**Pseudomonas fluorescens** ATCC 13525 Containing an Artificial Oxalate Operon and *Vitreoscilla* Hemoglobin Secretes Oxalic Acid and Solubilizes Rock Phosphate in Acidic Alfisols

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**Abstract**

Oxalate secretion was achieved in *Pseudomonas fluorescens* ATCC 13525 by incorporation of genes encoding *Aspergillus niger* oxaloacetate acetyl hydrolase (*oah*), *Fomitopsis plaustris* oxalate transporter (*FpOAR*) and *Vitreoscilla* hemoglobin (*vgb*) in various combinations. *Pf* (pKCN2) transformant containing *oah* alone accumulated 19 mM oxalic acid intracellularly but secreted 1.2 mM. However, in the presence of an artificial oxalate operon containing *oah* and *FpOAR* genes in plasmid pKCN4, *Pf* (pKCN4) secreted 13.6 mM oxalate in the medium while 3.6 mM remained inside. This transformant solubilized 509 μM of phosphorus from rock phosphate in alfisol which is 4.5 fold higher than the *Pf* (pKCN2) transformant. Genomic integrants of *P. fluorescens* (*Pf* int1 and *Pf* int2) containing artificial oxalate operon (*plac-FpOAR-oah*) and artificial oxalate gene cluster (*plac-FpOAR-oah, vgb, egfp*) secreted 4.8 mM and 5.4 mM oxalic acid, released 329 μM and 351 μM P, respectively, in alfisol. The integrants showed enhanced root colonization, improved growth and increased P content of *Vigna radiata* plants. This study demonstrates oxalic acid secretion in *P. fluorescens* by incorporation of an artificial operon constituted of genes for oxalate synthesis and transport, which imparts mineral phosphate solubilizing ability to the organism leading to enhanced growth and P content of *V. radiata* in alfisol soil.

**Introduction**

Acidic soils occupy about 30% of world’s ice-free land area and are considered important for future agricultural development, as presently only 24.2% of the total land area of the world is potentially arable [1,2]. Phosphorous (P) is the second major nutrient in the soil limiting plant growth after nitrogen (N). In acidic alfisols, plant growth and crop yields are limited by low P [3–5]. Due to the high reactivity of soluble inorganic phosphate (Pi) with Al, Fe and Ca, most soil P exists in the bound form and very low (<10 μM) amount of free P is available for plant growth in soil solution [6]. In acidic alfisols, P is mainly complexed with Fe and Al [7,8] which are difficult to dissolve by simple acidification. However, organic acids have a demonstrated chelation capacity, making them potentially ideal for releasing P from alfisols [5].

Application of rock phosphate (RP) as a P fertilizer to acidic soils is considered as an important strategy for enhancing plant P nutrition [9]. RP is rich in mineral phosphate complexes which could be solubilized in acidic conditions in alfisols. However, partially acidulated rock phosphate (PARP) obtained by mild acid treatment of RP, renders it more easily available for plants [10], indicating that mere addition of untreated RP to alfisols is not efficient. An alternative approach is to use organic acid secreting microorganisms to solubilize RP in alfisols [11,12]. Low molecular weight organic acids are known to be most effective in chelation of Fe and Al and thus solubilization of P [13–15]. Plants secreting piscidic, citric and oxalic acid in root exudates show increased growth and shoot P content [15–17]. Improved growth of pigeon pea plants as compared to other crops in alfisols is attributed to piscidic acid mediated FeP solubilization [7].

Although many microorganisms are known to solubilize mineral phosphates [6], majority of them solubilize Ca-P and very few microorganisms are known to solubilize Fe-P and Al-P [18]. As a result most phosphate solubilizing microorganisms are ineffective in supplying P to plants grown in alfisols. For instance, *Enterobacter asburiae* PSI3, a gluconic acid secreting bacterium, solubilizes P from alkaline vertisols [19] but does not release free P from alfisols supplemented with RP [5]. This has been attributed to the nature and amount of the organic acid secreted by the microorganism. It has been shown that P is released efficiently from alfisol amended with RP when treated with organic acids such as oxalic and citric as compared to gluconate, succinate and malate which are more effective in vertisol or alkaline soils [5]. About 5–10 mM oxalic acid solubilizes P from RP in acidic alfisol which could be accredited to the excellent chelating properties of this acid that
plausibly hinders refixation of P by chelation of Fe and Al ions [5,15,20]. Addition of oxalate to different phosphate rocks and soils resulted in efficient mineral phosphate solubilization [5,15].

Only a few bacterial strains belonging to Bacillus subtilis, Pseudomonas fluorescens, Arthrobacter spp. and Micrococcus spp. are known to secrete oxalic acid that too in very low amounts (≈ 2 mM) [21,22]. High levels of oxalic acid secretion are reported in fungi such as Aspergillus niger, A. fumigatus, Botrytis cinerea, Fumitopsis plautii and Penicillium spp. [21]. In fungi, oxalic acid synthesis is mediated by the cytosolic enzyme, oxaloacetate acetyl hydrolase (OAH), which breaks down oxaloacetate into oxalic and acetic acids [23–26]. A. niger OAH is a pH inducible enzyme belonging to the PEP mutase/isocitrate lyase super family and requires divalent metal ions for catalysis [23,25–27]. In bacteria such as Oxalobacter formigenes oxalate specific transporter (OxIT) is responsible for oxalate uptake and helps in ATP generation [28,29]. On the other hand, a high amount of oxalate secretion in fungi is mediated by efficient oxalate transporter [30]. F. plautii is a wood rotting fungus and degradation of wood is promoted by oxalic acid secretion with the help of an oxalate transporter encoded by FpOAR gene.

Oxygen is present in limited amounts in the rhizosphere which could limit the colonization and survival of rhizobacteria [31]. The obligate anaerobic bacterium, Vitreoscilla, synthesizes elevated quantities of homodimeric hemoglobin (VHb) under hypoxic growth conditions which allows improved growth under microaerobic conditions when dissolved oxygen is less than 2% of air saturation [32,33]. Expression of vgb gene encoding VHb protein in heterologous hosts often enhances growth and metabolism by facilitating oxygen transfer to the respiratory membranes [34]. Beneficial effect of vgb overexpression for improved bacterial growth has been demonstrated in plant associated bacteria [31].

Fluorescent pseudomonads are well-known plant-growth promoting rhizobacteria with root colonization and efficient biocontrol properties [35,36]. The present study deals with the genetic modification of Pseudomonas fluorescens ATCC 13525 for oxalic acid secretion by the incorporation of A. niger oah and F. plautii FpOAR genes and determination of its effect on its mineral phosphate solubilizing (MPS) ability and growth promotion of mung bean (Vigna radiata) plants in acidic alfisols. Additionally, vgb gene was incorporated as a part of an artificial operon containing the oah and FpOAR genes to enhance the survival and colonization of organisms in the soil environment. Incorporation of the artificial oxalate operon in P. fluorescens ATCC 13525 resulted in secretion of high amounts of oxalate which in turn released P from RP in acidic alfisols. Incorporation of vgb gene along with artificial oxalate operon resulted in better colonization and improved plant parameters in acidic alfisols.

Materials and Methods

Bacterial strains, plasmids and media

The plasmids, bacterial and fungal strains used in this study are shown in Table S1. Routine DNA manipulations were done with E. coli DH10B (Invitrogen, Carlsbad, CA, USA) as a host using standard molecular biology protocols [37]. pUC18T-mini-Tn7T-Gm-egfp was generously gifted by Dr. H. P. Schweizer, Colorado State University, USA (Table S1) [38]. P. fluorescens ATCC 13525 (Pf13525) and its plasmid derivatives were grown at 30°C and maintained on Pseudomonas agar (Hi Media, India) containing 50 μg/ml ampicillin and 10 μg/ml gentamycin as and when required. Fungal cultures were grown in minimal medium at 27°C [25,30].

Construction of artificial oxalate operon

RNA was isolated from A. niger by Trizol method (Sigma Aldrich, India) and oah gene was amplified from mRNA using gene specific primers (Integrated DNA Technology, USA) (Table S2). Amplicon was digested with BamHI/Pal and cloned in BamHI/ PstI digested plasmid pUCPM18Gm under lac promoter (Figure S1). The resultant construct was designated as pKCN2. The RNA isolated from F. plautii was amplified using gene specific primers of FpOAR gene (Table S2). Amplicon digested with SacI/BamHI was cloned upstream of oah gene, in SacI/BamHI digested plasmid pKCN2 under lac promoter to construct artificial oxalate operon, designated as pKCN4 (Figure S1). Amplicon of 2.8 kb containing lac-FpOAR-oah was amplified with forward lac primer (Table S2) and oah reverse primer from pKCN4, using XT-20 polymerase (Merck Geneli, India) and was cloned in Smal digested integration vector pUC18T-mini-Tn7T-Gm-egfp.

Construction of artificial oxalate gene cluster (plac-FpOAR-oah, vgb, egfp)

pUCVHb-egfp plasmid was digested with PstI to obtain 3.2 kb insert containing vgb and egfp genes. Insert was cloned in pKCN5 digested with Mdi and end filled using Klenow fragment (Thermo Scientific, USA). The resultant construct (artificial oxalate gene cluster) containing F. plautii FpOAR and A. niger oah genes under lac promoter, vgb gene under its natural oxygen sensitive promoter and egfp under rmb promoter in pUC18T-mini-Tn7T-Gm-egfp vector, was designated as pKCN7 (Figure S1). All the plasmids were transformed in Pf13525 using modified NaCl/CaCl2 method [39] and integration in the genome of Pf13525 was done by transformation method [40].

Physiological and analytical experiments

Bacterial inoculum was used to inoculate Tris rock phosphate (TRP) minimal medium [41] and alfisol soil medium (containing 0.5 g/ml alfisol in sterile medium containing 100 mM glucose, 10 mM KNO₃, micro nutrient cocktail and 30 mg RP/g of soil) for batch studies in 150 ml conical flask containing 30 ml of inoculated medium. The culture supernatants collected at the end were used for extracellular organic acid analysis by Prominance UFLC (Shimadzu, Japan) and P estimation [42]. Cell free extract was used for intracellular organic acid analysis.

MPS ability of Pf13525 transformants and integrants

MPS ability of Pf13525 transformants and integrants was determined on TRP minimal medium plates (containing 100 mM Tris buffer pH-8.0, 1% methyl red, 1.8% agar and 50 mM glucose) with RP as the sole P source (1mg/ml), respectively. Saline washed bacterial inoculum (5 μl) was spot inoculated on plates and incubated at 30°C. Phosphate solubilization and acid secretion was determined by monitoring the growth and red zone of acidification.

OAH assay

Cells grown in M9 minimal medium were used for cell free extract preparation and OAH enzyme activity measurements were done by the method described by Lenz et al (1976) [23]. OAH enzyme specific activity was expressed in nmole per minute per mg total protein. Total protein was estimated using a modified Lowry’s method [43]. One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute.
Plant experiments

Plant studies were done in Murashige and Skoog medium as well as in alfisol soil (containing 10 mg/g of RP). Soil analysis was done from Pulse Research Station (Anand Agriculture University, Vadodara) and was found to contain 0.085% organic carbon, 165.1 kg/ha total nitrogen, 262.4 kg/ha available K and 17.9 kg/ha available P.

Plant parameters such as lengths of the main root and shoot, dry weight and P content were monitored. Molybdate-blue method [45] was used to determine P content. Root colonization of Pf13525 integrant was observed on 5th and 10th day after inoculation and different sections of root were observed for bacterial colonization by using Confocal laser scanning microscopy (LSM 700 Carl Zeiss, GmbH).

Data analysis

Physiological experiments were done in three to four independent replicates for batch culture study. Data are expressed in mean with standard deviation. In plant experiments, three independent triplicate studies were carried out. Differences in mean values were determined using general analysis of variance (ANOVA) and linear regression analysis. The statistical analysis of all the parameters has been done using Graph Pad Prism (version 5.0) software.

Results

Effect of genetic modifications on OAH activity, growth and MPS phenotype of Pf13525

PF(pKCN2) and PF(pKCN4) [Table S1] transformants showed around 230 U/mg of OAH activity while integrants PFint1 and PFint2
int2 showed about 165 U/mg of OAH activity in M9 minimal medium containing 100 mM glucose (Figure 1A). On the other hand, Pf13525 and the vector control did not show detectable OAH activity as the gene is absent in the organism. Growth of Pf (pKCN4) transformant was not significantly different than the untransformed strain in 50 mM Tris-HCl (pH 8.0) medium containing 100 mM glucose as carbon source and RP as the sole P source (Figure 1B). However, the Pf (pKCN2) transformant and the integrants grew slowly and reached 0.28 O.D₆₀₀ nm after 168 h. Pf(pKCN4) transformant was most effective in acidification of the medium from pH 8.0 to 4.2 while Pf (pKCN2) did not decrease the pH to less than 7.0 (Figure 1C). Although growth of the integrants was slower as compared to the pKCN4 transformant, they were effective at reducing the pH of the medium indicating organic acid secretion. Further, organic acid mediated acidification by genetically modified Pf13525 strains was observed on TRP plates. Pf13525 and Pf (pKCN2) transformant did not show red zone of acidification while Pf(pKCN4) transformant, Pf int1 and Pf int2 acidified agar plate containing 100 mM glucose as carbon source and 50 mM Tris HCl (pH 8.0) (Figure 2A).

Effect of genetic modifications on organic acid secretion by Pf13525

To study the effect of increased OAH activity on organic acid secretion in genetically modified Pf13525 strains, the cell lysate and extracellular culture supernatants were analyzed for oxalic acid levels using HPLC. Pf (pKCN2) transformants carrying the oah gene showed the highest intracellular accumulation of oxalic acid up to 19.1 mM, but secreted a relatively less amount (1.2 mM) in the medium (Figure 2B). On the other hand, Pf (pKCN4) transformants possessing artificial oxalate operon, consisting of oah gene along with the fungal oxalate transporter, accumulated only 3.6 mM oxalic acid and secreted 13.6 mM in the medium. Genomic integrants of Pf13525, int1 and int2 secreted 4.1 and 4.7 mM of oxalic acid, respectively, while intracellular levels were 2.6 and 3.1 mM, respectively (Figure 2B).

In order to study the organic acid secretion in soil conditions, alfisol soil supplemented with 100 mM glucose and 30 mg RP/g of soil was inoculated with genetically modified Pf13525 strains and the oxalate secreted in the soil solution was estimated. As seen in Figure 2B, in agreement with the TRP medium studies, in alfisol Pf (pKCN4) secreted 15 mM of oxalate. Oxalate secretion

Figure 3. Root colonization study of V. radiata inoculated with Pf int2. (A) and (B) colonization study in Murashige-Skoog (MS) medium (hydroponics study) on 5th and 10th day respectively; (C) and (D) in alfisol soil (pot experiment) on 5th and 10th day, respectively, by Confocal Laser Scanning Microscopy (CLSM). Left panel shows fluorescence imaging, middle panel shows bright field images and rightmost panel shows overlapped images. doi:10.1371/journal.pone.0092400.g003
by integrants in alfisol was also comparable to that on TRP medium. Wild type Pf13525 secreted 2.0 mM gluconic acid under similar growth conditions but did not show either oxalate accumulation or secretion. All genetic modifications resulted in a decrease in gluconic acid secretion.

**Effect of genetic modifications of Pf13525 on MPS ability**

In TRP minimal medium, Pf[pKCN2], Pf[pKCN4], Pf int1 and Pf int2 released 62 µM, 217 µM, 152 µM and 155 µM of P from RP, respectively (Figure 2C). In alfisol soil medium containing 100 mM glucose as the carbon source and supplemented with 30 mg RP/g of soil, Pf[pKCN4], Pf int1 and Pf int2 released 509 µM, 329 µM and 352 µM levels of P, respectively (Figure 2C).

**Root colonization study**

Root colonization ability of Pf int2 was observed in V. radiata plants in Murashige-Skoog’s medium and alfisol soil. On 5th day, in both media abundant colonization was seen on the root surfaces, root tips and at branching points and the colonization decreased on 10th day (Figure 3).

**Effect of inoculation of genetically modified Pf13525 strains on growth parameters of V. radiata**

Inoculation of V. radiata with Pf int1 and Pf int2 in pot experiments with unsterilized alfisol soil supplemented with RP showed better growth (root and shoot length) and increased root and shoot dry weight. Pf int1 and Pf int2 inoculations showed 1.7 and 1.9 fold increase in root length, 1.3 and 1.4 fold increase in shoot length of V. radiata, respectively, as compared to wild type inoculations (Figure 4A). Pf int1 and Pf int2 inoculations resulted in 1.5 and 2 fold increase in root dry weight and 1.3 and 1.4 fold increase in shoot dry weight, respectively (Figure 4B). P content in Pf int1 and Pf int2 inoculations increased in shoot and root by 1.8 and 2.1 fold, respectively, as compared to plants inoculated with wild type strain (Figure 4C). The improvement in plant parameters is correlated with the amount of oxalic acid secreted by Pf int1 and Pf int2.

**Discussion**

Growth and yields of crops in alfisols are low due to acidic pH, aluminum toxicity and limited amount of available P, with high P re fixation capacity of the soil [46]. P is strongly complexed with Fe and Al in alfisols, which are difficult to solubilize by mineral acids. Several lines of evidences have shown that certain low molecular weight organic acids can release P from Fe-P and Al-P complexes. Among several organic acids tested, oxalic and citric acid solubilized RP in alfisol [5]. Only few bacteria are known to naturally secrete oxalate but do so in very low amounts as compared to several fungi which secrete oxalate in molar amounts [21]. Thus, the present work was aimed at genetic modification of P. fluorescens so as to enable it to secrete high amounts of oxalic acid and render it proficient at solubilizing P from RP amended alfisol. This bacterium was chosen since fluorescent pseudomonads are recognized as plant growth promoting bacteria with efficient root colonizing ability [35,36]. The strategy used was to express the key enzyme oxaloacetate acetyl hydrolase (OAH) for oxalate synthesis from a fungal system. Presence of this enzyme for oxalate biosynthesis has not been reported so far from bacteria. In order to enable the OAH transformants to secrete the oxalate in the extracellular milieu, an oxalate transporter from another fungal system was deployed.

P. fluorescens ATCC 13525 transformant harboring oah gene alone (without the heterologous transporter system) secreted low amount of oxalic acid (Figure 5). The ability to secrete the organic acid in the cell free supernatant may be attributed to the resident dicarboxylate transporters (DctA and DctB) [47]. Secretion of oxalic acid by P. fluorescens ATCC 13525 has been shown in response to Al toxicity in presence of external citrate [48], suggesting that the resident dicarboxylate transporters are functional. Pf[pCNK4] transformant harboring artificial oxalate operon containing oah and the fungal transporter PpOAR showed enhanced oxalate secretion (Figure 5) indicating more efficient transport. The transformant harboring artificial oxalate operon also exhibited MPS phenotype in minimal medium containing RP as the P source and in alfisol. Attaining MPS phenotype in alfisol by oxalate secretion is supported by the fact that 5–10 mM oxalate solubilizes significant amount of RP in alfisols [5]. The oxalic acid secreted by the transformant is also expected to be effective in releasing P from alkaline vertisols which contain high amount of Ca-P [41].
Integration of genes into the genome is a preferred method for genetic manipulation of bacteria for environmental applications as compared to plasmid transformation, due to increased stability of the integrant, absence of antibiotic resistance genes, lack of horizontal transfer and reduced metabolic load [49]. However, genomic integrants have the limitation of single copy expression leading to weak phenotype. This was reflected in the decrease in levels of oxalate accumulation and secretion in the genomic integrants of artificial oxalate operon as compared to the plasmid transformants (Figure 5). However, the amount of oxalate secreted in the integrants was sufficient to solubilize RP in buffered medium as well as in alfisols.

Hydroponic and alfisol soil experiments of V. radiata inoculated with genomic integrants demonstrated enhanced root colonization.
and plant growth as compared to the vector control suggesting that genetic manipulation supported colonization and survival in the rhizosphere. On the other hand, E. asburiae PS13, which is efficient in releasing P from alkaline vertisols mediated by secretion of high levels of gluconic acid, did not improve the growth of V. radiata in alfisol [51]. Significant enhancement in plant growth parameters and P content in Pf int2 inoculations as compared to Pf int1 L could be attributed to the presence of VHBs which is known to improve the metabolism of bacteria under microaerobic conditions. Similarly, presence of VHB in Rhizobium etli increased nitrogenase activity and N content in bean plants [31]. This suggests that metabolism of bacteria in the rhizosphere corresponds to that under microoxic conditions. Since oxalate is also implicated in alleviation of Al toxicity to plants [50] and organic acid secreting rhizobacteria are known to promote plant growth by multiple processes [51], it may be hypothesized that the genetically modified strain could be beneficial to plants in diverse soil conditions.

To summarize, in this study we report the genetic manipulation of rhizosphere colonizing bacterium for the secretion of oxalic acid with the aim of imparting the ability to carry out mineral phosphate solubilization (MPS) from acidic alfisol to enhance P availability to plants. Pf13525 harboring oah gene resulted in intracellular accumulation of high amounts of oxalic acid while incorporation of an artificial oxalate operon, containing additionally an oxalate transporter, lead to the secretion of oxalate in the medium, which in turn resulted in MPS ability in the organism. A genomic integrant of artificial oxalate operon showed improved growth and increased P content of V. radiata in alfisol soil. Furthermore, presence of VHB contributed to improved root colonization and better survival of Pf13525 integrant in soil, thus, improved plant growth and P content. The present work demonstrates the potential of oxalic acid secretion in mineral phosphate solubilization by rhizobacteria.

Supporting Information

Figure S1 Schematic representation of arrangement of genes in plasmid constructs used in this study. (A) Expression plasmid and (B) Integration plasmid constructs. Squares denote the genes, operon and gene clusters cloned in the vector backbone. (TIF)

Table S1 Plasmids, bacterial and fungal strains used in this study. Underlined sequences represent restriction enzyme sites used for cloning and sequences in italics indicate universal ribosome binding site (RBS). (DOCX)

Table S2 List of primers used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: KY CK GA GNK. Performed the experiments: KY CK. Analyzed the data: KY CK GA GNK. Contributed reagents/materials/analysis tools: KY CK GNK. Wrote the paper: KY GA GNK.

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