCP and RP solubilization was checked on PVK and TRP medium containing succinate (100 mmol l⁻¹). The P solubilizing efficiency of isolate was estimated in PVK and TRP broth (with 50 mmol l⁻¹ glucose) inoculated with 1 ml freshly grown cells having 10⁷ cfu ml⁻¹, and incubated at 37 °C for 5 days. Samples were withdrawn at intervals of every 24 h for 5 days to determine the decrease in pH and culture supernatant was collected by centrifuging the samples at 11,000g for 10 min, for estimation of soluble P

using Ames method (Ames 1966). Repression of TCP and RP solubilization in presence of succinate was checked on repression medium containing combination of 25 mmol l⁻¹ glucose + 25 mmol l⁻¹ succinate and 50 mmol l⁻¹ succinate.

HPLC analyses

The HPLC analyses were carried out using culture supernatant of SK2 grown in M9 broth having 50 mmol l⁻¹ glucose. The culture supernatant was filtered through 0.22-μm Nylon filter to obtain cell-free supernatant which was subjected to HPLC system (Waters Alliance model: 2695 separation module with Waters 2996 Photodiode Array Detector) having organic acid-specific ion-exchange column, Supelcogel 610H (Supelco Analytical). 0.1% O-phosphoric acid in distilled water was used as mobile phase. The flow rate was maintained at 0.5 ml min⁻¹ with column temperature of 30 °C. Gluconate, oxalate and malate were used as standards. Retention time and area of each peak in test samples were evaluated with standards to identify the type of organic acid.

Effect of glucose and succinate on growth

To check the effect of glucose and succinate on growth, SK2 was grown in M9 minimal medium containing carbon sources (glucose and succinate) which were autoclaved separately prior to the addition to the medium. Growth studies were performed using M9 medium containing 50 mmol l⁻¹ of glucose or succinate (monoauxic) and 25 mmol l⁻¹ each of glucose and succinate (diauxic) inoculated with approximately 10⁷ cfu ml⁻¹ of overnight grown culture. The culture flasks were incubated at 37 °C on rotary shaker at 150 rpm, and culture samples were aseptically withdrawn at regular intervals for measuring growth and glucose utilization. Growth was assayed by measuring absorbance at 600 nm,

Table 1 Primers used in the study

Genes	Name of primer	Relevant characteristics (5' → 3')	PCR condition	Amplicon size (bp)
16S rRNA	F27 R1492	TACGGTTACCTTGTTACGACTT AGAGTTTGATCATGGCTCAG	94 °C for 4 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C with final extension at 72 °C for 10 min	1500
<i>gdhA</i>	<i>gdhA</i> _FP <i>gdhA</i> _RP	TTCAAGATCAGGTTAACGACTT TGATTTCACCATTAATTTCTTGCG	94 °C for 4 min, followed by 35 cycles of 45 s at 94 °C, 1 min at 50 °C and 30 s at 72 °C with final extension at 72 °C for 10 min	400
<i>gdhB</i>	<i>gdhB</i> -FP <i>gdhB</i> -RP	TCTGAATGGACTGGTAAAACTTT CACATCACGATAACGGTTGT	94 °C for 4 min, followed by 35 cycles of 45 s at 94 °C, 1 min at 50 °C and 30 s at 72 °C with final extension at 72 °C for 10 min	300
<i>gyrB</i>	<i>gyrB</i> _FP <i>gyrB</i> _RP	ATGAGTTCAGAGTCTCAATCAG ACGCACGTTTTCTTGATAAC	94 °C for 4 min, followed by 35 cycles of 45 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C with final extension at 72 °C for 10 min	800

while glucose utilization was measured by Glucose-SLR Reagent (Lab-Care Diagnostics).

Effect of succinate on expression of *gdhA* (mGDH) and *gdhB* (sGDH) by reverse transcription PCR

The effect of succinate on the expression of both the GDH genes was ascertained by isolation of total RNA from 1.5 ml of culture grown in M9 media in different carbon sources using the Hybrid-R™ Kit (GeneAll®), as per manufacturer's instructions. The purified RNA was eluted in 50 µl nuclease-free water and stored at -80°C . RNA concentrations were quantified spectrophotometrically by measuring absorbance at 260 nm and purity was confirmed by electrophoresis on 2% agarose gel. Residual DNA contaminations were removed by DNase I treatment (Invitrogen) followed by DNase inactivation by adding 2 µl stop solution containing EDTA and heating at 65°C for 10 min. The DNase-treated RNA (200 ng) was used to synthesize cDNA using a PrimeScript™ 1st strand cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's instructions. cDNA was used as template for analyzing expression of *gdhA* and *gdhB* genes in glucose, succinate and glucose + succinate (repression medium) grown cells. DNA Gyrase A (*gyrA*) served as internal control.

The PCR mixture constituted 200 ng template cDNA, 20 pmol of forward and reverse primer each, 1X EmeraldAmp GT PCR master mix (2X premix) (Clontech Laboratories, Inc., Takara Bio Company), in a final volume of 20 µl. Amplification of target genes was carried out using gene-specific primers on the Nexus Gradient Mastercycler (Eppendorf, Germany) (Table 1).

Glucose dehydrogenase activity

A chromogenic assay involving 2, 6-dichlorophenolindophenol (DCIP, HiMedia) and phenazine methosulfate (PMS, SRL chemicals, India) was used for membrane and soluble glucose dehydrogenase activity. The enzyme activity was measured as the initial reduction rate of DCIP monitored by a UV/visible spectrophotometer (Bio-spectrometer-Eppendorf Pvt. Ltd.) at 600 nm.

Preparation of cell membrane and soluble fractions

The mid-log phase cells grown in M9 with 50 mmol l^{-1} glucose; 50 mmol l^{-1} succinate and 25 mmol l^{-1} glucose + 25 mmol l^{-1} succinate were harvested by centrifugation at $12,000g$ for 5 min at 4°C . The cells were washed with 50 mmol l^{-1} potassium phosphate buffer (pH 7.5) and resuspended in the same buffer. The resuspended pellet was used as a crude membrane fraction for mGDH assay. For soluble fraction, the pellet was resuspended in small volume

of same buffer containing 20% glycerol, 1 mmol l^{-1} DTT and sonicated (Syclon Ultrasonic Cell Crusher) for 5 min at a pulse rate of 30 s at 500 Hz on ice. Cell debris was removed by centrifugation at $11,000g$ at 4°C for 30 min. The supernatant was separated into a crude soluble fraction by centrifugation at $120,000g$ for 90 min and immediately used for sGDH assay (Matsushita et al. 1989).

The unit of glucose dehydrogenase activity was defined as nmol of DCIP reduced per minute. The specific activity of enzyme was defined as unit mg^{-1} total protein. Protein quantification was done by Lowry method (Lowry et al. 1951).

Characterization of isolate for in vitro plant growth promoting (PGP) activities

IAA production

For estimation of IAA, isolate was inoculated in tryptophan broth and incubated at 37°C for 48 h. The concentration of IAA produced was determined spectrophotometrically at 530 nm using Salkowski's reagent (Gordon and Weber 1951).

Siderophore production

To check the siderophore production, quantitative spectrophotometric assay was performed using CAS reagent as described (Schwyn and Neilands 1987).

Ammonia and hydrogen cyanide production

Ammonia production was checked by growing isolate in peptone water at 37°C for 48–72 h and using Nessler's reagent (Hansen 1930).

To check the HCN production ability, isolate was streaked on nutrient agar added with glycine (4.4 g l^{-1}). The agar was covered with a Whatman number 1 filter paper previously soaked in a solution containing 0.5% picric acid and 2% sodium carbonate (w/v). Plates were sealed with parafilm and incubated at 37°C for 3 days. The appearance of orange or red color indicated the production of HCN (Khan et al. 2014).

Zinc and potassium solubilization

The zinc (Zn) solubilizing ability of isolate was checked using plate assay (Sharma et al. 2012) with minor modifications. The inoculated minimal medium plates were supplied with 0.1% zinc oxide. The inoculated plates were incubated in dark at 37°C for 3 days and observed for production of clear zones around the colonies.

Potassium (K) solubilizing ability was tested on Alexandrov medium (5 g Glucose, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g

CaCO_3 , 0.006 g FeCl_3 , 2 g Ca_3PO_4 , 3 g insoluble potassium source (Feldspar) and 20 g agar in 1 l of deionized water). The pH of the medium was maintained at 7.5 (Setiawati and Mutmainnah 2016). The inoculated plates were incubated at 37 °C for 3–4 days and observed for production of clear zones around colonies. The diameter of the zones was recorded and solubilization (SI) was calculated.

Plant growth experiments

The soil used for plant growth experiments was clay and loamy with pH 7.3. The soil was sterilized by three cycles (1 h each) of autoclaving. 1 kg of sterilized soil was filled in each pot. The *V. radiata* seeds were surface sterilized with 0.1% HgCl_2 and allowed to imbibe in pure culture (10^8 cfu ml^{-1}) overnight. The seeds were placed at a depth of 5 cm and were watered with sterile water. The pots bearing inoculated and uninoculated plants were maintained under greenhouse conditions (10 h day and 14 h night cycle) at 25 °C. Post-inoculation, plants were gently uprooted on 60th day and scored for root length (cm), shoot length (cm), root:shoot ratio and dry mass (g).

Statistical analysis

All the experiments were performed in triplicates and result values are expressed as mean $\pm$ standard deviation. An analysis of variance (ANOVA) was performed using the statistical program GraphPad Prism® Version 6.07.

Results

Isolation and identification of isolate with strong MPS phenotype

The rhizosphere isolate of *V. radiata* (mung bean) was Gram-negative, non-sporulating, non-motile short rods bearing typical biochemical features of *Acinetobacter* genus. It tested positive for catalase and negative for oxidase (Bergey's Manual of Determinative Bacteriology) (Bergey et al. 1984). Amplification and sequencing of 16S rRNA gene confirmed that the isolate belonged to genus *Acinetobacter* bearing 98.57% similarity to its closest match *Acinetobacter pittii* strain AP43 and it was regarded as *Acinetobacter* sp. SK2. The 16S rRNA sequence of the isolate has been submitted to NCBI (accession number MG847098) and the pure culture of isolate has been submitted to the National Centre for Microbial Resource (NCMR, Pune, Maharashtra) as MCC 3675.

Acinetobacter sp. SK2 was characterized for MPS phenotype. On PVK agar, the isolate produced clear zone around the colony after 48 h of incubation indicating TCP

solubilization (Fig. 1a). RP solubilization was indicated by pink zone around the colony due to acidification of TRP medium upon 48 h of incubation. RP solubilization under buffered condition, therefore, constituted strong MPS phenotype (Fig. 1c) when glucose was the sole carbon source.

MPS by *Acinetobacter* sp. SK2 and its repression

The soluble P release by SK2 was 682 $\mu\text{g ml}^{-1}$ and 86 $\mu\text{g ml}^{-1}$ in PVK and TRP broth, respectively. A concomitant decrease in pH of the media from neutral to less than 4 correlated with the findings that acidification of the medium due to organic acid production led to MPS. However, the isolate failed to solubilize TCP and RP in PVK and TRP medium supplemented with succinate (Fig. 1b, d). The repression of P solubilization in PVK medium was 90–91% in glucose + succinate or succinate containing medium (Fig. 2a). The quantity of soluble P estimated during the course of growth in glucose + succinate and succinate was insignificant. Similar trend was observed for RP solubilization where soluble P decreased up to 97–99% in glucose + succinate and succinate (Fig. 2b).

HPLC analyses

HPLC analyses of glucose-grown cells showed gluconate as the main organic acid present in culture supernatant. 20 mmol l^{-1} gluconate was produced from 50 mmol l^{-1} glucose, confirming organic acid-mediated MPS (Supplementary Figure 1).

Fig. 1 MPS phenotype of *Acinetobacter* sp. SK2 on **a** PVK agar with glucose, **b** PVK agar with glucose + succinate, **c** TRP agar with glucose, **d** TRP agar with glucose + succinate

Fig. 2 Estimation of P solubilization and its repression in **a** PVK, **b** TRP broth. Figures indicate P release (bars) and pH change (lines) in PVK and TRP broth with glucose (G), glucose+succinate (GS) by SK2. PVK and TRP broth were supplemented with a total of

50 mmol l⁻¹ glucose (G), 25 mmol l⁻¹ glucose+25 mmol l⁻¹ succinate (GS) and 50 mmol l⁻¹ succinate (S). Values are mean ± SD of three to four independent observations

Effect of succinate on growth, gene expression and enzyme activity

A monoauxic growth pattern (Fig. 3a) was observed when SK2 was grown in minimal M9 medium containing 50 mmol l⁻¹ glucose or succinate as a sole carbon source with rapid growth on succinate as compared to glucose. The glucose utilization during diauxic growth in repression medium started in second log phase, after sixth hour of incubation, until which glucose remained unutilized (Fig. 3b). This indicated that succinate repressed glucose uptake and, hence, activity of enzymes and expression of genes contributing to MPS were carried out in the presence of glucose, glucose+succinate and succinate.

The specific activity of GDH was carried out to determine glucose oxidation in glucose, glucose+succinate and

succinate grown cells. The mGDH activity of cells grown in glucose was 164 U (mg protein)⁻¹ which was significantly higher than that in glucose+succinate (18.4 U (mg protein)⁻¹) and succinate (15 U (mg protein)⁻¹) grown cells. Significant repression, 89% and 91%, respectively, was observed in mGDH activity of cells grown in glucose+succinate and succinate. Incidentally, sGDH activity from soluble fraction of glucose-grown cells which was 10 U (mg protein)⁻¹, significantly decreased by 82% in succinate and 86% in glucose+succinate grown cells (Fig. 4c). The expression level of the *gdhA* and *gdhB* genes encoding mGDH and sGDH, respectively, varied significantly with the carbon source (Fig. 4a). The expression of both the genes was high in glucose, negligible in glucose+succinate and completely repressed in succinate, which is consistent with the results of GDH activity. Based on these findings, a metabolic scheme

Fig. 3 Effect of glucose and succinate on growth of *Acinetobacter* sp. SK2. **a** Monoauxic growth in 50 mmol l⁻¹ glucose (G) and 50 mmol l⁻¹ succinate (S), **b** diauxic growth in glucose+succinate

(25 mmol l⁻¹ each) and utilization of glucose. Values are mean ± SD of four to six independent observations

Fig. 4 Enzyme activities of *Acinetobacter* sp. SK2 grown on glucose, glucose + succinate and succinate. **a** Membrane glucose dehydrogenase (mGDH), **b** soluble glucose dehydrogenase (sGDH) (values are

mean $\pm$ SD of four to six independent observations). **c** Expression profile of *gdhA* and *gdhB* genes in glucose (G), glucose + succinate (GS) and succinate (S)

is proposed that explains the role of GDH enzymes in MPS phenotype of the isolate when grown on glucose and glucose + succinate (or succinate) (Fig. 5). This could explain involvement of sGDH in glucose oxidation which also appears to be under the catabolite repression by succinate. Further, the differences in expression suggest that the genes are likely being regulated at the transcription level due to the presence of succinate.

Characterization of in vitro PGP properties

The results of PGP properties of the isolate are described in Table 2. IAA production was indicated by pink

coloration of the cell-free supernatant upon the addition of Salkowski's reagent. The isolate SK2 produced $117 \mu\text{g ml}^{-1}$ IAA and 87% siderophore units. SK2 also showed Zn and K solubilizing ability (zone of clearance around colonies), besides producing ammonia as indicated by brown coloration of the medium upon addition of Nessler's reagent.

Fig. 5 Proposed pathways for MPS in *Acinetobacter* sp. SK2 and repression by succinate. **a** Glucose is oxidized to gluconate in periplasmic space by mGDH (*gdhA*) or taken up via OprB porin to enter the cells through ABC transporter where it is oxidized by sGDH (*gdhB*) to produce gluconate. Gluconate secreted by either or both steps can attribute to the MPS by chelating cations (Ca^{2+}) and releasing soluble P from complex insoluble phosphate (here $\text{Ca}_3(\text{PO}_4)_2$),

making it available for plant growth. **b** In the presence of succinate, the expression and hence activity of mGDH and sGDH are repressed diminishing MPS. The genes encoding the enzymes/proteins involved are given for each step: *oprB* outer membrane porin B, *gdhA* membrane glucose dehydrogenase, *gdhB* soluble glucose dehydrogenase, OM outer membrane, PS periplasmic space, IM inner membrane, ABC transporter

Table 2 Characterization of P solubilizing isolate SK2 for in vitro plant growth promoting traits

IAA ^a production (µg ml ⁻¹)	Siderophore production ^b (% siderophore units)	Potassium solubilization ^c (SI)	Zinc oxide solubilization ^d (SI)	HCN production ^e	Ammonia production ^f	Accession no. ^g
117 ± 1.3	87 ± 1.77	2.5 ± 0.07	2.8 ± 0.18	+Ve	+Ve	MCC 3675

Values are mean ± standard deviation of three independent observations

^aIAA production quantified by spectrophotometric method and indicated in µg ml⁻¹

^bSiderophore production quantified by CAS assay and indicated in % siderophore units

^cPotassium solubilization detected by plate assay by Solubilization Index (SI) on Alexandrov agar plates

^dZinc oxide solubilization detected by plate assay using Solubilization Index (SI) on minimal agar plates with zinc oxide

^eHCN production detected on nutrient agar medium added with glycine, on the basis of color change of Whatman paper previously soaked in a solution containing picric acid and sodium carbonate

^fAmmonia production detected in peptone water using Nessler's reagent

^gIsolate was identified on the basis of 16S rRNA gene amplification and sequencing and submitted to NCMR, Pune, Maharashtra, India

Discussion

Isolation, characterization and identification of P solubilizing isolate

Rhizosphere is a favourable place for various types of microorganisms including a class of bacteria that directly or indirectly benefit the plant growth. These bacteria are known as PGPR as they execute different plant growth promoting activities such as mineral provision, nitrogen fixation, phytohormone production or bio-control. Phosphorous is an important macronutrient for almost all plant process, but its availability is limited owing to its fixation in insoluble forms immediately after addition in soil (Rodriguez and Fraga 1999). Microbe-mediated MPS is an important option to make P available to plants. Various studies report isolation and characterization of PSBs from rhizosphere in order to use them as efficient P biofertilizer. The presence of a variety of nutrients in the rhizosphere influences the activity and existence of many microorganisms which may influence plant growth. Succinate is one of the most important organic acids generally found in root exudates in rhizosphere of plants and is known to repress glucose utilization in several species of bacteria. This study was aimed at understanding biochemical basis and effect of succinate on P solubilization in *Acinetobacter* sp. SK2.

MPS and its repression

TCP and RP solubilized by SK2 was 682 µg ml⁻¹ and 86 µg ml⁻¹, respectively, which is higher compared to many reported PSBs such as *Burkholderia cepacia* (51 µg ml⁻¹), *Klebsiella* sp. (110–130 µg ml⁻¹) (Rajput et al. 2013), *Rhizobium* sp. Td3 (423 µg ml⁻¹), SN1 (428 µg ml⁻¹) (Iyer and Rajkumar 2019), *Rhizobium* sp. RM (653 µg ml⁻¹) and RS (602 µg ml⁻¹) (Joshi et al. 2019). The reported P release

from RP is 63–76 µg ml⁻¹ by *P. aeruginosa* (Patel et al. 2011) and 50–77 µg ml⁻¹ in *Citrobacter* sp. (Gyaneshwar et al. 1999; Patel et al. 2011). Thus, SK2 was an efficient P solubilizer with an ability to release significant amounts of soluble P from TCP and especially RP in buffered conditions (Figs. 1a, c, 2). When succinate was added with glucose or individually, it repressed P solubilizing ability of the isolate. The repression of MPS phenotype mediated by succinate was significant (Figs. 1b, d, 2).

Organic acid-mediated MPS phenotype (HPLC analyses)

MPS in SK2 was due to gluconate production which was also reported to be the most abundant organic acid present in culture supernatant of various PSBs such as *Klebsiella pneumoniae*, *Rahnella aquatilis*, *Erwinia herbicola*, *Pseudomonas cepacia*, *Pseudomonas aeruginosa* and *Enterobacter asburiae* PSI3. (Rajput et al. 2013; Iyer et al. 2017; Patel et al. 2011; Gyaneshwar et al. 1999; Rodriguez and Fraga 1999). *Acinetobacter rhizosphaerae* strain BIHB723 is also reported to solubilize P by producing varieties of organic acids depending on the phosphorous sources available (Gulati et al. 2010).

Effect of succinate on growth, enzyme activity and gene expression

SK2 preferred succinate over glucose and showed diauxic growth on medium containing glucose and succinate (Fig. 3). Though succinate was preferentially utilized over glucose, it repressed MPS phenotype when present. The direct glucose oxidation pathway involving mGDH is reported to play a key role in the P solubilizing ability of *Enterobacteriaceae* and *Pseudomonadaceae* family (Patel et al. 2011). However, the isolate is exceptional

Fig. 6 Effect of *Acinetobacter* sp. SK2 inoculation on various plant growth parameters in comparison to uninoculated control. **a** Root length and shoot length (cm). **b** Fresh and dry weight (g). Values are mean $\pm$ standard deviation of six plants

since it contains a second type of enzyme known as sGDH, which was first reported in *A. calcoaceticus* (Duine et al. 1982; Matsushita et al. 1989); however, the role of sGDH in glucose oxidation lacks confirmation (Matsushita et al. 1995). sGDH from *A. calcoaceticus* is used commercially in glucose sensors as it has a high turnover number and the electrochemical regeneration of cofactor is not affected by the presence of O_2 unlike glucose oxidase (Flexer and Mano 2014). Therefore, it was important to evaluate the role of sGDH in glucose oxidation along with mGDH in SK2. Whether sGDH activity also contributes to MPS further remains to be explored.

Succinate repressed expression as well as activity of enzymes under the study. Repression of *gdhA* and *gdhB* expression (Fig. 4a) as well as enzyme activities (Fig. 4b) in the presence of succinate suggests that P solubilization is under SMCR. The glucose catabolic enzymes were found to be repressed by succinate in *Rhizobium* and *Bradyrhizobium* (Mandal and Chakrabarty 1993). Intracellular phosphorylative pathway for glucose utilization was also suppressed in *P. putida* CSV86 when grown in the presence of succinate (Basu et al. 2006). In *P. aeruginosa* M3 and SP1, succinate and malate repressed glucose dehydrogenase enzyme required for glucose oxidation (Patel et al. 2011). Succinate-mediated repression of MPS was also reported in *Klebsiella pneumonia* SM6 and SM11 (Rajput et al. 2013) and *Rhizobium* sp. RM and RS (Joshi et al. 2019). The repression of P solubilization by succinate in SK2 was due to repression of the mGDH enzyme and probably sGDH (Fig. 4). The preferential utilization of carbon sources in *Pseudomonas* and related species is controlled at a post-transcriptional level by catabolite repression control (Crc) protein (Moreno et al. 2007). Whether a similar post-transcriptional repression of mGDH or sGDH, the key enzyme of glucose oxidation is under the regulation of Crc needs further investigation.

In vitro plant growth promoting (PGP) activities

Many rhizospheric bacteria can produce IAA as a secondary metabolite and are vital for plant development and growth. The concentration of IAA produced by SK2 was better compared to the previous reports on *Acinetobacter* sp. PUCM1007 ($13 \mu\text{g ml}^{-1}$), PUCM10029 ($10 \mu\text{g ml}^{-1}$) (Rokhbakhsh-Zamin et al. 2011) and *Acinetobacter* sp. RS4 ($29 \mu\text{g ml}^{-1}$) (Iyer et al. 2017). SK2 also produced siderophore, ammonia, HCN and solubilized Zn and K (Table 2). Siderophore production was considered as one of the mechanisms of MPS (Rodriguez and Fraga 1999). It was found that apart from antagonistic activity, HCN helped in increasing P availability for rhizobacteria and plant hosts in oligotrophic alpine environments (Rijavec and Lapanje 2016). K helped in increasing resistance against diseases, pests and abiotic stresses. K was found to be important for activation of various enzymes responsible for energy metabolism and helped in plant growth promotion (Etesami et al. 2017). Zinc deficiency led to delayed shoot growth and root development, and increased susceptibility to heat, light and fungal diseases (Kamran et al. 2017). Plant growth experiments in pots with *V. radiata* indicated that SK2 inoculation improved almost all the plant growth parameters studied (Fig. 6). The growth promotion can be attributed to the inoculated SK2 assisted growth enhancement as it may have made P bioavailable to the plant and also provided other PGP traits such as IAA, siderophore production, etc. The *Acinetobacter* sp. SK2 can further explored for its all PGP potential to develop a sustainable multitrait biofertilizer.

Conclusion

This study was undertaken to understand the mechanism of P solubilization and its repression by succinate in *Acinetobacter* sp. SK2. We have demonstrated that the MPS phenotype was the result of gluconate production via periplasmic oxidation of glucose which catalyzed by mGDH and sGDH too may have some role in glucose oxidation. Succinate repressed P solubilization by repressing the *gdhA* and *gdhB* genes at the transcriptional level and/or post-transcriptional level. Significant repression of mGDH and sGDH activity in the presence of succinate indicated that the P solubilizing ability of this rhizobacteria can be compromised due to the preferential utilization of organic acids over glucose in the rhizosphere. Therefore, the regulation of the carbohydrate utilization pathway and its consequences needs to be considered prior to application of PSBs in fields. Furthermore, understanding the levels of repression by succinate may help plan strategies for development of SMCR-relieved strains of PSBs.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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