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ORIGINAL PAPER

Sucrose dependent mineral phosphate solubilization in *Enterobacter asburiae* PSI3 by heterologous overexpression of periplasmic invertases

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Abstract *Enterobacter asburiae* PSI3 solubilizes mineral phosphates in the presence of glucose by the secretion of gluconic acid generated by the action of a periplasmic pyrroloquinoline quinone dependent glucose dehydrogenase. In order to achieve mineral phosphate solubilization phenotype in the presence of sucrose, plasmids pCNK4 and pCNK5 containing genes encoding the invertase enzyme of *Zymomonas mobilis* (*invB*) and of *Saccharomyces cerevisiae* (*suc2*) under constitutive promoters were constructed with *malE* signal sequence (in case of *invB* alone as the *suc2* is secreted natively). When introduced into *E. asburiae* PSI3, *E. a.* (pCNK4) and *E. a.* (pCNK5) transformants secreted 21.65 ± 0.94 and 22 ± 1.3 mM gluconic acid, respectively, in the presence of 75 mM sucrose and they also solubilized 180 ± 4.3 and 438 ± 7.3 µM P from the rock phosphate. In the presence of a mixture of 50 mM sucrose and 25 mM glucose, *E. a.* (pCNK5) secreted 34 ± 2.3 mM gluconic acid and released 479 ± 8.1 µM P. Moreover, in the presence of a mixture of eight sugars (10 mM each) in the medium, *E. a.* (pCNK5) released 414 ± 5.3 µM P in the buffered medium. Thus, this study demonstrates incorporation of periplasmic invertase imparted P solubilization ability to *E. asburiae* PSI3 in the presence of sucrose and mixture of sugars.

Keywords *Enterobacter asburiae* PSI3 · Periplasmic invertase · Mineral phosphate solubilization · Gluconic acid

Introduction

Rhizospheric microorganisms possessing the ability to solubilize the bound phosphates have been considered agriculturally important (Gyaneshwar et al. 2002; Zaidi et al. 2009). Most phosphate solubilizing microorganism (PSMs) poorly solubilize soil mineral phosphates by the acidification of the soil through secretion of organic acids (Archana et al. 2012). PSMs secreting gluconic acid or 2-ketogluconic acid have been well characterized and these acids have been established as the basis of mineral phosphate solubilizing (MPS) phenotype dependent on the action of glucose dehydrogenase (GDH) and gluconate dehydrogenase (GAD) (Kim et al. 2003a; Sashidhar and Podile 2010; Sharma et al. 2013; Adhya et al. 2015). Recently our laboratory has reported many genetic modification approaches to allow rhizobacteria to secrete gluconic, 2-ketogluconic, oxalic and citric acids by the incorporation of specific genes involved in organic acid biosynthesis (Kumar et al. 2013; Adhikary et al. 2014; Wagh et al. 2014a, b, 2016; Yadav et al. 2014a, b).

Gluconic acid is the prominent organic acid produced by direct oxidation pathway via membrane-bound quinoprotein glucose dehydrogenase (GDH), which catalyzes the first step of direct glucose oxidation pathway resulting in the conversion of glucose to gluconic acid (Goldstein et al. 1993). Depending on the substrate specificity of the enzyme, GDH may also oxidize various aldoses including

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disaccharides to their corresponding aldonic acids (Sharma et al. 2005). The quinoprotein GDH controls the unique step in direct oxidation, where it transfers electrons from aldose sugars to electron transport chain via two electrons, two proton oxidations mediated by the cofactor PQQ. GDH mediated periplasmic oxidation of aldose sugars can contribute electrons directly to the respiratory electron transport pathway (Goldstein 1995). GDH enzyme mechanism and properties are well characterized in various organisms (Goodwin and Anthony 1998; Elias et al. 2004). In addition, protons generated from these oxidations can contribute directly to the trans-membrane proton motive force. GDH is an enzyme with diverse functions, one of which is its role in MPS owing to its ability to produce of organic acid such as gluconic acid (Goldstein 1995). GDH enzyme is known to exhibit broad substrate specificity in many organisms including *Enterobacter asburiae* PSI3 which efficiently solubilizes mineral phosphates by phosphate starvation-inducible GDH (Gyaneshwar et al. 1998). The ability to oxidize several aldose sugars is of advantage in P solubilization since the PSM can show MPS phenotype not only when growing on different aldosugars but also on a mixture of several sugars which can be cumulatively used for enhanced acidification and P solubilization (Sharma et al. 2005).

Root exudates contain a substantial amount of reduced carbon compounds which are secreted in the rhizosphere (Bais et al. 2006; Terzano et al. 2015). Rhizosphere microorganisms utilize root exudates as their major carbon source which is responsible for root colonization. Apart from glucose and fructose, sucrose is one of the most abundant sugars present. High levels of sucrose were found at the root tips of annual grass *Avena barbata* (Jaeger et al. 1999). Sucrose has been detected in large amounts in the soil near the root tip and large numbers of bacteria occur near the root area, with the highest sucrose and tryptophan exudation. Cowpea root exudates contain arabinose, ribose, glucose, and sucrose as the main constituents (Odunfa and Werner 1981). Glucose and fructose were the major components in all growth stages of stone wool-grown tomato (Kamilova et al. 2006). Roots of *Umbelliferae* family plants *Peucedanum alsaticum* and *Peucedanum cervaria* exude fructose, glucose, mannitol and sucrose which are available to root associated microorganisms (Hadacek and Kraus 2002).

One of the drawbacks of gluconic acid producing bacteria as PSMs is their inability to show efficient P solubilization using sucrose or fructose as the carbon sources since they are not GDH substrates. Organic acid secretion by PSMs in the presence of sugars which are not substrates of GDH is of interest because it broadens the range of C sources used for P solubilization. *Enterobacter hormaechei* DHRSS (previously denoted as *Citrobacter* spp. DHRSS) shows cytoplasmic invertase activity and solubilizes P using sucrose as the carbon source by the fermentative breakdown of the sugar leading to acetic acid secretion (Patel et al. 2008). Since acetic acid is a weak acid and is required in large amount for P solubilization, stronger acids like gluconic or 2-ketogluconic would be preferred (Gyaneshwar et al. 1998). It could be hypothesized that a periplasmic invertase could produce glucose from sucrose extracellularly and this could be coupled to the direct oxidation pathway involving GDH and GAD leading to gluconic and 2-ketogluconic acid secretion resulting in more efficient P solubilization using sucrose as the C source. Bacteria possessing invertase gene could also utilize raffinose as a substrate due to its structural similarity (Pollock, 1986). The present study tested the above hypothesis by heterologous cloning of the invertase encoding gene in *E. asburiae* PSI3, which natively possesses GDH enzyme and the PQQ synthesizing machinery. The modified strain successfully utilized sucrose to produce gluconic acid and demonstrated MPS ability on sucrose thus broadening the range of C sources on which its MPS phenotype is seen. Rhizobacteria demonstrating MPS ability using sucrose along with other sugars as carbon sources for P solubilization could be very effective in field conditions wherein root exudates provide multiple carbon sources.

Materials and methods

Bacterial strains, plasmids and bacteriological media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH10B (Invitrogen, USA) was used as intermediate cloning host during plasmid construction. *E. coli* DH10B, *E. asburiae* PSI3 (*E. a.* PSI3) and its plasmid derivatives were grown on Luria–Bertani (LB) medium (Hi Media, India) at 37 °C containing 20 µg/ml streptomycin, 50 µg/ml ampicillin, 50 µg/ml kanamycin and 10 µg/ml gentamycin as and when required. *E a* PSI3 has been deposited in Microbial Type Culture Collection (NCCS, Pune) for public access and allocated the strain number MCC 2292.

Construction of constitutive expressing periplasmic invertases

Routine molecular biology experiments such as plasmid preparations, transformation and in vitro manipulation of DNA were done according to standard molecular biology protocols (Sambrook and Russell 2001). All restriction enzyme used were purchased from Thermo Scientific, USA. dNTPs were purchased from Sigma, USA. For

Table 1 Bacterial strains and plasmid used in this study

Bacterial strains	Genotype	Reference
E. coli DH10B	F⁻*endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)* λ^-	Invitrogen, USA
E. coli BL 21 (DE3)	F⁻*ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) *λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Sambrook and Russell (2001)
E. asburiae PSI3	*Cajanus cajan* rhizosphere isolate	Gyaneshwar et al. (1998), Sharma et al. (2005)
Plasmids		
pTTQGm^r	pUC18 *ori,* Amp^r, Gm^r, *tac*	Kumar et al. (2013)
pMal-p2	Amp^r, *tac, malE*	New England Biolabs, USA
pUZE3	Amp^r, *lac*, pUC ori, *Zymomonas mobilis invB* gene	Yanase et al. (1998)
pRT23.04	pGEM-2 origin, Amp^r, *Saccharomyces cerevisiae* invertase (*suc*2) gene	Roitsch and Lehle (1988)
pCNK3	pET22b(+), Amp^r, *Zymomonas mobilis* invertase gene *invB* (periplasmic expression vector)	This study
pCNK4	pBBR1MCS-2, Kan^r, **lac malE-invB,*	This study
pCNK5	pTTQGm^r, Amp^r, Gm^r, **tac*, *suc*2	This study

* Constitutive promoter

constitutive expression of invertases, a *lac* and *tac* promoter with a abolish *lac* repressor binding site was inserted upstream of the start codon (Datta et al. 2002). DNA fragment from pUZE3 containing 1.5 kb *invB* gene was excised out by digestion with EcoRI and HindII restriction enzymes (RE) and cloned into periplasmic expression vector pET22b(+) within the same RE sites to obtain pCNK3. *MalE* signal sequence was amplified from plasmid pMal-p2 (New England Biolabs, USA) using forward (lac MBP FP) and reverse primers (MBP RP) (Integrated DNA technology, USA) (Table S1) with the help of *Pfu* polymerase (Thermo Scientific, USA). For constitutive gene expression under the *lac* promoter, a second set of primer (lac1)s was used to amplify ~200 bp region of the *E.coli* lac promoter which is denoted as the *lac** promoter along with the cloned with the *malE* signal sequence. 1.2 kb *invB* was amplified with gene-specific primers ZE3 FP and ZE3RP using plasmid pUZE3 as the template. Recombinant PCR was done with primers lac FP and ZE3 RP to join constitutive *lac** promoter with the *malE* signal sequence with the *invB* gene to obtained 1.4 kb PCR product. This PCR product was cloned in EcoRV digested pBBRMCS-2 vector. Clone was confirmed by restriction digestion and plasmid was designated as pCNK-4.

The 1.6 kb *suc2* gene was PCR amplified by gene specific primers Suc2 FP and Suc2 RP primers (Table S1) using plasmid pRT23.04 as a template. Constitutive *tac* promoter sequence was added to the amplified PCR product by performing second PCR amplification using FP Tac and Suc2 RP primers. Amplified PCR product was cloned in pTTQGm under SmaI RE site. Obtained clone was named as pCNK5.

Physiological experiments

Wild-type *E. a.* PSI3 and its transformants were grown overnight in LB with respective antibiotics at 37 °C on an Orbitek rotary shaker (Scigenics, India) at under 200 rpm shaking conditions. Cultures were washed thrice in normal saline (0.85 % NaCl) and used to inoculate Tris rock phosphate (TRP) minimal medium containing glucose, sucrose or mixture of both and mixture of eight sugars includes glucose, sucrose, xylose, arabinose, galactose, cellobiose, mannose and maltose. Batch culture studies were done in 150 ml conical flasks containing 30 ml of TRP medium having 1 % inoculant and grown at 37 °C under shaking conditions. The cultures were analyzed for growth, pH changes during growth, P solubilization and organic acid secretion as described below.

MPS ability of recombinant *E. a.* PSI3 strains was determined qualitatively on TRP minimal medium plates (50, 75 and 100 mM Tris buffer pH-8.0, 1 mg/ml Senegal Rock Phosphate (RP), 1 % methyl red, 1.8 % agar and varied concentration of glucose or sucrose as or when required). About 3–5 µl of saline washed bacterial inoculum was spotted on TRP plates and incubated at 37 °C. Phosphate solubilization on TRP plates was determined by monitoring the red zone of acidification.

Invertase assay

Wild-type and recombinant cultures were grown overnight in M9 minimal medium with sucrose as the carbon source. Cells were harvested by centrifugation at 5000x*g* for 10 min. Whole cells as well as toluenized cells, were used

for enzyme assay. The assay system (2 ml) consisted of 0.01 M sucrose, 0.1 M buffer (either aetate buffer at pH 5.0 or phosphate buffer, pH 7.0, or Tris HCl buffer, pH 8.0), with an appropriate amount of cells as the source of enzyme. The reaction system was incubated at 37 °C for 30 min and the reducing sugar produced in cell free supernatant was measured by GOD-POD kit (Enzopak, Reckon Diagnostics). One unit of activity is defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmol of glucose per h. Specific activity is defined as units per milligram of total protein. Total protein was estimated using a modified Lowry's method (Peterson 1979).

GDH assay

GDH Activity (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm (Quay et al. 1972). Whole cells as well as toluenized cells, were used for enzyme assay. The molar absorbance of DCIP was taken as 15.1 mM^{-1} cm^{-1} at pH 8.75. The reaction mixture included: Tris–Cl buffer (pH 8.75), 16.66 mM; D-glucose, 66 mM; DCIP, sodium salt, 0.05 mM; phenazine methosulfate, 0.66 mM; sodium azide, 4 mM; whole cells, and distilled water to 3.0 ml. One unit of GDH activity was defined as μmoles of 2,6- dichlorophenolindophenol reduced per minute. Specific activity was defined as units per mg total protein estimated by a modified Lowry's method (Peterson 1979).

Analytical methods

All the physiological experiments were done with the initial cell density of ~0.025 OD at 600 nm as monitored spectrophotometrically (path length of 1 cm; Shimadzu UV-1700 spectrophotometer). Growth was monitored as an increase in absorbance at 600 nm and pH of the medium was monitored at 12 h time intervals to monitor acidification of medium. Samples (2 ml) were taken from flasks when the pH of the medium reached below 5 and were centrifuged at 9200×*g* for 10 min at 4 °C and the culture supernatants collected were used to estimate organic acid and inorganic phosphate (Pi). Pi estimation was done by Ames method (Ames 1964). Organic acids were identified and quantity determined by HPLC analysis for which the culture supernatant was passed through 0.2 μm nylon membranes (Pall Life Sciences, India) before injecting into the RP C-18 column of the Prominence UFLC (Shimadzu corporation, Japan) equipment. The column was operated at room temperature using mobile phase of 0.02 % sulphuric acid at a flow rate of 1.0 ml min–1 and the column effluents were monitored using a UV detector at 210 nm. Standards of organic acids (Sigma, USA) were prepared in double distilled water, filtered using 0.2 μm nylon membranes and were subjected to chromatography under similar conditions for determining the individual retention time. Comparison of peak area with external standards was used for quantification of organic acids.

Data analysis

Physiological experiments were done in four to six independent experiments. Data are expressed in mean ± standard deviation. The statistical analysis of all the parameters has been done using Graph Pad Prism (version 5.0) software.

Results

Functionality of the cloned invertases was evident from the good growth of *E. coli* (pCNK4) and (pCNK5) transformants on M9 minimal medium containing sucrose and raffinose (Fig. 1) while wild-type strain and vector control (containing empty plasmid) failed to grow on these sugars. Periplasmic invertase specific activity of *E. a.*(pCNK4) and *E. coli* (pCNK4) were 43.8 ± 0.03 mU and 40.3 ± 0.05 mU, respectively, while *E. coli* (pCNK5) and *E. a.*(pCNK5) had 18.4 ± 0.02 mU and 29.3 ± 0.04 mU/mg (Table 2). As expected *E. coli* transformants did not show GDH activity whereas the GDH activity of *E. asburiae* PSI3 was unaffected due to the presence of plasmids (Table 2).

Effect of overexpression of periplasmic invertases on sucrose-dependent MPS ability of *E. asburiae* PSI3

E. a. PSI3 (pCNK4) showed acidification and MPS ability on TRP plates containing 75 mM sucrose with 75 mM Tris buffer pH-8.0 (Fig. 2a-1) and 50 mM sucrose with 50 mM Tris buffer pH-8.0 (Fig. 2a-2) while *E. a.* PSI3 wild type and *E. a.* PSI3 (pBBR1MCS-2) did not show acidification and MPS ability. In liquid TRP medium, the growth of *E. a.* PSI3 (pCNK4) reached up to 0.4 O.D. and acidified the medium to pH 4.8 in 96 h (Fig. 2b, c). The Pi release was found to be 180 ± 4.3 μM (Table 3). *E. a.* PSI3 wild type did not secrete gluconic acid when sucrose provided as a sole carbon source thus did not show acidification of medium while *E. a.* PSI3 (pCNK4) produced 21.65 ± 0.94 mM gluconic acid (Table 3).

E. a. PSI3 (pCNK5) showed MPS ability on TRP agar plates containing varied amount of sucrose (sucrose and glucose) and buffering condition while vector control failed to show any MPS ability in these conditions (Fig. 3a). In TRP liquid medium containing 75 mM sucrose and 75 mM

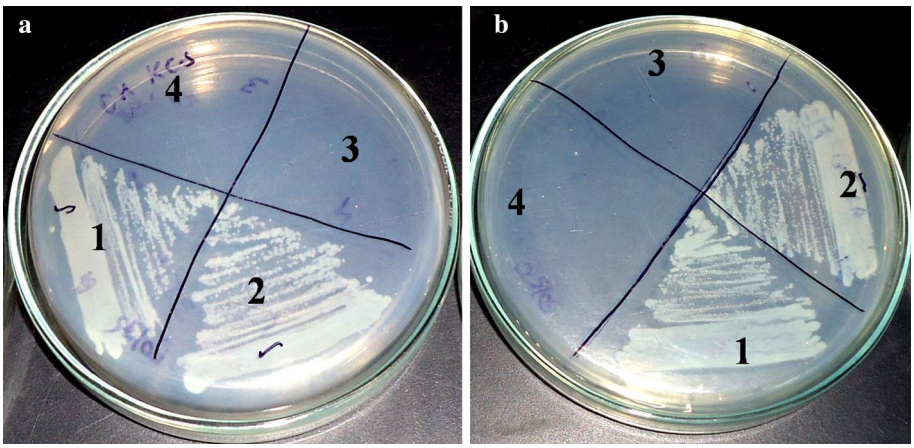

Fig. 1 Functional expression of pCNK4 and pCNK5 in *E. coli* transformants as seen by growth on M9 medium containing **a** sucrose and **b** raffinose as the sole carbon source. *1E. coli* BL21 (DE3) (pCNK4), *2 E. coli* BL21 (DE3) (pCNK5), *3 E. coli* BL21 (DE3) (pBBR1MCS-2) and *4 E. coli* BL21 (DE3) (pTTQGm)

Table 2 Specific enzyme activity of invertase and GDH

Transformants	Invertase activity (mU/mg protein)	GDH activity (U/mg protein)
E.coli (pBBRMCS-2)	UD	UD
E.coli (pCNK4)	43.8 ± 0.03	UD
E.coli (pTTQGm)	UD	UD
E.coli (pCNK5)	18.4 ± 0.02	UD
E.a. (pBBRMCS-2)	UD	0.980 ± 0.028
E. a.(pCNK4)	40.3 ± 0.05	0.965 ± 0.021
E.a. (pTTQGm)	UD	0.960 ± 0.014
E.a. (pCNK5)	29.3 ± 0.04	0.975 ± 0.035

UD undetectable

Tris pH-8.0, significant growth and pH drop was observed in *E. a.* PSI3 (pCNK5) compared to vector control (Fig. 3b, c). *E. a.* PSI3 (pCNK5) produced 22 mM gluconic acid and the Pi released was found to be 438 ± 7.3 µM (Table 3). When 25 mM glucose and 50 mM sucrose with 100 mM Tris buffered pH-8.0 TRP medium were used, *E. a.* PSI3 (pCNK5) showed similar growth and pH drop pattern with improved MPS ability (Table 3). The Pi released was 479 ± 8.1 µM and produced 34 mM of gluconic acid (Table 3). A mixture of sugars was used to study the P solubilization by *E. a.* PSI3 (pCNK5) due to its high ability to release P as compared to *E. a.* PSI3 (pCNK4). When the mixture of eight sugars at 10 mM each was used in 100 mM Tris RP liquid medium, the *Ea* (pCNK5) grew up 0.6 O.D. and decreased pH to approximately 4 (Fig. 4a, b) with a simultaneous release of 414 ± 5.3 µM P in the medium (Table 3). The vector transformant did not release any P into the medium due to insufficient pH drop.

Discussion

Most PSMs release P from calcium phosphate complexes in the presence of high levels of glucose (~100 mM). However, in field conditions, where root exudates are the main source of carbon, the PSMs are faced with a variety of carbon sources but in low amounts (Bais et al. 2006; Terzano et al. 2015). Although the dynamics of the root exudation is not clear, the PSMs which could be effective using a wide variety of sugars as carbon sources for not only growth but also for expressing the MPS phenotype, are expected to have a greater potential in being successful in field conditions. In the present work we have broadened the range of sugars that can be utilized for displaying MPS phenotype in *E. asburiae* PSI3. The MPS ability of this organism is attributed to the oxidation of aldosugars to their corresponding acids by the action of a broad-substrate specific glucose dehydrogenase (GDH). Sucrose, an important sugar component of root exudates, does not contribute towards organic acid secretion mediated by GDH as it is not a substrate of GDH. The present study describes the effects of incorporation of two different periplasmic invertases in *E. asburiae* PSI3 which enabled this organism to secrete gluconic acid while growing on sucrose as the sole carbon source by the action of GDH on the glucose generated by the invertase. Expression of *Z. mobilis* periplasmic *invB* gene under constitutive *lac** promoter in *E. asburiae* PSI3 showed four fold lower enzyme activity compared to earlier reported *invB* expression from the pET vector in *E. coli* BL21 (DE3)

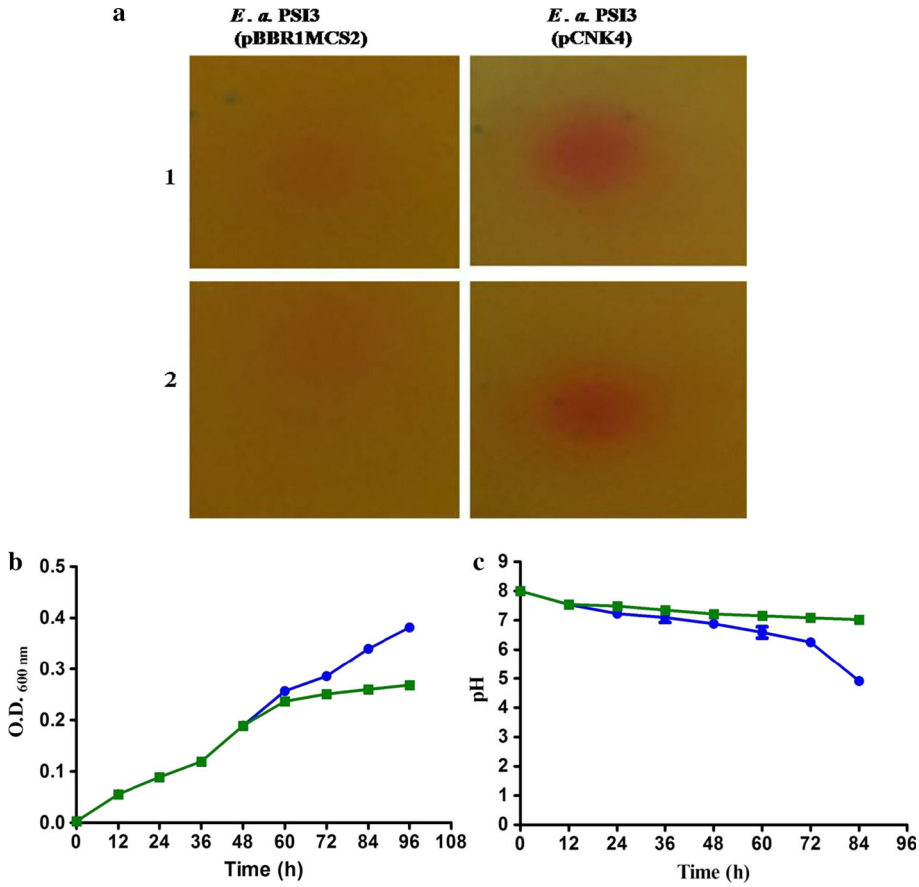

Fig. 2 Mineral phosphate solubilizing ability *E. a.* PSI3 (pCNK4) transformants on **a** sucrose containing TRP agar plate as indicated by red zone of acidification and **b** growth and **c** acidification on TRP minimal medium with 75 mM sucrose and 75 mM Tris–Cl pH 8.0. *Numbers* in panel **a** indicate *1* 50 mM Tris HCl (pH 8) containing 50 mM sucrose and *2* 75 mM Tris HCl (pH 8) containing 75 mM sucrose. Values for each time point are represented as mean ± SD of four to six independent observations. *Closed circles E. a.* PSI3 (pCNK4) and *Closed squares E. a.* PSI3 (pBBRMCS-2)

Table 3 Gluconic acid secretion and Pi released in the supernatant by *E. a.* PSI3 transfromants after 96 h of growth in TRP medium containing different buffer strength and amounts of sucrose and glucose

Plasmid carried by the *E. a.* PSI3 transformant	Tris (pH-8.0) + sucrose + glucose (mM)	Final $O.D._{600}$	pH	Gluconic acid (mM)	Pi (μM)
pBBR-MCS-2	75 + 75 + 0	0.21 ± 0.01	7.4 ± 0.06	ND	UD
pCNK4	75 + 75 + 0	0.31 ± 0.03	4.8 ± 0.04	21.65 ± 0.94	180 ± 4.3
pTTQGm	100 + 50 + 0	0.24 ± 0.02	7.8 ± 0.06	ND	UD
pCNK5	100 + 50 + 0	0.41 ± 0.03	6.4 ± 0.05	8.3 ± 0.82	UD
pTTQGm	75 + 50 + 0	0.31 ± 0.007	7.2 ± 0.07	ND	UD
pCNK5	75 + 50 + 0	0.44 ± 0.003	4.7 ± 0.04	22 ± 1.3	438 ± 7.3
pTTQGm	100 + 50 +25	0.34 ± 0.008	6.6 ± 0.09	9.4 ± 1.6	UD
pCNK5	100 + 50 +25	0.52 ± 0.02	4.2 ± 0.05	34 ± 2.3	479 ± 8.1
pTTQGm	100 + Mixture of 8 sugars* 10 mM each	0.36 ± 0.01	6.8 ± 0.07	ND	UD
pCNK5	100 + Mixture of 8 sugars* 10 mM each	0.62 ± 0.02	4.5 ± 0.03	ND	414 ± 5.3

UD undetected, *ND* not determined

* Composition given in text

under 10 L fermentation process (Vásquez-Bahena et al. 2006). However, *Z. mobilis invB* expression under *tac* promoter showed invertase activity that was ~22 % less as compared to *lac* promoter in *E. coli* (Yanase et al. 1998) so expression under *lac* promoter is preferred over *tac* for better expression. However, *suc2* gene expression in *E. asburiae* PSI3 under *tac* promoter showed two-fold higher periplasmic invertase activity compared to *S. cerevisiae* expressing from its own promoter (Rothe and Lehle 1998).

Enterobacter asburiae PSI3 containing periplasmic invertase produced gluconic acid leading to good MPS ability under the buffered condition which further

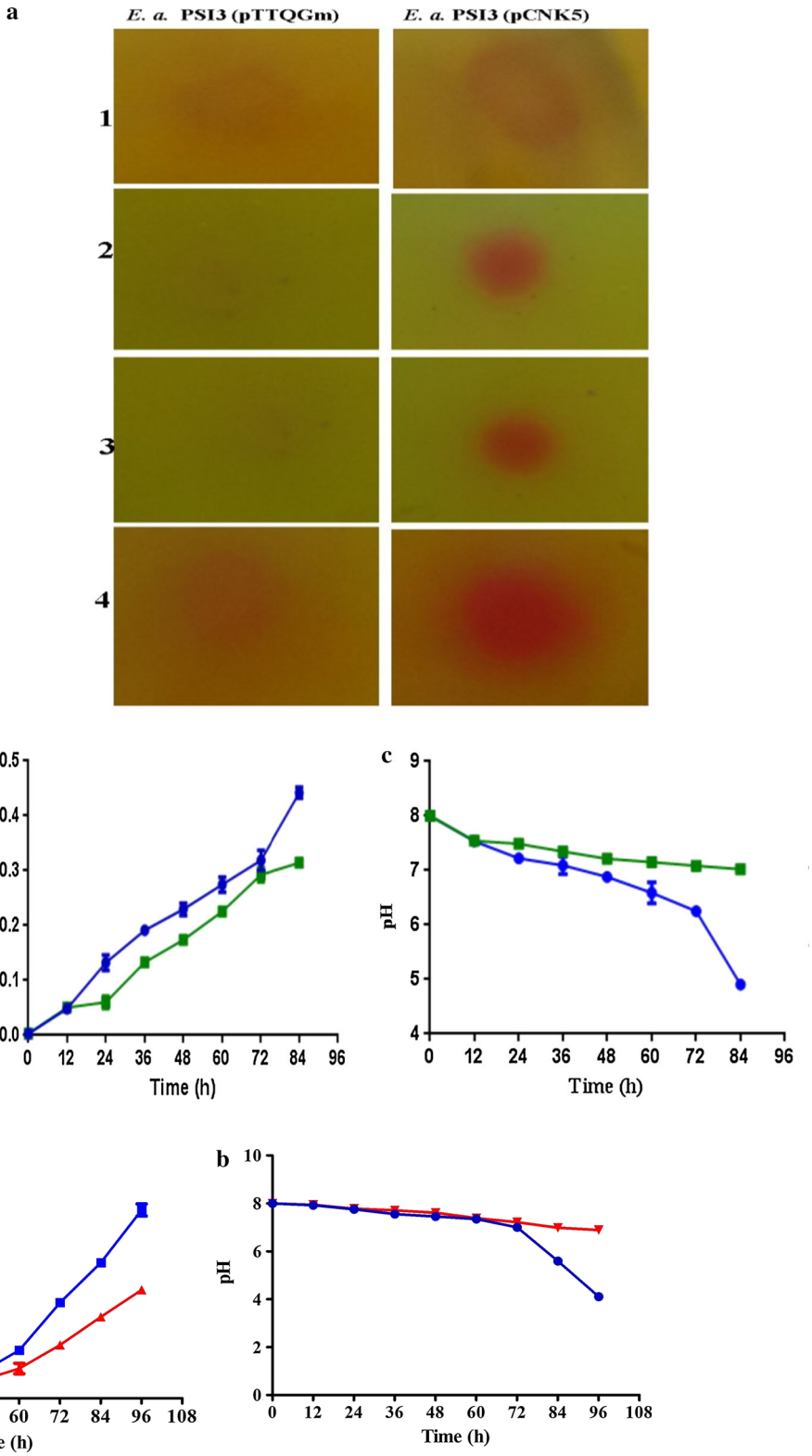

Fig. 3 Mineral phosphate solubilizing ability *E. a.* PSI3 (pCNK5) transformants on **a** sucrose containing TRP agar plate as indicated by red zone of acidification and **b** growth and **c** acidification on TRP minimal medium with 75 mM sucrose and 75 mM Tris–Cl pH 8.0. *Numbers* in panel **a** indicate *1* 100 mM Tris (pH-8.0) and 50 mM sucrose; *2* 50 mM Tris (pH-8.0) and 50 mM sucrose; *3* 75 mM Tris (pH-8.0) and 50 mM sucrose and *4* 100 mM Tris (pH-8.0), 50 mM sucrose and 25 mM glucose. *Closed circles E. a.* PSI3 (pCNK5); *Closed squares E. a.* PSI3 (pTTQGm). Values for each time point are represented as mean ± SD of four to six independent observations

Fig. 4 Growth (**a**) and pH profile (**b**) of *E. a.* PSI3 (pCNK5) transformants on TRP minimal medium containing mixture of 8 sugars 10 mM each and 100 mM buffer. *Closed circles E. a.* PSI3 (pCNK5) **b** *Inverted triangle E. a.* PSI3 (pTTQGm). Values for each time point are represented as mean ± SD of four to six independent observations

improved when sucrose and glucose were used together. Many fungi have been reported to show MPS ability on sucrose as carbon source. For instance, *Aspergillus aculeatus* solubilized Sonari rock phosphate and released 4.4 mg/100 ml P_2O_5 (Narsian and Patel 2000). *Penicillium rugulosum* showed better MPS ability on sucrose as compared to glucose or maltose as the carbon source in the presence of hydroxyapatite as P source. Similarly, *Penicillium oxallicum* CBPS-Tsa released 563 mg/L P in the medium when 5 g/L sucrose and CaP was provided and P release was enhanced to 824 mg/L in the presence of sodium nitrate as nitrogen source (Kim et al. 2003b). *Aspergillus niger* BHUAS01 and *Penicillium citrinum* solubilized tricalcium phosphate in the presence of sucrose and released 421 µg/ml P after 21 days of incubation (Yadav et al. 2011). *Penicillium bilaii* also solubilizes CaHPO4 in the presence of sucrose as the carbon source (Cunningham and Kuiack 1992). For many of P-solubilising fingi the organic acids secreted while growing on sucrose are well-characterized. *Penicillium bilaii* produces 10 mM each of citric and oxalic acid (Cunningham and Kuiack 1992). *Penicillium rugulosum* secretes gluconic and citric acid in the presence of sucrose as the carbon source (Reyes et al. 1999a). A mutant strain of *Penicillium rugulosum* produced 14.3 mM citric and 7.7 mM gluconic acids in the presence of $FePO_4$ and nitrate as nitrogen source (Reyes et al. 1999b). However, when $FePO_4$ was replaced by hydroxyapatite, it produced 90 mM gluconic and 0.28 mM citric acid.

Rhizospheric bacteria producing high amount of organic acid are expected to be more effective than fungi due to better colonisation ability. Among bacteria *Azotobacter chroococcum* isolated from wheat rhizosphere and *Citrobacter* DHRSS isolated from sugar cane showed MPS ability in the presence of sucrose (Kumar and Narula 1999; Patel et al. 2008). While the acid produced by *A. chroococcum* is not reported, *Citrobacter* DHRSS produced high amount of acetic acid and released P in the medium (Patel et al. 2008). In present study heterologous incorporation of two different invertases successfully resulted in the production of gluconic acid while using sucrose as the carbon source, which resulted in MPS phenotype in buffered conditions while growing in the presence of sucrose.

The levels of carbon sources used usually for demonstrating MPS ability in the laboratory are much higher than the realistic levels found in root exudates. First, the absolute concentration is much lower than that used in artificial laboratory media and secondly different sugars are present as a mixtures in the root exudates, which together could provide a substantial amount of carbon source, if the organism has the versatility to use them. We have earlier reported that *E. asbuirae* PSI3 solubilized rock phosphate under buffered conditions (mimicking conditions in alkaline vertisol) while growing on a mixture of aldose sugars (Sharma et al. 2005). The concentration of each sugar in the mixture was 15 mM, whereas independently each sugar was required at 75 mM for displaying P solubilization phenotype. The genetic modification that is reported in this work has allowed the expansion of the range of sugars that *E. asbuirae* PSI3 could use for P-solubilization and it was demonstrated that eight sugars could be used each at 10 mM for efficient P-solubilization. Taking into consideration that the exudation of sugars by plant roots is similar to a steady state-steady flow condition, the ability to use low concentrations of various sugars for efficient MPS activity is highly indicative of the improve efficacy in field conditions.

Conclusion

Enterobacter asburiae PSI3, a gluconic acid producing, P-solubilizing rhizobacterium was genetically modified so as to express invertase enzyme in the periplasm by heterologous cloning of the invertase-encoding genes from two different sources viz. *Zymomonas mobilis* and *Saccharomyces cerevisiae*. The transformants of both the genes showed high invertase activity in *E. coli* as well as *E. asburiae* while the native strains of both organisms are invertase negative. In *E. asburiae* PSI3 transformants there was higher acidification of the medium due to gluconic acid production using sucrose as the carbon source indicating that the combined action of invertase and the resident GDH of the organism resulted in gluconic acid when sucrose was the carbon source, whereas the native organism produced gluconic acid only when provided with glucose in the medium. In *E. coli* this phenomenon was not observed since the resident GDH of *E. coli* is inactive due to lack of biosynthesis of its cofactor PQQ. To the best of our knowledge this is the first report of gluconic acid production by soil bacterium using sucrose as the carbon source.

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