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Glucose and arabinose dependent mineral phosphate solubilization and its succinate-mediated catabolite repression in *Rhizobium* sp. RM and RS

Ekta Joshi, Bhagya Iyer, and Shalini Rajkumar*

Institute of Science, Nirma University, Sarkhej-Gandhinagar Highway, Ahmedabad, Gujarat 382481, India

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***Rhizobium* sp. RM and RS are *Vigna radiata* root nodule isolates with the ability to solubilize tricalcium phosphate and rock phosphate under 50 mM Tris-Cl buffering conditions. *Rhizobium* sp. RM and RS were unique as they could produce two different organic acids, gluconic acid and oxalic acid using glucose and arabinose, respectively, which are two of the most prominent sugars present in the rhizospheric soil. However, P solubilization in these isolates was repressed in the presence of succinate resembling the phenomenon of catabolite repression. RM and RS produced 24 mM and 20 mM gluconic acid in presence of glucose which was repressed to 10 mM and 8 mM, respectively, in glucose + succinate conditions. Similarly, RM and RS produced 28 mM and 23 mM oxalic acid in arabinose containing media which was repressed to 9 mM and 8 mM, respectively, in the presence of arabinose + succinate. Results of enzyme activities indicated 66% repression of quinoprotein glucose dehydrogenase in glucose + succinate compared to glucose grown cells and 84% repression of glyoxylate oxidase in arabinose + succinate compared to arabinose grown cells. This is perhaps the first report on mechanism of P solubilization in rhizobia through utilization of two different sugars, glucose and arabinose and its repression by succinate. Succinate-mediated catabolite repression of arabinose is the unique aspect of this study.**

[**Key words:** Arabinose; Mineral phosphate solubilization; Glucose dehydrogenase; Arabinose dehydrogenase; 2-Keto-3-deoxyarabonate aldolase; Glycolaldehyde dehydrogenase; Glyoxylate oxidase]

Apart from the use of chemical fertilizers, microbial phosphate solubilization and mineralization is the only feasible way to enhance plant available phosphate. Phosphorus, the second most important plant growth limiting macronutrient after nitrogen is abundantly available in soil in both organic and inorganic forms (1,2). Majority of the soil phosphorus is in a form unavailable for plant uptake but can be converted to plant available forms by phosphate solubilizing microorganisms (3,4). Mineral phosphate solubilization (MPS) is one of the most important plant growth promoting activity shown by plant growth promoting rhizobacteria and it contributes significantly to the plant growth and development.

Bacteria belonging to various genera like *Pseudomonas*, *Rhizobium*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Bacillus*, *Erwinia*, *Burkholderia* and *Micrococcus* are well known for solubilizing insoluble inorganic phosphate (P) compounds such as tricalcium phosphate (TCP), dicalcium phosphate and rock phosphate (RP) (5,6). P solubilization is achieved through the secretion of low molecular weight organic acids like gluconate, ketogluconate, malate, succinate, fumarate, oxalate, and citrate, which cause acidification and release of free P in a form available for plant uptake (5,7). Gluconic acid produced by gram-negative bacteria through periplasmic glucose oxidation pathway via pyrroloquinoline quinone dependent glucose dehydrogenase (Gcd) is shown to result in most efficient P solubilization (8,9). Gluconic acid seems to be the most frequent agent for MPS in several P solubilizing bacteria such as *Pseudomonas* sp. (2), *Erwinia herbicola* (10) and *Pseudomonas cepacia* (11).

Majority of the rhizobacteria reported so far have been shown to solubilize P only when grown on glucose. Other rhizospheric sugars have received little or no attention in this regard. L-Arabinose is a carbon source widely used by microorganisms including diazotrophs such as *Azospirillum* sp. (12), *Bradyrhizobium* sp. (13) and *Rhizobium* sp. (14). Arabinose is also reported to be present in the root exudates of many plants at physiologically relevant concentrations making it a prominent sugar present in the rhizospheric soil (15). Arabinose is a substrate that could be used by rhizobia in the rhizosphere; is a component of the plant cell wall and is present in arabinogalactan proteins secreted by plant root cells (16). Role of a pentose such as arabinose on plant growth promoting traits of bacteria especially P solubilization has not been explored. L-Arabinose is a superior carbon source and is metabolized by distinct pathways that distinguish rhizobia into slow and fast growers (17). In the slow-growing rhizobia (*Bradyrhizobium japonicum* and *Rhizobium* sp. 32H1), L-arabinose is metabolized through non-phosphorylated intermediates leading to the production of pyruvate and glycolaldehyde (13). In this pathway, L-arabinose metabolism begins with oxidation to L-arabino-γ-lactone, followed by hydration forming L-arabonate which undergoes dehydration to L-2-keto-3-deoxyarabonate. It is split by an aldolase reaction to pyruvate and glycolaldehyde (13). Glycolaldehyde is subsequently

* Corresponding author. Tel.: +91 79 30642757; fax: +91 2717 241916.
E-mail address: shalini.rjk@nirmauni.ac.in (S. Rajkumar).

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metabolized through an oxalate degradation pathway (18). In contrast, L-arabinose metabolism produces L-2-keto-3-deoxyarabonate in fast growing rhizobia (*Sinorhizobium meliloti* and *Rhizobium leguminosarum*) which is oxidized by α-ketoglutarate semialdehyde dehydrogenase to α-ketoglutarate that enters TCA cycle (19).

Succinate on the other hand is one of the major organic acid present in plant root exudates which is known to repress glucose utilization by a mechanism termed as succinate-mediated catabolite repression (20). Organic acid mediated MPS phenotype has been shown to be repressed by succinate in *Pseudomonas* (21), *Klebsiella pneumoniae* (22) and *Rhizobium* sp. Td3 (23). Even though phosphate solubilization by bacteria has been reported since long, failure of these PSBs in field conditions due to succinate-mediated catabolite repression may restrict their use as biofertilizers. The present study is aimed at determining the exact biochemical basis of MPS phenotype in rhizobia using arabinose and glucose as carbon source and its succinate-mediated repression.

MATERIALS AND METHODS

Bacterial species: characteristics and growth condition *Rhizobium* sp. RM and RS used for this study are *Vigna radiata* root nodule isolates which were maintained routinely on yeast extract mannitol agar (YEMA) (HiMedia Laboratories Private Limited, Mumbai, India) and preserved at −20°C as glycerol stocks. For characterization and identification, bacterial isolates were subjected to a series of standard morphological and biochemical tests as per the Bergey's manual of systemic bacteriology (24).

Molecular characterization based on 16S rRNA gene sequence Molecular identification was carried out through sequencing of 16S rRNA gene following amplification using universal primers F27 (5′-AGA GTT TGA TCA TGG CTC AG-3′) (25) and R1492 (5′-TAC GGT TAC CTTGTTACG ACT T-3′) (26). Both end sequencing was carried out using BigDye_Terminator v 3.1 (BDTv3.1) and Applied Biosystems 37309l DNA Analyze (ABI3730XL). Closely related sequences from NCBI database were used for constructing a phylogenetic tree using Phylogeny (27).

Determining MPS phenotype of rhizobial isolates The MPS phenotype of the rhizobial isolates was determined by spot inoculation on Pikovskaya's agar (28) and 50 mM Tris buffered rock phosphate (TRP) agar of pH 8 containing glucose and arabinose individually at 100 mM concentration. TRP agar contained methyl red to monitor change in color of media on production of organic acid (29). TCP and RP solubilizing efficiency of RM and RS were quantitatively estimated in Pikovskaya's and TRP broth inoculated with freshly grown cells incubated at 28°C (22). Samples were withdrawn at 24 h interval for 5 days, centrifuged at 7830 ×*g* for 10 min to obtain cell free supernatant which was used to determine the change in pH and P release (30). Cell free supernatant of mid-end log phase of day 4 were used for quantification of organic acids.

Determining succinate-mediated repression of MPS phenotype of rhizobial isolates Repression in P solubilization was qualitatively checked on TRP agar. For repression studies, 100 mM succinate, equimolar mixture of glucose + succinate and arabinose + succinate (50 mM each) were used as carbon source. Succinate-mediated repression of TCP and RP solubilization was quantitatively estimated as mentioned above in Pikovskaya's broth and TRP broth, respectively. To study the effect of succinate concentration on RP solubilization, isolates were grown in TRP broth with 10, 20, 30, 40 and 50 mM succinate. Total carbon source concentration was made 100 mM with glucose/arabinose. RP solubilization was estimated as mentioned above.

Growth experiments For growth studies, RM and RS were grown in M9 minimal medium (HiMedia Laboratories) containing 20 mM glucose, 20 mM arabinose and 20 mM succinate. The culture flasks were incubated at 28°C on rotary shaker. Samples were withdrawn at regular intervals for estimation of growth by measuring absorbance at 600 nm.

High performance liquid chromatography analysis for determination of organic acids Cell free supernatants of both the rhizobial isolates were filter sterilized using 0.22-μm Nylon filter prior to estimation. High performance liquid chromatography (HPLC) was carried out in Waters Alliance model 2695 separation module with Waters 2996 Photodiode Array Detector having organic acid specific ion-exchange C_{18}-column. *O*-Phosphoric acid (0.1%) in distilled water was used as mobile phase with a flow rate of 0.5 ml min^{-1} and column temperature was kept at 28°C. Gluconate, oxalate, malate, citrate, acetate and succinate were taken as standards.

Enzyme assays Cell free supernatant of cell lysates were used as crude enzyme source except for Gcd assay where whole cells were used. Cell lysis was carried out with mid-log phase cells grown in M9 minimal medium containing 20 mM glucose, 20 mM arabinose, 20 mM succinate, 10 mM glucose + 10 mM succinate and 10 mM arabinose + 10 mM succinate. Cells were harvested by centrifugation at 11,270 ×*g* for 5 min at 4°C (31) and were washed with 80 mM phosphate buffer (pH 7.5). Cell pellet was resuspended in small volume of the same buffer containing 20% glycerol and 1 mM DTT and sonicated (Syclon Ultrasonic Cell Crusher, Ningbo Haishu Sklon Electronic Instrument Co., Ltd., Ningbo, China) for 2–3 min at a pulse rate of 30 s at 500 Hz on ice. Cell debris was removed by centrifugation at 11,270 ×*g* at 4°C for 30 min and the supernatant was directly used for enzyme assays. For Gcd assay, whole cells were washed thrice with N saline, resuspended in N saline and used directly (32). All the enzyme assays were performed at 28°C. Protein quantification was done by Lowry method (33). One unit of enzyme activity was defined as the amount of protein required to convert 1 μmol of substrate to product per minute.

Glucose dehydrogenase (Gcd) (EC 1.1.5.2) Gcd (EC 1.1.5.2) assay was performed by determining the spectrophotometric reduction in the absorbance of 2,6-dichlorophenolindophenol (DCIP) at 600 nm as described by Rajput et al. (22). The specific activity of enzyme was defined as IU (mg protein)$^{-1}$.

Arabinose dehydrogenase (EC 1.1.1.46) Arabinose dehydrogenase (EC 1.1.1.46) assay was carried out by measuring the formation of NADPH continuously at 340 nm in a spectrophotometer. The assay mixture (1 ml) contained 10 mM L-arabinose, 100 mM Tris-Cl (pH 9.0), 10 mM NADP and sonicated cells. Enzyme activity was measured every 30 s for 6 min (14).

L-2-Keto-3-deoxyarabonate aldolase (L-KDA aldolase) (EC 4.1.2.18) L-2-Keto-3-deoxyarabonate aldolase (EC 4.1.2.18) assay was carried out as described by Pedrosa and Zancan (13) with some modifications. The reaction mixture contained 40 mM HEPES buffer (pH 8.0), 10 μM $MgCl_2$, 7.5 μM sodium pyruvate, 10 μM KDA and sonicated cells. The L-KDA aldolase activity was estimated by thiobarbituric acid method (34). Enzyme activity was measured every 30 s for 8 min.

Glycolaldehyde dehydrogenase (EC 1.2.1.21) Glycolaldehyde dehydrogenase (EC 1.2.1.21) assay was carried out as described by Duncan (14) with some modifications. The reaction mixture contained 1 mM glycolaldehyde, 2.5 mM NAD, 100 mM sodium glycine buffer (pH 9.5) and sonicated cells. Enzyme activity was measured every 30 s for 10 min.

Glyoxylate oxidase (EC 1.2.3.5) Glyoxylate oxidase (EC 1.2.3.5) assay was carried out using DCIP linked reaction system (35). The assay was performed as described by Rajput et al. (22).

All the experiments were performed in three independent triplicates and result values were expressed as mean ± standard deviation of all the observations.

RESULTS AND DISCUSSION

Identification and characterization of rhizobial isolates for MPS phenotype As phosphate plays an important role in virtually all plant process but its availability is limited in soil, we attempted to isolate P solubilizing rhizobia from the root nodules of *V. radiata.* Two gram-negative phosphate solubilizing isolates RM and RS with the ability to grow on yeast extract mannitol agar with congo red were obtained. They fermented lactose, produced phenylalanine deaminase and utilized citrate. Based on 16S rRNA sequencing, both the isolates were shown to belong to genus *Rhizobium* (Fig. 1). The 16S rRNA sequences of *Rhizobium* sp. RM and RS have been submitted to NCBI under accession number MH046067 and MH046069, respectively.

Mineral phosphate solubilization by *Rhizobium* sp. RM and RS On Pikovskaya's agar *Rhizobium* sp. RM and RS produced clear halo zone around the colony after 48 h of incubation indicating TCP solubilization (Fig. S1A). On TRP agar with 100 mM glucose and 100 mM arabinose, RP solubilization was seen as pink zone around the colony indicating acidification of medium after 48 h of incubation (Fig. S1C and E). In Pikovskaya's broth containing glucose, RM and RS achieved a minimum pH of 3.4 and 3.5 with P release of 653 μg ml^{-1} and 602 μg ml^{-1} while a minimum pH of 3.5 and maximum P release of 592 μg ml^{-1} and 553 μg ml^{-1}, respectively, was obtained in the presence of arabinose (Fig. 2). TCP solubilization up to 159 μg ml^{-1} in *Bacillus megaterium* Y99 (36), 51 μg ml^{-1} in *Burkholderia cepacia* (9) and 110–130 μg ml^{-1} in *Klebsiella* sp. (22) has been recorded. RP solubilization by RM and RS grown in glucose was 132 μg ml^{-1} and 108 μg ml^{-1} with a minimum pH of 3.8 and 4, respectively (Fig. 3A and C). In TRP broth containing arabinose, RM and RS

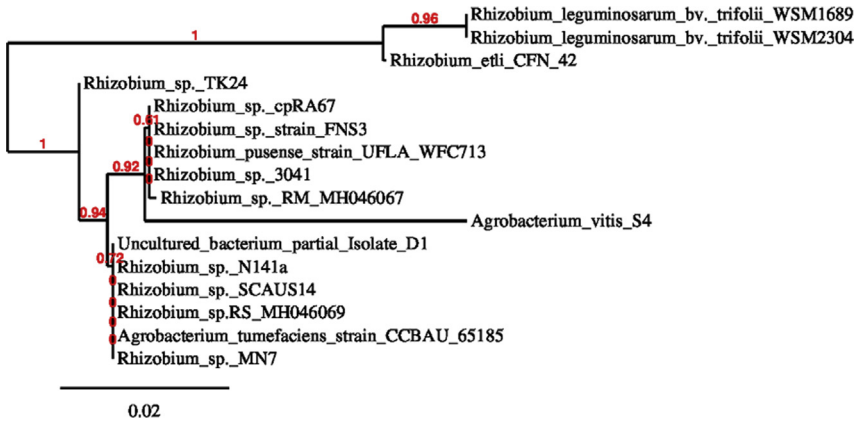

FIG. 1. Phylogenetic tree of 16S rRNA gene homology. Maximum likelihood tree based on 16S rRNA gene sequences of RM and RS with their GeneBank accession numbers.

decreased media pH to 3.8 and 4 with P release of 106 μg ml^{-1} and 98 μg ml^{-1}, respectively (Fig. 3B and D). RP solubilization up to 77 μg ml^{-1} in *Enterobacter asburiae* PSI3 (29), 76.12 μg ml^{-1} in *Pseudomonas aeruginosa* M3, 63.45 μg ml^{-1} in *P. aeruginosa* SP1 (21) and 23 μg ml^{-1} in *Rhizobium* sp. Td3 (23) has been reported. *Pseudomonas striata* released 22 μg ml^{-1} while *B. cepacia* has been reported to release 35 μg ml^{-1} of P from RP (9). In Pikovskaya's broth, TCP is the P source that can be solubilized by many bacterial species. This medium has some amount of free P (up to 80–100 μg ml^{-1}) which was detected even in uninoculated control or day 0. However, TRP broth contains water washed RP which is difficult to solubilize and is present in undetectable amount as free P. RP solubilization therefore constitutes strong MPS phenotype and was recorded after cells entered the exponential phase. P release increased gradually with decrease in pH and declined after 3–4 days due to cell lysis and nutrient depletion. The obtained results were comparable with the earlier reports of bacterial TCP and RP solubilization (22). So far none of the rhizobial species have been reported for MPS phenotype using both glucose and arabinose as sole carbon source.

Succinate-mediated repression of MPS by *Rhizobium* sp. RM and RS RM and RS failed to solubilize P when grown on Pikovskaya's agar and TRP agar supplemented with succinate and

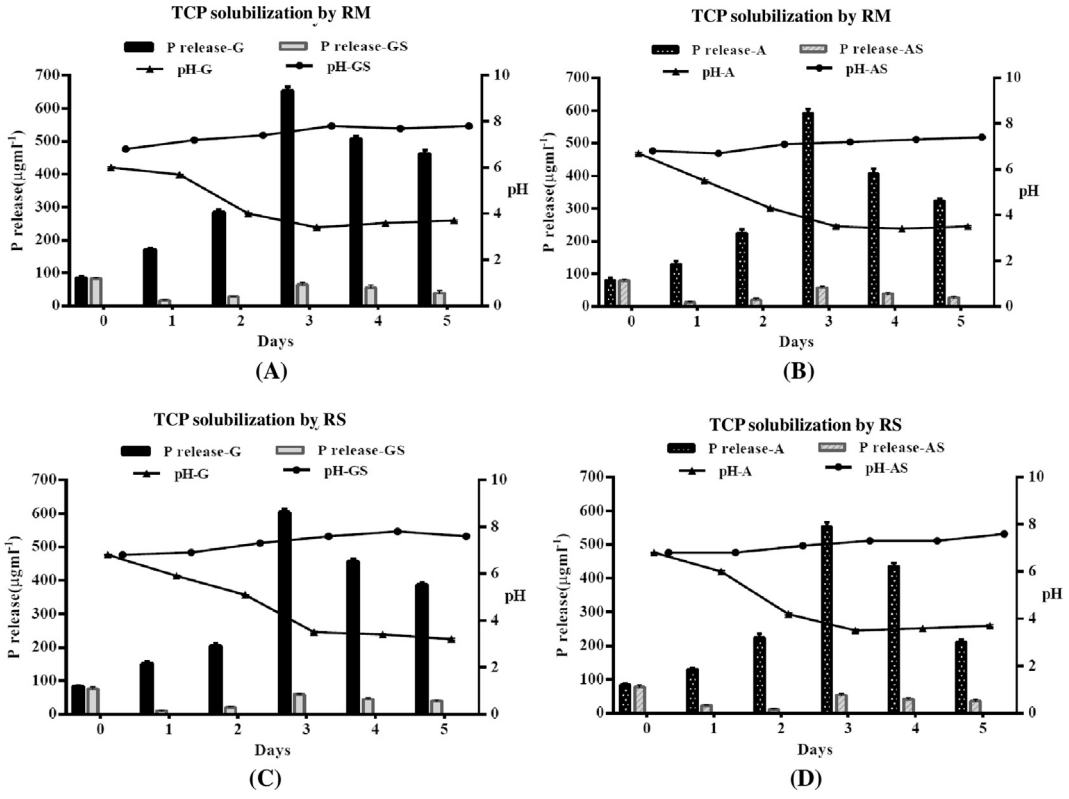

FIG. 2. TCP solubilization. By RM: (A) glucose and glucose+succinate, (B) arabinose and arabinose + succinate; by RS: (C) glucose and glucose + succinate, (D) arabinose and arabinose + succinate. Bars in the graph indicate P release on each day and lines in the graph indicate pH. Values are mean ± standard deviation of three independent observations in triplicates.

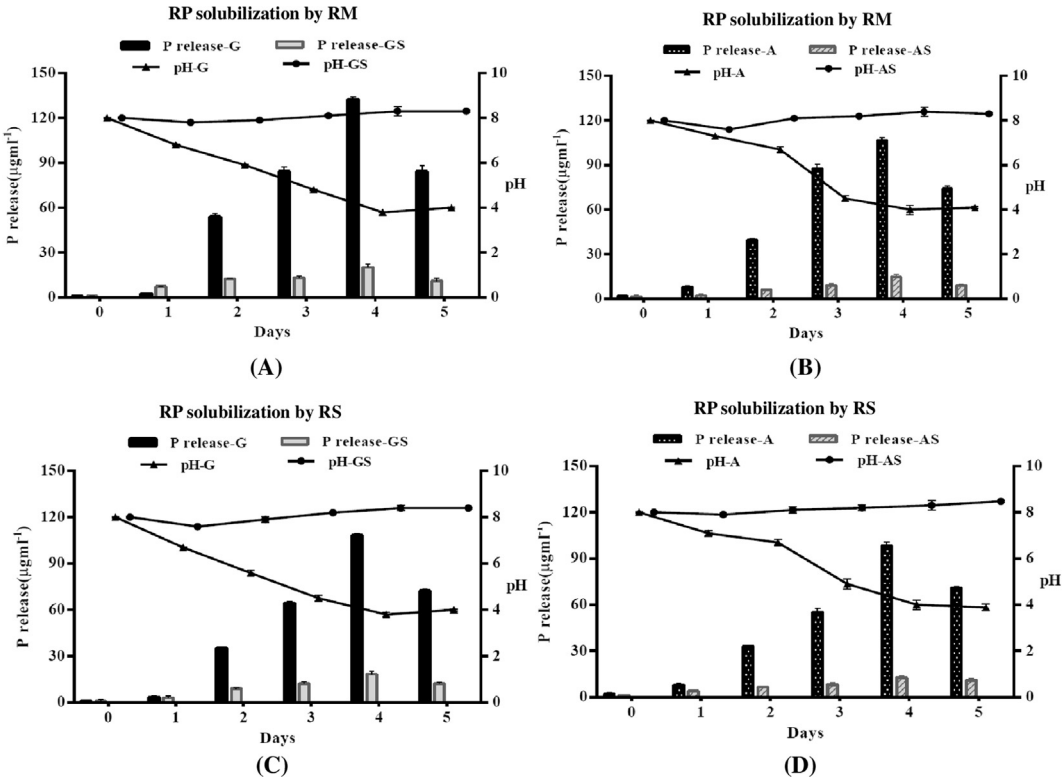

FIG. 3. RP solubilization. By RM: (A) glucose and glucose + succinate, (B) arabinose and arabinose + succinate; by RS: (C) glucose and glucose + succinate, (D) arabinose and arabinose + succinate. Bars in the graph indicate P release on each day and lines in the graph indicate pH. Values are mean ± standard deviation of three independent observations in triplicates.

in combination of C sources (50 mM arabinose/glucose + 50 mM succinate) even after prolonged incubation (Fig. S1B, D, and F). In fact, the region around the bacterial colony turned slightly black indicating that the cells were experiencing nutritional stress and were unable to grow possibly due to P deprivation (21). TCP solubilization by RM significantly decreased to 63 μg ml^{-1} (pH 7.8) in presence of glucose + succinate and 58 μg ml^{-1} (pH 7.2) in presence of arabinose + succinate (Fig. 2A and B). In case of RS, TCP solubilization decreased to 58 μg ml^{-1} (pH 7.6) in presence glucose + succinate and 54 μg ml^{-1} (pH 7.3) in presence of arabinose + succinate (Fig. 2C and D). In presence of glucose + succinate, RP solubilization by RM significantly decreased to 19.8 μg ml^{-1} with pH 8.3 and in arabinose + succinate it decreased to 14.8 μg ml^{-1} with pH 8.4 (Fig. 3A and B). Similarly in case of RS, RP solubilization significantly decreased to 18.4 μg ml^{-1} with pH 8.4 in glucose + succinate and 12.7 μg ml^{-1} with pH 8.3 in arabinose + succinate (Fig. 3C and D). This data suggested that succinate significantly repressed P solubilization in RM and RS irrespective of sugar being glucose or arabinose (Figs. 2 and 3). C_4 acids like succinate present in the plant root exudates may cause repression of P solubilization which could compromise performance of PSBs *in vivo* even though they are promising *in vitro* (37). Succinate-mediated repression of glucose utilization and MPS phenotype in *P. aeruginosa* and *K. pneumoniae* has already been reported (21,22). Compared to the P release obtained with 100 mM glucose, 85% and 86% repression was obtained for RM and RS, respectively, in the presence of 50 mM glucose + 50 mM succinate. Similarly, compared to the P release obtained with 100 mM arabinose, 83% and 87% repression was obtained for RM and RS, respectively, in the presence of 50 mM arabinose + 50 mM succinate. Our laboratory has previously reported succinate-mediated repression of TCP and RP solubilization in *Pseudomonas* sp., *Erwinia* sp., *Acinetobacter* sp., *Enterobacter* sp. (37) and *K. pneumoniae* (22). So far no rhizobia with the ability to solubilize P using two different sugars, arabinose and glucose and its repression by succinate have been reported.

Effect of succinate concentration on P solubilization Fig. S2 depicts the effect of succinate concentration on RP solubilization in RM and RS. In glucose containing media with varying concentration (10–50 mM) of succinate, 53–90% repression was obtained for RM and 51–82% repression was obtained for RS. In arabinose containing media with similar varying concentration of succinate, 47–85% repression was obtained for RM and 55–89% repression was obtained for RS. These results suggested succinate concentration dependent repression of P solubilization in RM and RS.

Growth experiments RM and RS grown on 20 mM glucose, arabinose and succinate showed typical monoauxic growth pattern. The maximum OD_{600} obtained with RM was 2.3, 1.8 and 1.2 in presence of glucose, arabinose and succinate, respectively, after 18 h, 21 h and 24 h of incubation. Similarly, the maximum OD_{600} obtained with RS was 2.1, 1.7 and 1 in presence of glucose, arabinose and succinate, respectively, following 20 h, 24 h and 26 h of incubation. In RM and RS, glucose supported maximum growth followed by arabinose and succinate (Fig. S3).

Determination of organic acid HPLC analysis of cell free supernatants identified the production of gluconic acid and oxalic acid by both the species RM and RS when grown on glucose and arabinose as sole C source, respectively. RM and RS produced 24 mM and 20 mM gluconic acid from 100 mM glucose which was reduced to 10 mM and 8 mM, respectively, in the presence of 50 mM glucose + 50 mM succinate. This indicated a 58% and 60% reduction in acid production (data not shown). Secretion of organic

acids mainly gluconic acid (*Pseudomonas* sp., *E. herbicola* and *B. cepacia*), 2-ketogluconic acid (*Rhizobium meliloti, R. leguminosarum* and *Bacillus firmus*) and mixture of acids like lactic, and acetic acid (*Bacillus amyloliquefaciens* and *Bacillus licheniformis*) involved in P solubilization has been frequently reported (9,38,39). RM and RS produced 28 mM and 23 mM oxalic acid from 100 mM arabinose which was significantly repressed to 9 mM and 8 mM in the presence of 50 mM arabinose + 50 mM succinate, indicating 68% and 65% repression, respectively. Oxalic acid mediated glucose dependent MPS phenotype in *Klebsiella* has been previously reported (22). Both the isolates were far better than other reported PSBs because none of the rhizobacteria reported till date could produce gluconic acid as well as oxalic acid by using glucose and arabinose, respectively. Oxalic acid mediated P solubilization using arabinose as C source and its repression by succinate is unique to these isolates.

Glucose dehydrogenase (Gcd) (EC 1.1.5.2) activity and its repression Gluconic acid production is catalyzed by periplasmic oxidation of glucose by Gcd in *Enterobacter* 60-2G (40). Gcd activity of 44 IU (mg protein)$^{-1}$ in RM (Fig. 4A) and 36 IU (mg protein)$^{-1}$ in RS when grown in glucose was significantly repressed to 65% and 66%, respectively, in glucose + succinate and up to 90% when grown in succinate. Our observations are in accordance with the earlier reports that showed succinate-mediated repression of glucose dehydrogenase in *Klebsiella* sp. (22) and succinate and malate mediated repression of Gcd in *P. aeruginosa* M3 and SP1 (21).

Enzymes involved in arabinose metabolism and its link with MPS phenotype Activities of arabinose dehydrogenase, L-KDA aldolase, glycolaldehyde dehydrogenase and glyoxylate oxidase were checked in presence of arabinose, succinate and arabinose + succinate. The conversion of L-arabinose to L-arabino-γ-lactone was measured by reduction of $NADP^+$ spectrophotometrically. In presence of arabinose, activity of arabinose dehydrogenase in RM was 125 IU (mg protein)$^{-1}$ which was significantly repressed by 77% in arabinose + succinate (Fig. 4B). When grown in minimal medium supplemented with 0.1% arabinose, arabinose dehydrogenase activity in *R. meliloti*, *Rhizobium trifolli*, *R. leguminosarum* and *Rhizobium* sp. 32H1 was found to be 14, 184, 42 and 34 IU (mg protein)$^{-1}$, respectively (14). In presence of arabinose, activity of L-KDA aldolase in RM was 102 IU (mg protein)$^{-1}$ which was significantly repressed by 82% in arabinose + succinate (Fig. 4C). When grown in minimal medium supplemented with 0.1% arabinose, L-KDA aldolase activity in *R. meliloti*, *R. trifolli*, *R. leguminosarum* and *Rhizobium* sp. 32H1 was found to be 15, 0.5, 0.5, and 42 IU (mg protein)$^{-1}$, respectively (14). In presence of arabinose, glycolaldehyde dehydrogenase activity in RM was 96 IU (mg protein)$^{-1}$ which was significantly repressed by 87% in arabinose + succinate (Fig. 4D). When grown in minimal medium supplemented with 0.1% arabinose, glycolaldehyde dehydrogenase activity in *R. meliloti*, *R. trifolli*, *R. leguminosarum* and *Rhizobium* sp. 32H1 was found to be 6, 0.5, 3 and 25 IU (mg protein)$^{-1}$, respectively (14). Glyoxylate oxidase activity was checked in a DCIP-linked reaction system. The activity of glyoxylate oxidase in RM was 83 IU (mg protein)$^{-1}$ in arabinose which was significantly repressed by 84% in arabinose + succinate (Fig. 4E). When grown in succinate as sole carbon source, 93–95% repression in activities of all the estimated enzymes was observed. In presence of arabinose, activity of arabinose dehydrogenase, L-KDA aldolase, glycolaldehyde dehydrogenase and glyoxylate oxidase of RS was 118, 96, 86 and 80 IU (mg protein)$^{-1}$, respectively, which was significantly repressed by 70–80% in presence of arabinose + succinate.

L-Arabinose is a good carbon source for the growth of rhizobia and is metabolized by distinct pathways in slow and fast growing rhizobial species. In both groups, the first two enzymatic reactions are conserved following which the pathways diverged to produce pyruvate and glycolaldehyde in the slow growing rhizobia and α-ketoglutarate in the fast growing rhizobia (41). In slow growing rhizobia (our case), pyruvate is most likely oxidized via TCA cycle

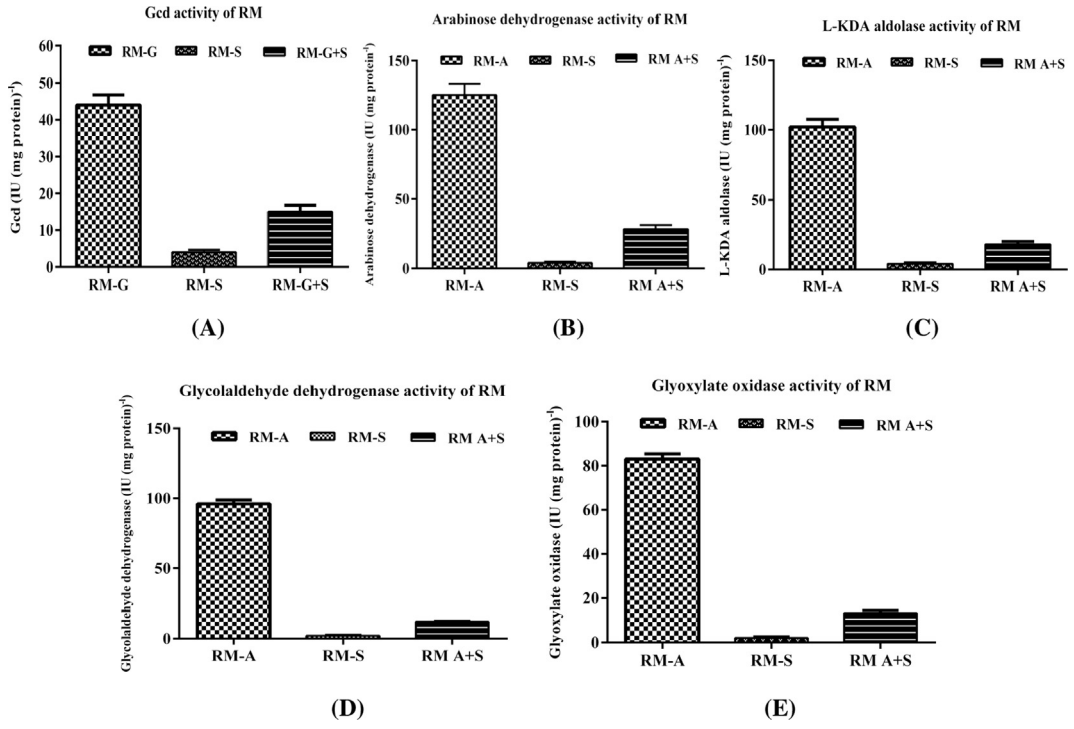

FIG. 4. Enzyme activities by RM. Activities of periplasmic glucose oxidation and arabinose metabolism enzymes of RM: (A) glucose dehydrogenase, (B) arabinose dehydrogenase, (C) L-KDA aldolase, (D) glycolaldehyde dehydrogenase and (E) glyoxylate oxidase. Enzyme activities are expressed in IU (mg protein^{-1}) and the values are mean ± standard deviation of three independent observations in triplicates.

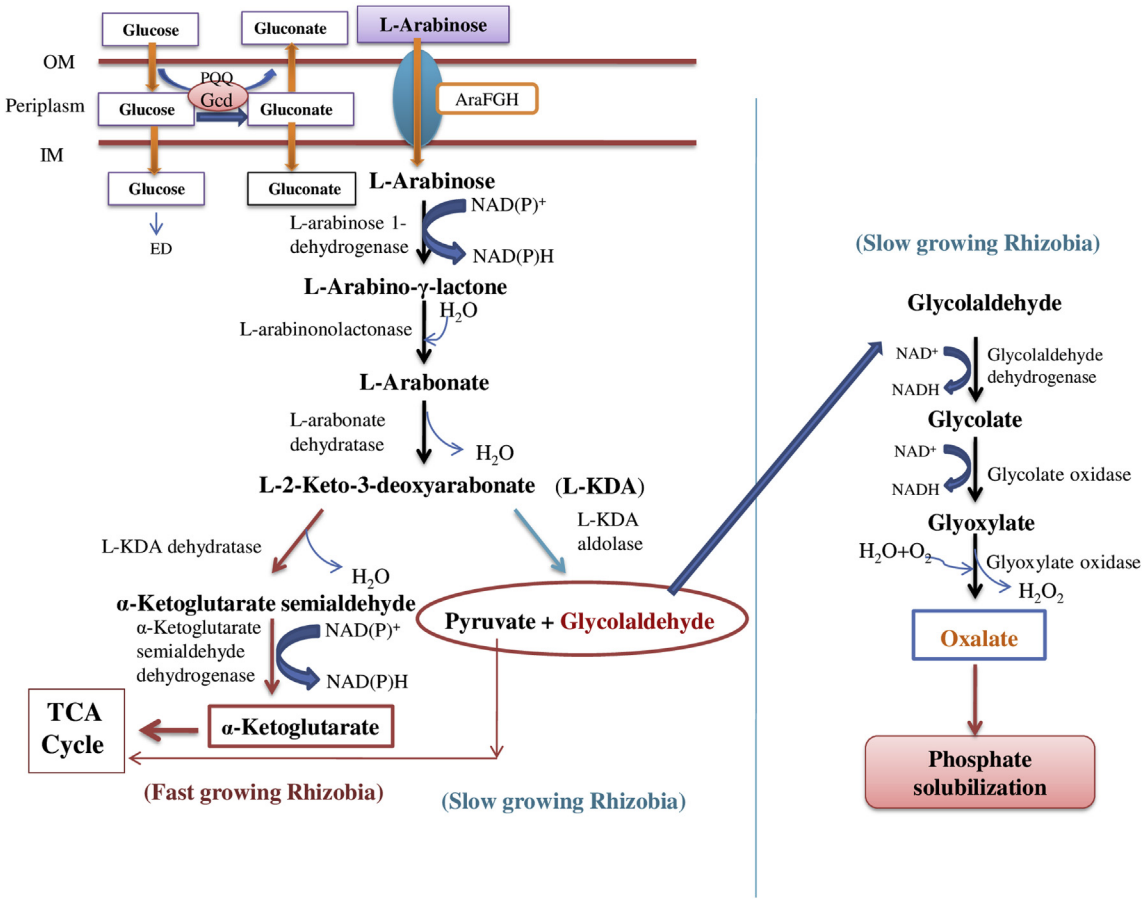

FIG. 5. Proposed pathway linking arabinose utilization and P solubilization in *Rhizobium* sp. RM. Arabinose metabolism differentiates fast and slow growing rhizobia. L-Arabinose is converted into α-ketoglutarate in the case of fast growing rhizobia and to glycolaldehyde and pyruvate in the case of slow growing rhizobia. Glycolaldehyde was oxidized to oxalate by series of enzymes leading to MPS.

but the fate of glycolaldehyde needed to be resolved with respect to P solubilization. Based on our results, glycolaldehyde might have been oxidized to oxalate by series of enzymatic reactions (Fig. 5). Koch et al. (18) reported arabinose utilization leading to oxalate production with further conversion of oxalate to formate in *B. japonicum*. Based on our findings and earlier reports, a metabolic scheme representing the biochemical link of enzymes of glucose and arabinose metabolism with organic acid production leading to phosphate solubilization has been proposed (Fig. 5).

In this study, the mechanism of P solubilization in RM and RS has been investigated by linking arabinose metabolism to oxalic acid production. Gluconate production from glucose has already been established in several bacterial species. In slow growing rhizobia (our isolates), glycolaldehyde formed glycolate which was oxidized to oxalate leading to arabinose dependent P solubilization. Gluconic acid was produced from glucose via direct glucose oxidation pathway. Gcd responsible for gluconic acid production and arabinose dehydrogenase and glyoxylate oxidase important for oxalic acid production were repressed by succinate in *Rhizobium* sp. RM and RS (Fig. 4).

So, here we conclude that rhizobial isolates RM and RS are unique as they secreted oxalic acid up to 28 mM, sufficient for RP solubilization. These *Rhizobium* species secreting both gluconic and oxalic acids in high amounts are ideal for MPS in alkaline vertisols. Unraveling the mechanism of P solubilization and its repression in these rhizobia will help in understanding the complex relation between the carbon sources arabinose, glucose and succinate and their effect on MPS phenotype. Here, we conclude that RM and RS followed distinct pathway for oxalic acid production when arabinose was the sole carbon source. This study would certainly help in understanding one of the factors that may influence efficiency of these PSBs in field conditions and possibly help in drawing a strategy to overcome it.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.04.020.

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