Plant Soil (2016) 409:51–64
DOI 10.1007/s11104-016-2937-7

REGULAR ARTICLE

Inoculation of genetically modified endophytic *Herbaspirillum seropedicae* Z67 endowed with gluconic and 2-ketogluconic acid secretion, confers beneficial effects on rice (*Oriza sativa*) plants

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Received: 30 December 2015 / Accepted: 23 May 2016 / Published online: 9 June 2016

Abstract
Background and aims *Herbaspirillum seropedicae* (*Hs*) Z67 a diazotrophic endophyte was genetically engineered for secretion of 2-keto-D-gluconic acid by heterologous expression of genes for *pqq* synthesis and gluconate dehydrogenase to study its beneficial effect on plants.
Methods Two plasmids, pJNK5, containing a 5.1 Kb *pqq* gene cluster of *Acinetobacter calcoaceticus* and pJNK6, carrying in addition the *Pseudomonas putida* KT2440 gluconate dehydrogenase (gad) operon were constructed in pUCPM18Gm^r under P*lac* promoter. *H. seropedicae* Z67 transformants were monitored for P and K solubilization, cadmium (Cd) tolerance and rice growth promotion.
Results *Hs* (pJNK5) secreted 23.5 mM gluconic acid and *Hs* (pJNK6) secreted 3.79 mM gluconic acid and 15.8 mM 2-ketogluconic acid respectively. Under aerobic conditions, *Hs* (pJNK5) and *Hs* (pJNK6) solubilized 239.7 μM and 457.7 μM P on HEPES rock phosphate and, 76.7 μM and 222.7 μM K on HRPF (feldspar), respectively, in minimal medium containing 50 mM glucose. Under N free minimal medium, similar effects of P and K solubilization were obtained. *Hs* (pJNK5) and *Hs* (pJNK6) inoculation increased the biomass, N, P, K content of rice plants (Gujarat – 17). These plants also accumulated 0.73 ng/g PQQ, and had improved growth and tolerance to $CdCl_2$.
Conclusions Incorporation of *pqq* and *gad* gene clusters in *H. seropedicae* Z67 imparted additional plant growth promoting traits of P and K solubilization and ability to alleviate Cd toxicity to the host plant.

Keywords *pqq* gene cluster · *gad* operon · 2-ketogluconic acid · Cadmium tolerance · Mineral phosphate solubilization · Potassium solubilization

Responsible Editor: Stéphane Compant .

Electronic supplementary material The online version of this article (doi:10.1007/s11104-016-2937-7) contains supplementary material, which is available to authorized users.

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Introduction

Herbaspirillum seropedicae Z67 is a nitrogen-fixing bacterium which colonizes as an endophyte economically important crops such as rice, sugarcane, and wheat (da Silva et al. 2003). Inoculation of this bacterium has been shown to increase biomass and N content of rice plants equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani 1995). It is well known that N fixation by endophytic or symbiotic bacteria is limited by the availability of P and that co-inoculation of N fixing bacteria with phosphate solubilizing

microorganisms (PSMs) results in improved N status of plants (Elkoca et al. 2007; Valverde et al. 2007).

PSMs increase the availability of P by secreting low molecular weight organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, etc. which account for their mineral phosphate solubilizing (MPS) ability (Gyaneshwar et al. 2002). Solubilization of mineral P from soils depends on the nature and amount of the organic acid, which varies from 10 to 100 mM from strong to weak organic acids (Archana et al.2012). One of the most well-studied mechanisms of organic acid mediated P solubilization is the secretion of high levels of gluconic acid synthesized by the direct glucose oxidation pathway involving pyrroloquinoline quinone (PQQ)-dependent periplasmic glucose dehydrogenase (GDH) (Goldstein 1995). Many bacteria possess the apo-GDH but lack the ability to synthesize its cofactor PQQ. Heterologous expression of PQQ biosynthesis gene clusters from *Pseudomonas.*, *Burkholderia*, *Azospirilluim brasilense*, *Deinococcus radiodurans* and *Serratia marcescens* in *Escherichia coli* have been reported to confer upon *E. coli* the ability to secrete gluconic acid (Krishnaraj and Dahale 2014). Coexpression of genes for the cofactor (PQQ) and the apoenzyme (GDH) from *Serratia marcescens* has been reported to confer high MPS ability to *E. coli* (Farhat et al. 2013). The GDH enzyme and the PQQ biosynthesis pathway have been targeted for genetic manipulation to improve MPS ability in several Gram-negative bacteria (Sashidhar and Podile 2010). We have recently reported the heterologous expression of PQQ biosynthesis operon in the *H. seropedicae* Z67 (Wagh et al. 2014a). Interestingly, *pqq*E alone is not sufficient for gluconic acid secretion in *H. seropedicae* Z67 (as against in *E. coli*), but the cluster consisting of *pqq*ABCDE resulted in high PQQ levels and the appearance of GDH activity and gluconic acid secretion in this bacterium conferring upon it the MPS ability (Wagh et al. 2014a).

2-Keto-D-gluconic acid (2KDG) is a stronger acid than gluconic acid and can solubilize mineral phosphates more efficiently by chelation of calcium ions from Ca-phosphates in soils (Moghimi et al. 1978). Certain plant growth promoting bacteria are known to secrete 2KDG (Gulati et al. 2010; Park et al. 2010). The production of 2KDG is attributed to the gluconate dehydrogenase enzyme which is part of the direct glucose oxidation pathway. Yum et al. 1997 reported overexpression of *Erwinia cypripedii* ATCC 29267 *gad* operon encoding three subunits of membrane-bound GADH in *E. coli*. The holoenzyme glucose dehydrogenase of *E. coli* was reconstituted by addition of pyrroloquinoline quinone to the culture medium, and this led to the conversion of D-glucose to 2KDG by recombinant *E. coli* harboring the cloned GADH gene. Overexpression of *Pseudomonas putida* KT 2440 *gad* operon in *Enterobacter asburiae* PSI3, a P-solubilizing rhizobacterium proficient at gluconic acid secretion, resulted in the extracellular conversion of gluconic acid to 2KDG with enhanced MPS ability (Kumar et al. 2013).

The role of organic acids in solubilizing insoluble K containing minerals in addition to mineral P solubilization is becoming significant. Several bacterial strains solubilize minerals, such as feldspar, biotite, illite etc. by excreting organic acids that either directly dissolve rock K or chelate silicon ions to bring the K in to solution (Sheng and He 2006) and can supply K to plants (Sheng 2005; Basak and Biswas 2009). Organic acid mediated changes in metal mobility are important for alleviation of metal phytotoxicity (Archana et al. 2012). For instance, citric, malic and oxalic acids are implicated in the sequestration and detoxification of Al^{3+} (Singh and Chauhan 2011). *Enterobacter asburiae* PSI3, a gluconic acid secreting rhizobacterium could enhance growth of mung bean seedlings in the presence of phytotoxic levels of Cd^{2+} (Kavita et al. 2008), while multimetal-resistant, gluconic acid secreting *Enterobacter* sp. C1D promoted plant growth in the presence of toxic levels of Cr^{3+}. Since 2KDG is a stronger acid (pKa 2.66) than gluconic acid (pKa 3.8), it is of interest to develop strategies to incorporate 2KDG production in plant-beneficial bacteria and study the plant growth promotion ability of the engineered strain.

This present study reports the overexpression of *Acinetobacter calcoaceticus pqq* gene cluster and *Pseudomonas putida* KT2440 *gad* operon in *H. seropedicae* Z67 resulting in higher 2KDG secretion imparting upon the engineered strain superior MPS and K solubilizing ability, tolerance to Cd toxicity there by promoting growth of rice plants.

Materials and methods

Bacterial strains, plasmids, growth media, and culture conditions

Bacterial strains and plasmids used for this work are listed in Table 1. DNA manipulation and plasmid constructions were performed using *E. coli* DH10B as the host strain

Table 1 Bacterial strains and plasmids used in this study

Plasmid/Strains	Characteristics[a]	Source orReference
Plasmids		
pUCPM18-Gm	pUC18 derived Broad-Host-Range vector containing gentamycin resistance gene; Amp^r(100 μg/ml); Gm^r(20 μg/ml)	Hester et al. 2000; Adhikary et al. 2014
pSS2	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *Acinetobacter calcoaceticus.* Tet^r(40 μg/ml)	Goosen et al. 1989
pCNK12	pJET2.1 with *gad* operon.Amp^r(100 μg/ml)	Kumar et al. 2013
pJNK5	pUCPM18-Gm with 5.1 Kb *pqq* gene cluster of *Acinetobacter calcoaceticus*. Gm^r(20 μg/ml)	This study
pJNK6	pJNK5 with 3.8 Kb *gad* operon of *Pseudomonas putida* KT 2440.Gm^r(20 μg/ml)	This study
Bacterial Strains		
E. coli DH10B	Host strain for routine DNA manipulation experiments and plasmid maintenance. Str^r(40 μg/ml)	Invitrogen, USA
HerbaspirillumseropedicaeZ67	Nitrogen fixing rice endophyte, Nal^r(20 μg/ml)	Baldani et al. 1986
Hs(pUCPM18) Gm^r	*H. seropedicae* Z67 with (pUCPM18-Gm) Gm^r(30 μg/ml), Nal^r(20 μg/ml)	This study
Hs(pJNK5)	*H. seropedicae* Z67 with pJNK5, Gm^r(30 μg/ml), Nal^r(20 μg/ml)	This study
Hs (pJNK6)	*H. seropedicae* Z67 with pJNK6, Gm^r(30 μg/ml), Nal^r(20 μg/ml)	This study

Concentrations of antibiotics used given in parenthesis

Amp^r Ampicillin, *Gm*^{r} Gentamicin, *Tet*^{r} Tetracycline, *Str*^{r} Streptomycin, *Nal*^{r} Nalidixic

using standard protocols (Sambrook and Russell 2001). Plasmid bearing derivatives of *E. coli* DH10B were routinely grown at 37 °C on Luria Bertani broth (LB) and maintained on Luria Bertani Agar (LA) plates with 40 μg ml^{-1} streptomycin. *H. seropedicae* Z67 was routinely grown at 30 °C on LB and maintained on semisolid JNFb medium (pH 5.8) plates containing 20 μg ml^{-1}nalidixic acid at 30 °C (Baldani et al. 1986). For growth of *H. seropedicae* Z67 and its plasmid bearing derivatives under nitrogen fixing conditions, JNFb medium was prepared devoid of a nitrogen source.

Cloning of *A. calcoaceticus pqq* gene cluster in the broad host range vector pUCPM18Gm under *lac* promoter

Plasmid pSS2 containing *pqq* gene cluster of *A. calcoaceticus* was obtained as a generous gift from Dr. N. Goosen, Department of Molecular Genetics, University of Leiden, Netherlands. pSS2 was digested with *Eco*R1 and BamHI and the 5.1 Kb insert was purified by using the Invitrogen gel purification Kit (Carlsbad, CA, USA) and ligated with the broad host range vector pUCPM18Gm digested with same restriction endonucleases (REs), which resulted in pJNK5 plasmid. The ligation mixture was transformed in to *E. coli* DH10B Str^r; the screening was done on the basis of antibiotic resistance. The plasmid was isolated and confirmed by RE digestions.

Cloning of *gad* operon in pJNK5 under *lac* promoter

Plasmid pCNK12 containing *gad* operon of *P. putida* KT 2440 (Kumar et al. 2013) was digested by BamHI and XbaI. The 3.8 Kb fragment containing the *gad* operon was purified using the Invitrogen gel purification Kit. Plasmid pJNK5 containing the *pqq* gene cluster of *A. calcoaceticus* was digested with BamHI and XbaI and the 3.8 Kb fragment was ligated with pJNK5 which resulted in pJNK6 plasmid. The ligation mixture was transformed in to *E. coli* DH10B Str^r; screening was done on the basis of antibiotic resistance. Plasmid was isolated and confirmed by RE digestion. Further, plasmids pUCPM18Gm as vector control, pJNK5, pJNK6

were transformed by electroporation in *H. seropedicae* Z67 as described by (Unge et al. 1996).

P-solubilizing assay

Fresh cultures of *H. seropedicae* Z67 and its transformants, obtained by inoculating a single colony in 3 ml LB and growing under shaking conditions at 30 °C, were dispensed in 1.5 ml sterile tubes, centrifuged at 9200 x *g* and washed thrice with sterile normal saline (0.85 % NaCl). P-solubilizing ability was monitored as di-calcium phosphate (DCP) solubilization on Pikovaskya's (PVK) agar (Pikovskaya 1948) and acid secretion recorded on rock phosphate containing buffered minimal agar (HRP medium) plates with methyl red as a pH indicator. HRP medium is similar to TRP medium (Buch et al. 2008) with 100 mM HEPES buffer of pH 8.0 instead of 100 mM Tris-Cl pH 8.0 (Wagh et al. 2014a). The culture suspension was aseptically spot inoculated on the HRP agar plates and allowed to dry completely followed by incubation at 30 °C for 2–4 days. P solubilization was determined by monitoring the zone of clearance on PVK agar and red zone on the HRP Methyl red agar plates.

Potassium solublization assay

Washed and resuspended culture suspensions as mentioned above were used to characterize K-solubilizing ability using Feldspar as the mineral K source (Aleksandrov agar plate) (Hu et al. 2006). Culture suspensions were aseptically spot inoculated on the plates and left to dry completely followed by incubation at 30 °C for 2–4 days. K solubilization was determined by monitoring the zone of clearance.

GDH and GADH assays

H. seropedicae Z67 wild type as well as transformants were grown on M9 and HRP minimal medium with 50 mM glucose as the C source and 50 mM HEPES buffer pH 8.0. Cells were harvested (5000 x*g* for 10 min) after the pH of the culture supernatant was reduced to below 5.0, washed with sterile saline, and resuspended in 50 mM Tris-HCl, pH 8.75, and the whole-cell suspension was employed as the source of the enzyme. Spectrophotometric method was followed for estimation of GDH activity, according to (Matsushita and Ameyama 1982). GADH enzyme activity was measured using D – gluconate as substrate in the assay mixture (Matsushita et al. 1982). GDH and GADH units were defined as nanomoles of 2, 6-dichlorophenol indophenol (DCIP) reduced per minute using glucose and gluconate as substrates, respectively.

PQQ determination

PQQ levels of *H. seropedicae* Z67 transformants was estimated as described earlier (Wagh et al. 2014a).

Growth characteristics

Growth profile and pH reduction of the spent medium were monitored for wild type as well as *H. seropedicae* Z67 (*Hs*) transformants using HRP medium and P released in the supernatant was determined as a measure of rock phosphate solubilization ability (Gyaneshwar et al. 1998). HRP medium was supplemented with 1 mg/ml Feldspar (HRPF) for monitoring K solubilization. Growth curves and pH profiling were performed in 150 ml conical flasks containing 30 ml of relevant medium (Wagh et al. 2014a).

Minimum inhibitory concentration (MIC) and maximum tolerance concentration (MTC) for cadmium of *Hs* transformants

The Minimal inhibitory concentration (MIC) and maximum tolerance concentration (MTC) of *Hs* transformants for Cd was determined by broth dilution method of Calomiris et al. 1984. Freshly grown cultures of *Hs* transformants were washed thrice with saline (0.85 %), resuspended in 1 ml saline and used for inoculation. Cultures were inoculated aseptically in 150 ml conical flask containing different concentrations of $CdCl_2$ (70 μM, 80 μM, 90 μM and 100 μM) and growth was monitored after incubation on a shaker at 30 °C.

Hydroponics experiment for studying the effect of Cd on rice plants

The hydroponic experiment was designed in three replicates according to Crestani et al. (2009). Rice (*Oryza sativa*, Gujarat-17) seeds were surface sterilized in 70 % ethanol for 1 min and in 1 % sodium hypochlorite (NaClO) for 20 min, rinsed 5–6 times with sterile distilled water and then allowed to germinate for 72 h on sterile moist filter paper in petri plates kept in the overnight at 30 °C. Uniformly grown seedlings with 5 mm

radicles were transferred to nylon nets placed on top of beakers containing 500 ml hydroponics nutrient solution for rice suggested by Miyamoto et al. 2001. $CdCl_2$ was added wherever required as independently sterilized solution to nutrient medium. PQQ (100 nM) was also added exogenously to a set of plants (Choi et al. 2008). Rice seedlings were raised for 10 days with $CdCl_2$ (1–10 μM in the ½ strength M.*S. medium*) and 100 nM PQQ with $CdCl_2$ (1–10 μM in the ½ strength M.*S. medium*). After 10 d of growth, plants were removed, rinsed with tap water and used for catalase (Cat) and superoxide dismutase (SOD) activity assay. Root length and shoot length were measured to ascertain the result of stress on rice plants.

Superoxide dismutase (SOD) assay

Extracts were prepared by using polyvinylpyrolidone (PVP) according to Costa et al. 2002. Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazolium (NBT). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract; riboflavin was added last (Van Rossum et al. 1997). One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50 % in a reaction mixture. An enzyme unit of SOD was calculated according to the formula given by Constantine and Stanley 1977.

Catalase (CAT) assay

Fresh samples were prepared for catalase assay using polyvinyl pyrolidone (PVP) and a pinch of activated charcoal according to Mahatma et al. (2011). Total catalase (EC 1.11.1.6) activity was determined in the supernatants by measuring the decrease in absorption at 240 nm as H_2O_2 was consumed according to the method of Aebi 1984 and enzyme activity expressed as mmol H_2O_2 oxidized min^{-1} g^{-1} protein. The 3 ml assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2 and 50 μl enzyme extract.

Plant inoculation experiments to study the effect of *Hs* transformants

Rice (*Oryza sativa*, Gujarat-17) seeds were surface sterilized in 70 % ethanol for 1 min and in 1 % sodium hypochlorite (NaClO) for 20 min, and then rinsed 5–6 times with sterile distilled water and then allowed to germinate for 24 h on moist filter paper in petri plates kept in the dark at 30 °C. Pure bacterial cultures were grown in nutrient broth at 30 °C, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. The plants were grown at 30 °C under natural daylight, while grown seeds were dipped with bacterial cultures in petri plates for 2 h and then seeds were rinsed with sterile water, treated seeds were irrigated with 50 mL of sterile distilled water every day. The experiment was conducted on six replicates (three bags per replicate, two plants per bag) for each treatment and was completely randomized. Plant growth parameters were followed as per the method described in Wagh et al. (2014b).

Analytical methods

Modified Lowry's method (Peterson 1979) was applied for total protein estimation. Cell growth of *H. seropedicae* Z67 wild type as well as transformants was determined as optical density at 600 nm (OD_{600}). Soluble K content in the supernatant of HRPF medium was determined by flame photometry (Sugumaran and Janarthanam 2007). Different concentrations of KCl were used to prepare a standard curve. HPLC analysis was used to detect organic acid levels and retention time was determined under above mentioned conditions for identification of the organic acid. Pure organic acids were used for quantification according to Wagh et al. 2014b. Physiological parameters like growth rate, biomass yield on glucose and specific glucose reduction rate were calculated as described by Buch et al. 2008, 2009. Graph Pad Prism (version 3.0) and Microsoft Excel were used for statistical analysis of the parameters. Each parameter has been represented as mean ± SD or mean ± SEM as specified in the figure captions.

Results

Growth and MPS ability of *Hs* transformants with pJNK5 and pJNK6 plasmids

The transformant containing the *pqq* cluster alone [*Hs* (pJNK5)] as well as the transformant carrying the *pqq* cluster conjunction with *gad* operon [*Hs* (pJNK6)] showed comparable P solubilization on Pikovaskya's agar and very good acidification zone on 100 mM

HEPES and 50 mM glucose containing HRP medium (Fig. S1). K solubilization was also apparent for both the transformants on Aleksandrov agar plate. The wild type strain and the transformant with the empty plasmid vector failed to display any proficiency at solubilization of mineral phosphates as well as mineral K (Fig. S1).

When inoculated in HRP minimal medium with or without feldspar, growth of the transformants and wild type strain was not significantly different. However, a drop in pH was observed in case of *Hs* (pJNK5) and *Hs* (pJNK6) bringing pH to 5.0 and 4.6, respectively, within 40 h indicating the secretion of organic acids (Figs. 1a and 1b). Growth under microaerobic conditions was slow for all the strains, however similar observations regarding lowering of pH could be made in case of the transformants carrying the *pqq* [*Hs* (pJNK5)] and *pqq*, *gad* [*Hs* (pJNK6)] clusters (Figs. 1b and 1c).It is apparent that the pH of the spent medium is lowered to a greater extent by *Hs* (pJNK6) than *Hs* (pJNK5) under aerobic as well as microaerobic conditions. A higher pH drop with pJNK6 than pJNK5 is also well substantiated in Fig. S1 with different media.

Quantitative growth parameters for the transformants and the wild type strain for aerobic growth on HRP medium are given in Table 2. Growth rates of *H. seropedicae* Z67 and the transformants *Hs* (pUCPM18), *Hs* (pJNK5) and *Hs* (pJNK6) were similar on HRP medium**.** Total glucose depletion of *Hs* (pJNK5) and *Hs* (pJNK6) was 2.8 fold higher as against wild type strain and *Hs* (pUCPM18), respectively, on HRP medium. On the other hand, the specific glucose utilization rate of *Hs* (pJNK5) and *Hs* (pJNK6) was 1.1

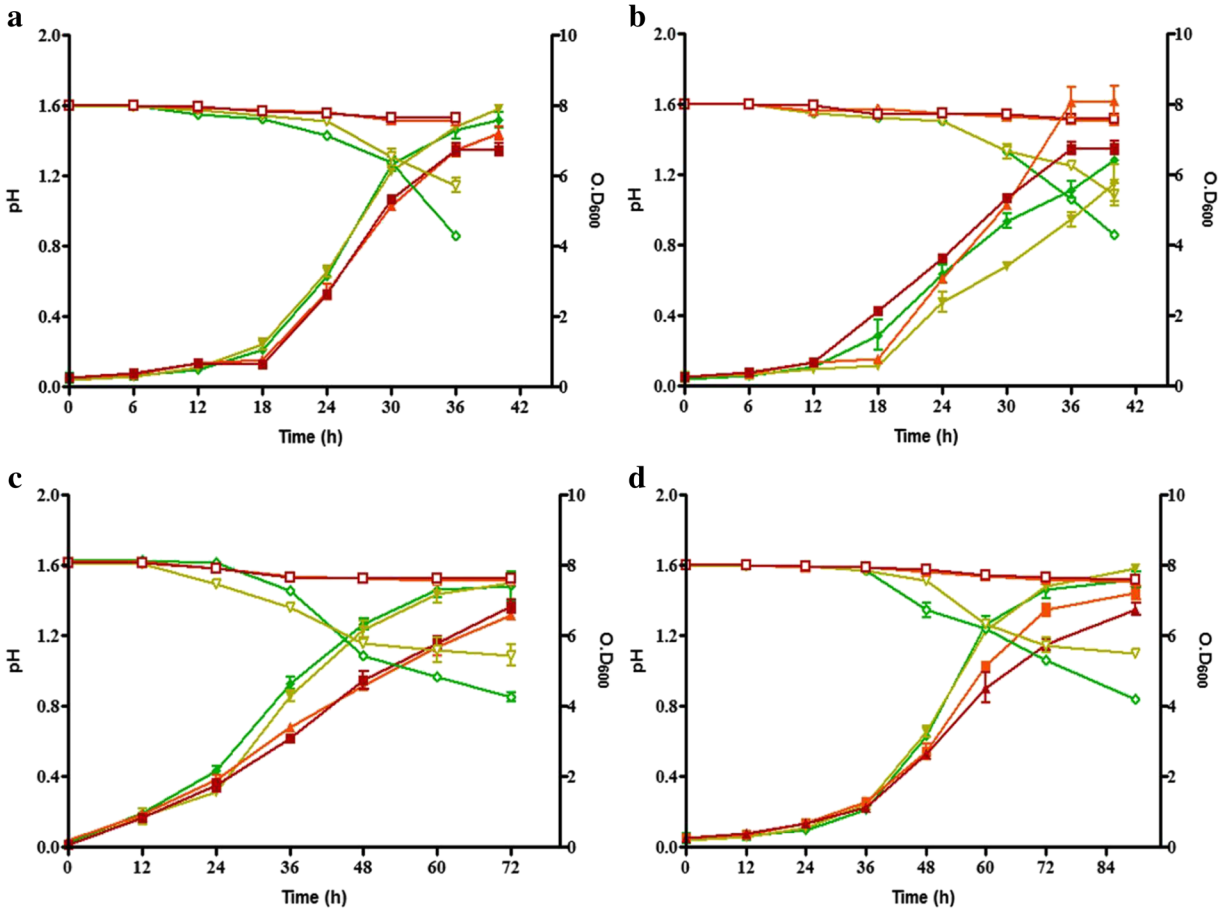


Fig. 1 Growth and pH profile of *H. seropedicae* Z67 and its transformants. (**a**, **c**- HRP medium and **b**, **d**- HRP medium supplemented with 1 mg/ml Feldspar). (**a**, **b** – Aerobic, N source supplemented condition and **c**, **d**- Microaerobic, N source-free condition) Growth in terms of OD_{600} is depicted as filled symbols and pH of the extracellular supernatant as open symbols, ■,□, *Hs* (wild type); △, ▲, *Hs* (pUCPM18Gm); ▽,▼, *Hs* (pJNK5); ◇, ◆, *Hs* (pJNK6), Values at each time point are represented as the mean ± SD of six independent observations

Table 2 Physiological parameters of *H. seropedicae* Z67 transformants grown on HRP minimal medium with 50 mM glucose as C source under aerobic conditions

Bacterial Strain	Growth rate μ (h^{-1})[a]	Total glucose depleted (mM)[b]	Glucose consumed (mM)[b]	Biomass yield Ydcw/Glc[a] (g/g)	Specific glucose depletion rate QGlc[a] (g.g $dcw^{-1}.h^{-1}$)
Hs	0.16 ± 0.003	12.20 ± 0.80	10.56 ± 0.41	0.042 ± 0.004	0.65 ± 0.06
Hs (pUCPM18)	0.15 ± 0.01	12.33 ± 0.75	10.60 ± 0.17	0.042 ± 0.01	0.67 ± 0.16
Hs(pJNK5)	0.149 ± 0.01	34.46 ± 0.85***	11.89 ± 0.59	0.041 ± 0.01	0.77 ± 0.28***
Hs (pJNK6)	0.16 ± 0.001***	34.16 ± 2.38***	14.53 ± 2.16***	0.021 ± 0.00***	1.31 ± 0.017***

The results are expressed as Mean ± SEM of readings from 3 independent observations

ns non-significant (as compared with wild type culture control)

*** $P < 0.001$

[a] Determined from mid log phase of each experiment

[b] Determined at the time of pH drop (36 h)

and 2 fold higher than wild type and *Hs* (pUCPM18), respectively, on HRP medium**.** However, glucose uptake was apparently similar in all *H. seropedicae* Z67 transformants when compared with wild type. In contrast, biomass yield of *Hs* (pJNK6) was decreased by 2 fold on HRP medium when compared with wild type. *Hs* (pJNK5) showed a similar biomass yield as the wild type strain**.** *Hs* (pJNK5) and *Hs* (pJNK6) released P up to 239.7 μM and 457.7 μM, respectively, using RP as the P source (Table 4).

Under N free HRP medium, growth rates of *Hs* (pUCPM18), *Hs* (pJNK5) and *Hs* (pJNK6) were similar as compared to the wild type strain **(**Table 3**)**. On the other hand, the specific glucose utilization rate of *Hs* (pJNK6) was 2 fold higher than wild type, *Hs* (pUCPM18), and *Hs* (pJNK5), respectively, on N free HRP medium**.** In contrast, biomass yield of *Hs* (pJNK6) was decreased by 2 fold on N free HRP medium when compared with wild type, *Hs* (pUCPM18) and *Hs* (pJNK4) showed similar pattern of biomass yield as compared with wild type strain**.** Wild type strain and *Hs* (pUCPM18) neither showed acidification nor solubilized RP whereas *Hs* (pJNK5) and *Hs* (pJNK6) released P up to 195.6 μM and 440.8 μM, respectively, on 50 mM glucose (Table 3). The extent of P release in microaerobic conditions is similar to that under aerobic conditions.

K solubilizing ability of *H. seropedicae*Z67 transformants

Hs (pJNK5) *Hs* (pJNK6) showed K solubilization, on Aleksandrov agar plates (Fig. S1). *H. seropedicae* Z6 and its transformants when inoculated in HRP medium supplemented with feldspar (HRPF) medium. No pH

Table 3 Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown under microaerobic conditions on Nitrogen free HRPF minimal medium with 50 mM glucose as C source

Bacterial Strain	Growth rate μ (h^{-1})[a]	Biomass yield $Y_{dcw/Glc}$[a] (g /g)	Specific glucose depletion rate Q_{Glc}[a] (g.g $dcw^{-1}.h^{-1}$)	Phosphate released P (μM)[b]	Potassium released, K (μM)[b,c]
Hs	0.084 ± 0.001	0.021 ± 0.002	0.32 ± 0.03	UD	UD
Hs(pUCPM18)	0.076 ± 0.00	0.021 ± 0.005	0.33 ± 0.08	UD	UD
Hs(pJNK5)	0.074 ± 0.00	0.020 ± 0.007	0.36 ± 0.11	195.56 ± 15.00***	63.66 ± 4.79***
Hs(pJNK6)	0.083 ± 0.000	0.010 ± 0.00***	0.65 ± 0.00***	440.76 ± 23.61***	198.18 ± 10.78***

The results are expressed as Mean ± SEM of readings from 3 independent observations

ns non-significant (as compared with wild type culture controls), *UD* undetectable

*** $P < 0.001$

[a] Determined from mid log phase of each experiment

[b] Determined at the time of pH drop (72 h)

[c] HRP medium containing 1 mg/ml feldspar

Table 4 Effect of *H. seropedicaeZ67* transformants on the enzyme activity, organic acid secretion and mineral phosphate and potassium solubilization in HRP medium containing 50 mM glucose as the carbon source

Bacterial Strain	Enzyme activity (U) HRP medium		Amount of organic acid secreted		P release (μM)[a]	K release (μM)[ab]	PQQ secreted in minimal medium (μM)
	GDH	GADH	Gluconic acid (mM)	2-keto-gluconic acid (mM)			
Hs	UD	UD	UD	UD	UD	UD	UD
Hs (pUCPM18)	UD	UD	UD	UD	UD	UD	UD
Hs(pJNK5)	221.66 ± 10.69***	UD	23.47 ± 3.93***	UD	239.66 ± 8.73***	76.66 ± 5.033***	1.15 ± 0.11***
Hs (pJNK6)	234.33 ± 8.08***	414.00 ± 9.84***	3.79 ± 0.27***	15.83 ± 0.42***	457.66 ± 22.05***	222.66 ± 19.08***	1.17 ± 0.06***

The results are expressed as Mean ± SEM of readings from 3 independent observations

ns non-significant as compared to wild type culture controls), *UD* undetectable

*** $P < 0.001$

[a] Determined at the time of pH drop (36 h)

[b] HRP medium containing 1 mg/ml feldspar

change in the case of *Hs* wild type and *H*s (pUCPM18) was observed but *Hs* (pJNK5) and *Hs* (pJNK6) showed good growth and a drop in pH up to 5.0 and 4.6, respectively, within 40 h in the presence of glucose (Fig. 1). *Hs* (pJNK5) and *Hs* (pJNK6) also released K up to 76.7 μM and 222.7 μM, respectively, on 50 mM glucose (Table 4).

The wild type strain and *Hs* (pUCPM18) neither showed acidification nor solubilized feldspar while *Hs* (pJNK5) and *Hs* (pJNK6) released K up to 63.7 μM and 198.2 μM, respectively, on 50 mM glucose under microaerobic conditions in N free HRPF medium (Table 3). The extent of K release in microaerobic condition is similar to that under aerobic conditions.

PQQ levels, GDH and GADH activities and organic acid secretion in *H. seropedicae* Z67 transformants

Hs wild type and *Hs* (pUCPM18) did not secrete PQQ while *Hs* (pJNK5) and *Hs* (pJNK6) secreted 1.15 μM and 1.17 μM PQQ, respectively, in M9 minimal medium. *Hs* wild type and *Hs* (pUCPM18) did not show any GDH activity in the HRP medium while *Hs* (pJNK5) and *Hs* (pJNK6) showed 221.5 U and 234 U of GDH activity, respectively (Table 4). On the other hand, GADH activity was undetectable in *Hs* wild type, *Hs* (pUCPM18) and *Hs* (pJNK5) whereas 414 U of GADH activity was found in *Hs* (pJNK6) when compared with *Hs* wild type strain. Organic acid secreted by *Hs* (pJNK5) was 23.47 mM and *Hs* (pJNK6) 3.79 mM, while *Hs* (pJNK5) was unable to produce 2KDG, *Hs* (pJNK6) secreted 15.83 mM 2KDG acid which is sufficient for RP solubilisation (Table 4) and **(**Fig 2**)**.

Effect of *Hs* transformants *Hs* (pJNK5) and *Hs* (pJNK6) on Cd tolerance

Since PQQ is known as antioxidant molecule, *Hs* transformants were monitored for minimum inhibitory and tolerance levels. *Hs* wild type and *Hs* (pUCPM18) were able to tolerate 40 μM of $CdCl_2$ and minimum inhibitory concentration (MIC) was 60 μM. However, *Hs* (pJNK5) and *Hs* (pJNK6) were able to grow and tolerate up to 100 μM $CdCl_2$**.**

Effect of PQQ on the growth and antioxidant enzyme status of rice seedling against Cd stress

Under hydroponic conditions, rice seedlings treated with 10 μM Cd showed growth inhibition of root and

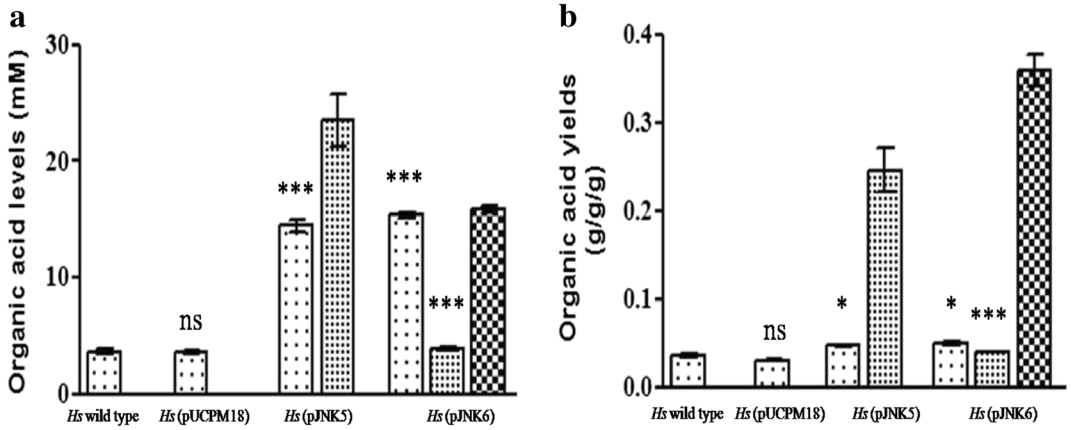


Fig. 2 Extracellular organic acid secretion by *H.* seropedicae Z67 wild type and its transformants on HRP medium. **a**: Organic acid levels (mM); **b**- Organic acid yields expressed as g/g/g indicating g of organic acid produced/g of glucose utilized/g dry cell weight. - Gluconic acid; 2 ketogluconic acid and Acetic acid. Values are for cultures grown for 36 h. Results are expressed as Mean ± SEM of 3 independent experiments. Wild type and vector control do not produce detectable levels of gluconic aid nor 2 keto gluconic acid. Values for acetic acid were compared with wild type strain and for gluconic acid in *Hs* (pJNK6) were compared with *Hs* (pJNK5). *** $P<0.001$, * $P<0.05$, ns=non-significant

shoot length by 70 % and 60 %, respectively, when compared with control seedlings while PQQ treated seedlings with 10 μM Cd had root and shoot length of rice similar to that of untreated seedlings **(**Fig. 3**).**

Cd treated rice seedlings exhibited a significant change in the activities of CAT and SOD. CAT activity of control seedlings was 18 U while seedling treated with 10 μM Cd showed a 3.4 fold increase in activity when compared with control plants without Cd. PQQ treated plants showed a significant decrease in CAT activity by 2 fold in presence of 10 μM Cd. SOD activity of 10 μM Cd treated seedlings was increased by 3.8 fold when compared with control plants without Cd. When PQQ was supplemented to plants treated with 10 μM Cd, SOD activity was significantly reduced by 2.3 fold as compared to 10 μM Cd treated seedlings (Fig. 4).

Effect of *Hs* (pJNK5) and *Hs* (pJNK6) on the nutrient status of rice plants

Shoot length, stem length, fresh weight, dry weight, N, P and K content were monitored in rice plants 30 days

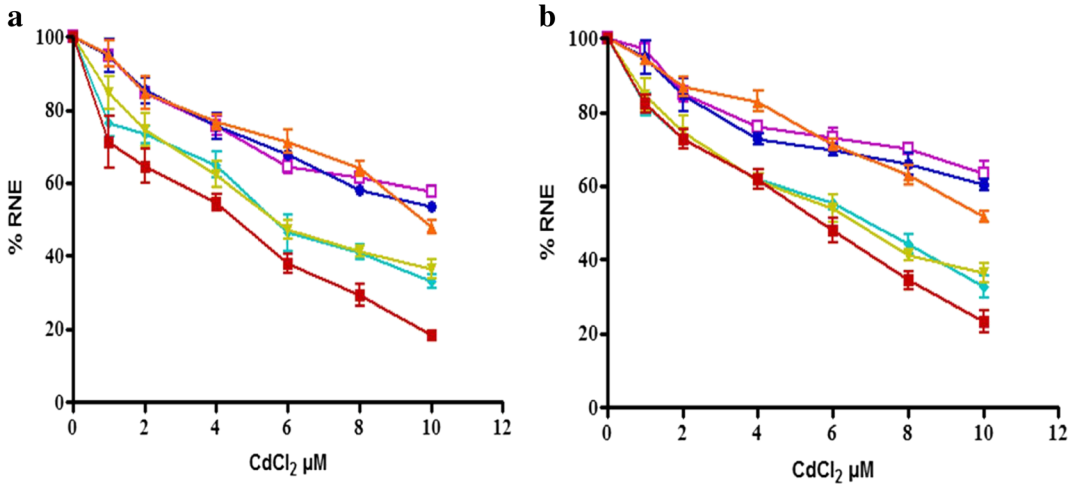
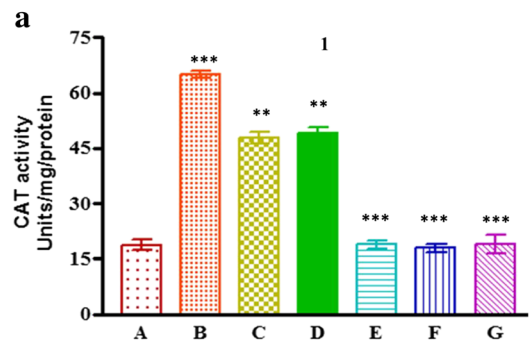


Fig. 3 Relative net elongation (RNE) (%) of root and shoot of rice seedling. (**a**, **b**- CdCl2 treated, **a**-% RNE of root; **b**-% RNE of shoot). (**a**, **b**) - CdCl2; - Exogenous PQQ; - *Hs* (wild type); - *Hs* (pUCPM18Gm^r); - *Hs* (pJNK5); - *Hs* (pJNK6). The results are expressed as Mean±SEM of readings from six plants of treatments *** $P<0.001$, ns=non-significant (as compared with wild type culture controls)



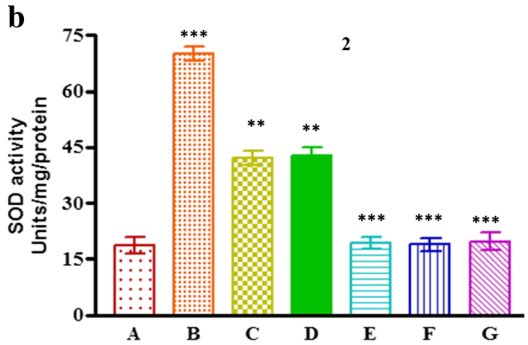


Fig. 4 Effect *H. seropedicae* Z67 transformants (pJNK5) and (pJNK6) on the antioxidant enzyme activity of rice seedlings exposed to cadmium after 10 DAI. **1**- CAT and **2** – SOD. -Control; -Cd 10 μM; - *Hs* Cd 10 μM; - *Hs* (pUCPM18 ^{Gm} Cd 10 μM); - *Hs* (pJNK5) Cd 10 μM; - *Hs* (pJNK6) Cd 10 μM; - PQQ Cd 10 μM. The results are expressed as Mean ± SEM of readings from six plants of treatments *** P<0.001, ns=non-significant (as compared with wild type culture controls)

after inoculation (DAI) (Table 5 and S1). *Hs* wild type strain and *Hs* (pUCPM18) inoculated rice plants showed 1.3 fold increase in shoot length as compared to control uninoculated plants, while *Hs* (pJNK5) and *Hs* (pJNK6) inoculations showed 1.7 and 2 fold increase as compared to the plants inoculated with the wild type strain. Root length was increased in both *Hs* and *Hs* (pUPM18) treatments by 2 fold when compared with uninoculated plants, while *Hs* (pJNK5) and *Hs* (pJNK6) showed 1.7 and 2 fold increase in root length as compared with *Hs* inoculated plants. Fresh weight of plants inoculated with *Hs* and *Hs* (pUCPM18) increased by 2 fold when compared with control set, while *Hs* (pJNK5) and *Hs* (pJNK6) showed a significant increase in fresh weight by 1.6 and 2 fold when compared with *Hs* treated plants. *Hs* and *Hs* (pUCPM18) treated plants showed 1.5 fold increased total chlorophyll content when compared with control plants, while both *Hs* (pJNK5) and *Hs* (pJNK6) increased chlorophyll content by 1.4 and 1.6 fold respectively when compared with wild type strain colonized plants.

Hs (pJNK5) and *Hs* (pJNK6) inoculated rice plants showed presence of PQQ in rice plants while control plants did not contain any PQQ. Plants inoculated with *Hs* (pJNK5) and *Hs* (pJNK6) showed 0.726 ng and 0.728 ng of PQQ, respectively **(**Table 5**).** Nitrogen content in plant leaves was significantly increased with *Hs* and *Hs* (pUCPM18) by 1.3 fold as compared with control plants while N levels in *Hs* (pJNK5) and *Hs* (pJNK6) treated plants showed 1.5 and 2 fold increase, respectively, when compared *Hs* plants. N levels in roots in *Hs* and *Hs* (pUCPM18) treated plants showed 2 fold increase against uninoculated control, while *Hs* (pJNK5) and *Hs* (pJNK6) treatment showed 1.7 and 2.2 fold increase when compared with *Hs* treatment. Phosphorus content in leaves and roots of both *Hs* (pJNK5) and *Hs* (pJNK6) treated plants increased by 2.2 and 3 fold respectively, when compared with *Hs* inoculated plants. K content of leaves and roots was increased in case of *Hs* (pJNK5) and *Hs* (pJNK6) inoculated plants by almost 1.3 and 1.6 fold respectively, when compared against *Hs* treated plants **(**Table S1**).**

Discussion

Phosphate solubilizing rhizobacteria primarily secrete gluconic acid by the direct glucose oxidation pathway mediated by periplasmic GDH which requires PQQ as a redox cofactor (Krishnaraj and Dahale 2014). Earlier studies from our laboratory showed that incorporation of 5.1 Kb *Acinetobacter calcoaceticus* and 13.4 Kb *Pseudomonas fluorescens* B16 *pqq* gene clusters independently in *H. seropedicae* Z67 resulted in high secretion of gluconic acid, leading to mineral phosphate solubilization under aerobic and nitrogen fixing microaerophilic conditions (Wagh et al. 2014a). The present study shows that cloning of the smaller *pqq* gene cluster of *A. calcoaceticus* along with the *gad* operon of *P. putida* in *H. seropedicae* Z67 resulted in secretion of gluconic acid as well as 2-keto D-gluconic acid. This work demonstrates the functional establishment of the direct glucose oxidative pathway by the heterologous expression of PQQ biosynthesis genes and GAD encoding operon which act in conjunction with the resident apo-GDH encoded by *H. seropedicae* Z67

Table 5 Effects of *H. seropedicae*Z67transformants on rice plant fresh weight, dry weight, plant height leaf chlorophyll content at 30 DAI

H. seropedicae Z67 transformants	Shoot length (cm)	Shoot/Root ratio	Root Length/Plant (cm)	Root/Shoot ratio	Total plant fresh weight /Plant (mg)	Total plant Dry weight /Plant (mg)	Total leaf chlorophyll content (mg/g fresh weight)	PQQ levels (ng/g of fresh weight /plant)
Control	21.66 ± 2.51	1.91 ± 048	11.66 ± 2.08	0.54 ± 0.16	109.66 ± 2.51	13.66 ± 0.57	2.86 ± 0.17	UD
Hs	29.66 ± 1.52	1.38 ± 0.39	22.33 ± 4.72	0.75 ± 0.19	210.66 ± 10.01	16.33 ± 0.57	3.31 ± 0.27	UD
Hs(pUCPM18)	29.66 ± 2.51	1.27 ± 0.21	23.66 ± 3.21	0.80 ± 0.129	204.33 ± 6.02	15.33 ± 1.52	3.02 ± 0.18	UD
Hs(pJNK5)	50.33 ± 2.51***	1.27 ± 0.10***	39.66 ± 2.08***	0.78 ± 0.06***	340.66 ± 4.04***	37.66 ± 1.52***	4.70 ± 0.35***	0.726 ± 0.03***
Hs(pJNK6)	63.00 ± 2.0***	1.11 ± 0.13***	57.00 ± 8.18***	0.90 ± 0.11***	423.33 ± 10.01***	46.66 ± 1.52***	5.55 ± 0.79***	0.728 ± 0.05***

The results are expressed as Mean ± SEM of readings from 6 plants of treatments

ns non-significant (as compared with wild type culture controls), *UD* undetectable

($n = 6$) *** $P < 0.001$

genome. The increased secretion of gluconic acid and 2KDG improved P solubilization and plant growth enhancement ability as compared to the native strain as well as only gluconic acid secreting transformant.

Hs (pJNK6) containing *P. putida* KT2440 *gad* operon secreted 15.83 mM 2KDG along with 3.79 mM gluconic acid. As compared to the present work, earlier reports from our lab showed that overexpression of *P. putida* KT2440 *gad* operon in *E. asburiae* PSI3 (which naturally has gluconic acid secreting ability) resulted in the secretiom of 11.63 mM 2KDG along with 21.65 mM gluconic acid (Kumar et al. 2013). GADH activity of *Hs* (pJNK6) was found to be 414 U in the HRP minimal medium and was similar to *E. asburiae* PSI3 *gad* transformant (Kumar et al. 2013). Over expression of *gad* in *E. coli* enhanced GADH activity by 2 fold (Yum et al. 1997). *Hs* (pJNK6) transformant released 2 fold higher P than *Hs* (pJNK5) indicating 2KDG secretion resulted in more efficient at P solubilization than GA secretion alone.

Interestingly, *Hs* transformants (pJNK5) and (pJNK6) were able to solubilize potassium minerals. The role of organic acid secretion like oxalic, citric, malic, succinic tartaric and acetic in P solubilizaion is well documented (Archana et al. 2012). A *Bacillus* sp. that solubilized phosphate also solubilized potassium minerals, the biochemical basis for latter is not fully understood (Hu et al. 2006). Three fold higher potassium solubilization in *Hs* (pJNK6) than *Hs* (pJNK5) strongly suggests a significant role of 2KDG in K solubilization which has been well substantiated by earlier observation of Duff et al. (1963) who showed 2KDG secretion by a *Pseudomonas* strain did result in K solubilization. Higher K solubilization by *Hs* (pJNK6) compared to the earlier report by Duff et al. (1963) may be due to the strain's ability to secrete gluconic acid in addition to 2-keto gluconic acid.

Hs (pJNK5) and *Hs* (pJNK6) produced 1.15 and 1.17 μM of PQQ in the medium after over expression but lesser than that of the *E. coli* transformant containing *Gluconobacter oxydans pqq* cluster which produced 6 μM PQQ (Yang et al. 2010), while overexpression of *pqq* under different promoters in *E. coli* and *Klebsiella pneumonia* showed two fold increased PQQ production (Sun et al. 2014). However, *Hs* transformants showed PQQ secretion in P deficient medium while *E. coli* transformants in Luria broth. Additionally, both *H. seropedicae* Z67 *pqq* and *gad* transformants showed

MPS ability under microaerobic nitrogen fixing conditions showed two fold increased (Wagh et al. 2014b). Incorporation of *pqq* and *gad* genes has maintained the biomass, specific glucose utilization and mineral K solubilization in *H. seropedicae Z67* under both aerobic and microaerobic conditions in HRP medium.

Phosphate solubilizing bacteria (PSB) are known to enhance yield in rice and maize. A similar increase in growth, yield, N, and P levels were found in wheat plants upon co-inoculation (mixture) of *Rhizobium* with PSB (Bhardwaj et al. 2014). Plant treatment with transformants *Hs* (pJNK5) and *Hs* (pJNK6) resulted in a significant increase in fresh weight dry weight, shoot length, root length and in chlorophyll in comparison with treatment with the control and wild type *Hs* strain. Increased plant growth promotion was observed by Bhardwaj et al. (2014) in plants that were co-inoculated with phosphate solubilizing bacteria along with nitrogen fixing bacteria. Similarly, overexpression of the citrate operon in *H. seropedicae* Z67 solubilised mineral phosphate and enhanced plant growth of rice (Wagh et al. 2014b). Rice plants inoculated in autoclaved soil with *Hs* (pJNK5) and *Hs* (pJNK6) accumulated PQQ, increased the biomass, N. P and K content. The similar effect of increase in plant growth was found upon inoculation with PQQ secreting *P. fluorescens* B16 but not with its *pqq* mutant (Choi et al. 2008). Significant increase in uptake of N, P and K status in plants by the transformants could be attributed to the cumulative effect of the organic acid secretion, nitrogen fixing and PQQ producing ability of bacteria.

Response to the oxidative stress generated by heavy metals leads to increase in the activity of oxidative enzymes, which play a major role in antioxidative defense system (Choi et al. 2008). Cd stress tolerance in rice seedlings is associated with a significant increase in the activities of these enzymes (Bhardwaj et al. 2014). Cd stress in this study showed increased SOD and CAT activities, but rice plants inoculated with *Hs* (pJNK5) and *Hs* (pJNK6) showed normal levels of CAT and SOD signifying that PQQ secreted by these transformants appear to be sufficient to counter the toxic effects of Cd. This is supported by the fact that PQQ as a potent water soluble antioxidant neutralizes reactive species by scavenging reactive oxygen species (ROS) and plays a key role in the DNA repair and reduces oxidative stress (Misra et al. 2012). Additionally, PQQ protects cells against oxidative stress caused by γ radiation.

In conclusion, *H. seropedicae* Z67 possessing *pqq* and *gad* gene clusters secretes high amount of PQQ, gluconic and 2-ketogluconic acids which confer MPS and potassium solubilization abilities. Inoculation of these transformants on rice plants promoted overall growth, increased the N and P status, and provided tolerance to Cd. Thus, the present study demonstrates that incorporation of 2KDG secretion in nitrogen fixing bacterial isolates could be a good strategy for enhancing their potential as biofertlizers.

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