Enhanced citric acid biosynthesis in *Pseudomonas fluorescens* ATCC 13525 by overexpression of the *Escherichia coli* citrate synthase gene

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Citric acid secretion by fluorescent pseudomonads has a distinct significance in microbial phosphate solubilization. The role of citrate synthase in citric acid biosynthesis and glucose catabolism in pseudomonads was investigated by overexpressing the *Escherichia coli* citrate synthase (*gltA*) gene in *Pseudomonas fluorescens* ATCC 13525. The resultant ~2-fold increase in citrate synthase activity in the *gltA*-overexpressing strain *P*(pAB7) enhanced the intracellular and extracellular citric acid yields during the stationary phase, by about 2- and 26-fold, respectively, as compared to the control, without affecting the growth rate, glucose depletion rate or biomass yield. Decreased glucose consumption was paralleled by increased gluconic acid production due to an increase in glucose dehydrogenase activity. While the extracellular acetic acid yield increased in *P*(pAB7), pyruvic acid secretion decreased, correlating with an increase in pyruvate carboxylase activity and suggesting an increased demand for the anabolic precursor oxaloacetate. Activities of two other key enzymes, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase, remained unaltered, and the contribution of phosphoenolpyruvate carboxylase and isocitrate lyase to glucose catabolism was negligible. Strain *P*(pAB7) demonstrated an enhanced phosphate-solubilizing ability compared to the control. Co-expression of the *Synechococcus elongatus* PCC 6301 phosphoenolpyruvate carboxylase and *E. coli* *gltA* genes in *P. fluorescens* ATCC 13525, so as to supplement oxaloacetate for citrate biosynthesis, neither significantly affected citrate biosynthesis nor caused any change in the other physiological and biochemical parameters measured, despite approximately 1.3- and 5-fold increases in citrate synthase and phosphoenolpyruvate carboxylase activities, respectively. Thus, our results demonstrate that citrate synthase is rate-limiting in enhancing citrate biosynthesis in *P. fluorescens* ATCC 13525. Significantly low extracellular citrate levels as compared to the intracellular levels in *P*(pAB7) suggested a probable limitation of efficient citrate transport.

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INTRODUCTION

Most phosphate-solubilizing bacteria (PSB) solubilize mineral phosphates by secreting a variety of organic acids, principally gluconic acid. However, the nature and amount of organic acids limit the efficacy of PSB in soils and in field conditions (Kucey *et al.*, 1989; Gyaneshwar *et al.*, 2002; Khan *et al.*, 2007; Srivastava *et al.*, 2006). Organic

Abbreviations: CS, citrate synthase; dcw, dry cell weight; G-6-PDH, glucose-6-phosphate dehydrogenase; GDH, glucose dehydrogenase; ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase; OAA, oxaloacetate; PPC, phosphoenolpyruvate carboxylase; PSB, phosphate-solubilizing bacteria; PYC, pyruvate carboxylase.

A supplementary figure showing the effect of *ppc-gltA* co-expression on organic acid secretion by *P. fluorescens* 13525 is available with the online version of this paper.

acids at concentrations ranging from 10 to 100 mM are required to release phosphate from alkaline soils, citric acid being the most effective (Gyaneshwar *et al.*, 1998; Srivastava *et al.*, 2006). Of the known PSB, several strains of *Bacillus* sp. and *Citrobacter koseri* have been reported to secrete citric acid along with various other organic acids (Gyaneshwar *et al.*, 1998). Apart from mineral phosphate solubilization, citric acid secretion by PSB could also be implicated in mediating aluminium tolerance, as in *Pseudomonas fluorescens* ATCC 13525 (Mailloux *et al.*, 2008), and as a siderophore, as in *Bradyrhizobium* and *Pseudomonas aeruginosa* (Guerinot *et al.*, 1990; Carson *et al.*, 1992; Marshall *et al.*, 2009).

Citric acid biosynthesis involves condensation of oxaloacetate (OAA) and acetyl-CoA catalysed by the ubiquitous

citrate synthase (CS), a key non-redundant enzyme governing the carbon flux into the tricarboxylic acid (TCA) cycle, with a dual function of producing cellular energy and biosynthetic precursors (Park *et al.*, 1994). However, despite its key position, no direct correlation has been demonstrated between CS activity and bacterial citric acid accumulation. Genetic modifications leading to citric acid accumulation in bacteria include isocitrate dehydrogenase (ICDH) mutation in *Escherichia coli* (K and B strains) and *Bacillus subtilis* (in early stationary phase) as well as aconitase mutation in *Streptomyces coelicolor* (Lakshmi & Helling, 1976; Matsuno *et al.*, 1999; Viollier *et al.*, 2001; Aoshima *et al.*, 2003; Kabir & Shimizu, 2004).

Fluorescent pseudomonads are well-known plant-growth-promoting rhizobacteria with high biocontrol efficacy as well as phosphate-solubilizing ability (Rodríguez & Fraga, 1999; Haas & Défago, 2005). Distinct metabolic features in pseudomonads make them an attractive model for genetic modifications targeting citric acid accumulation. Unlike citric-acid-accumulating bacteria such as *E. coli* and *Bacillus*, which utilize glucose via the Embden–Meyerhoff–Parnas pathway, pseudomonads utilize glucose by two different routes: the periplasmic direct oxidation pathway mediated by pyrroloquinoline-quinone-dependent glucose dehydrogenase (PQQ-GDH) and intracellular phosphorylative oxidation mediated by glucokinase and glucose-6-phosphate dehydrogenase (G-6-PDH), ultimately followed by the Entner–Doudoroff pathway (Lessie & Phibbs, 1984). The control of glycolytic flux mediated by allosterically regulated phosphofructokinase is not possible in pseudomonads, because they lack this enzyme (Lessie & Phibbs, 1984). Other metabolic advantages include high carbon flux through the TCA cycle resulting in lower acetate overflow and increased capacity to produce OAA due to the presence of pyruvate carboxylase (PYC) and/or phosphoenolpyruvate carboxylase (PPC) at the anaplerotic node, availability of a citrate transporter and absence of glucose-mediated catabolite repression (Lessie & Phibbs, 1984; Stover *et al.*, 2000; Nelson *et al.*, 2002; Basu *et al.*, 2006). Thus, CS activity could limit citric acid biosynthesis in pseudomonads.

This paper describes the effect of overexpression of the *E. coli* citrate synthase (*gltA*) gene on glucose metabolism and citrate levels in *P. fluorescens* ATCC 13525.

METHODS

Materials and chemicals. Luria–Bertani broth and *Pseudomonas* agar for culture maintenance; D-glucose, Tris base, phenazine methosulphate (for GDH assay), cysteine.HCl, phenylhydrazine, oxaloacetate, 5,5'-dithiobis(2-nitrobenzoic acid), Folin–Ciocalteu reagent, HPLC-grade acetonitrile and other routine analytical-grade salts and organic acids were procured from Sisco Research Laboratories, Qualigens Fine Chemicals and Merck. NADH, NADP, acetyl-CoA (sodium salt), phosphoenolpyruvate (monopotassium salt), isocitrate, 2,6-dichlorophenolindophenol (sodium salt; for GDH assay), glucose 6-phosphate (for G-6-PDH assay), and the enzymes malate dehydrogenase (porcine heart), lactate dehydrogenase and

citrate lyase were obtained from Sigma. Restriction enzymes and T4 DNA ligase were purchased from Bangalore Genei and used according to the manufacturers' instructions.

Bacterial strains, plasmids and culture conditions. *E. coli* and *P. fluorescens* strains used for this work are listed in Table 1. *E. coli* JM101 was used as the host strain for all the DNA manipulation and molecular biology experiments, using standard protocols (Sambrook & Russell, 2001). The *gltA* (citrate synthase gene) mutant of *E. coli*, W620, was obtained from the *E. coli* Genetic Stock Center (CGSC), Yale University, USA. The *E. coli* *gltA* gene cloned in pBluescriptSK(–) was a generous gift from Dr E. Delhaize, CSIRO Plant Industry, Australia; plasmid pYanni3 was kindly gifted by Dr D. W. Wackernagel, University of Oldenburg, Germany. The *Pseudomonas* stable pUCPM18 plasmid was generously gifted by Dr J. Sokatch, University of Oklahoma Health Sciences, USA, and was used to derive recombinant plasmids pAB7 and pAB8 (Table 1). Physiological studies were carried out using *P. fluorescens* ATCC 13525.

E. coli plasmid transformants were routinely grown at 37 °C and maintained on Luria–Bertani agar; *P. fluorescens* ATCC 13525 and its plasmid-bearing derivatives were grown at 30 °C and maintained on *Pseudomonas* agar. For both *E. coli* and *P. fluorescens*, the final concentrations of antibiotics were 30 µg tetracycline ml^{–1} and 50 µg kanamycin ml^{–1}, respectively, as and when required.

Construction of pAB7 with the *E. coli* *gltA* gene under the *lac* promoter. The *E. coli* *gltA* gene was obtained as a 1312 bp DNA fragment by digesting plasmid pCS-Ec (4237 bp) with *SacI* and *Bam*HI. The fragment was gel eluted, purified and ligated to purified *SacI*/*Bam*HI-digested plasmid pUCPM18 (5349 bp). The resultant plasmid pAB6 (6646 bp) containing the inserted *gltA* gene in the correct orientation with respect to the *lac* promoter of pUCPM18 was identified by restriction pattern. Subsequently, a kanamycin resistance gene (*nptII*) was incorporated in pAB6 in order to provide an appropriate selection marker for *Pseudomonas* transformants. The requisite *nptII* gene was obtained as a purified 1637 bp DNA fragment by digesting plasmid pYanni3 (7535 bp) with *XhoI*/*Hind*III and was ligated to *Sall*/*Hind*III-digested pAB6 to yield pAB7 (8265 bp). The desired plasmid was selected as kanamycin-resistant colonies on Luria–Bertani agar and was confirmed by restriction digestion pattern. Plasmid pAB7 was separately digested with *XbaI* to release a 1294 bp *gltA* gene fragment and the remaining plasmid backbone was self-ligated to obtain pAB8 (6971 bp), containing only the *nptII* gene in pUCPM18; pAB8 was used as the control plasmid for all experiments.

Development of *P. fluorescens* ATCC 13525 harbouring the *gltA* gene independently and in combination with the *Synechococcus elongatus* PCC 6301 *ppc* gene. *P. fluorescens* ATCC 13525 was independently transformed with plasmids pAB7 and pAB8 by using the NaCl/CaCl₂ method (Cohen *et al.*, 1972) to obtain strain Pf(pAB7), expressing *E. coli* *gltA*, and its corresponding plasmid control, Pf(pAB8). *P. fluorescens* ATCC 13525 co-expressing the *ppc* and *gltA* genes, Pf(pAB37), and its double transformant control, Pf(pAB48), were developed by similarly transforming Pf(pAB3), expressing *S. elongatus* PCC 6301 *ppc*, and its control Pf(pAB4), with plasmids pAB7 and pAB8, respectively. The single transformants were selected on *Pseudomonas* agar containing kanamycin while double transformants were similarly selected on kanamycin and tetracycline.

Physiological experiments and phosphate solubilization. Fresh cell cultures grown with overnight shaking at 30 °C in 3 ml Luria–Bertani broth were aseptically harvested, repeatedly washed with sterile 0.9 % (w/v) saline and resuspended in 1 ml of the same under sterile conditions. The resultant bacterial cell suspension was used to inoculate M9 minimal medium supplemented with 100 mM glucose and micronutrient cocktail (Sambrook & Russell, 2001) to give an

Table 1. Bacterial strains and plasmids used in this study

Ap, ampicillin; Km, kanamycin; Str, streptomycin; Tc, tetracycline.

Plasmid/strain	Relevant characteristics	Source/reference
Plasmids		
pUCPM18	Broad-host-range cloning vector, Ap ^r	Hester <i>et al.</i> (2000)
pYanni3	Cloning vector, Km ^r	W. Wackernagel
pCS-Ec	<i>E. coli</i> <i>gltA</i> cloned in pBluescriptSK(-), Ap ^r	E. Delhaize
pAB8	pUCPM18 with <i>nptII</i> gene, Ap ^r /Km ^r	This study
pAB7	<i>gltA</i> gene of <i>E. coli</i> in pAB8, Ap ^r /Km ^r	This study
Strains		
<i>E. coli</i>		
JM101	F' <i>traD36 proA⁺ B⁺ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV thi</i>	Sambrook & Russell (2001)
W620	CGSC 4278 <i>glnV44 gltA6 galK30 LAM-pyrD36 relA1 rpsL129 thi-1, Str^r</i>	<i>E. coli</i> Genetic Stock Center
<i>P. fluorescens</i>		
13525	Wild-type	ATCC
Pf(pAB3)	<i>P. fluorescens</i> 13525 with pAB3 containing <i>S. elongatus</i> PCC 6301 <i>ppc</i> gene under <i>lac</i> promoter, Tc ^r	Authors' unpublished results
Pf(pAB4)	<i>P. fluorescens</i> 13525 with pAB4 control plasmid, Tc ^r	Authors' unpublished results
Pf(pAB8)	<i>P. fluorescens</i> 13525 with pAB8, Km ^r	This study
Pf(pAB7)	<i>P. fluorescens</i> 13525 with pAB7, Km ^r	This study
Pf(pAB48)	<i>P. fluorescens</i> 13525 with pAB8 and pAB4, Km ^r /Tc ^r	This study
Pf(pAB37)	<i>P. fluorescens</i> 13525 with pAB7 and pAB3, Km ^r /Tc ^r	This study

initial cell density of OD₆₀₀ 0.01–0.03 ($\sim 2 \times 10^6$ c.f.u. ml⁻¹). Batch culture studies were performed by shaking 150 ml conical flasks with 30 ml of the inoculated medium on an Orbitek rotary shaker at 30 °C and 200 r.p.m. Antibiotics were added to a final concentration of 12.5 µg kanamycin ml⁻¹ and 7.5 µg tetracycline ml⁻¹, as and when required.

Dicalcium-phosphate-solubilizing ability of the *gltA* transformants of *P. fluorescens* ATCC 13525 was tested on Pikovskaya's agar (Pikovskaya, 1948). Three microlitres of the bacterial inoculum was aseptically spotted on the agar plates and was allowed to dry completely followed by incubation at 30 °C. Ability to release inorganic phosphate from rock phosphate was monitored using TRP minimal medium as described by Buch *et al.* (2008), with the modification that 25 mM Tris/HCl (pH 8.0) was used instead of 100 mM Tris/HCl (pH 8.0). Preparation of bacterial inocula for phosphate-solubilization studies was similar to that for the above-mentioned physiological experiments. Phosphate solubilization on Pikovskaya's agar was determined by monitoring the zone of clearance, and a decrease in the pH of the medium from 8.0 to <5 in TRP broth was used to indicate rock phosphate solubilization.

Analytical techniques. Change in cell density, determined as OD₆₀₀ (Helios γ spectrophotometer, Thermo Spectronics), was used as the measure of growth while decrease in pH of the medium was used as the measure of acid production. The observations were continued until the medium pH was <5. Samples (1 ml), withdrawn aseptically at regular time intervals, were centrifuged at 9200 g for 1 min at 4 °C and were immediately frozen at -20 °C until further used for biochemical estimations. The culture supernatants derived from these samples were used to measure residual glucose concentration in the medium using a GOD-POD kit (Enzopak, Reckon Diagnostics). The culture supernatants derived at the end-point of the experiment (pH <5) were passed through 0.2 µm pore-size nylon membranes (MDI Advanced Microdevices) and used for organic acid analysis by HPLC. The secreted organic acids were identified and quantified using a Varian Microsorb Rphosphate-18 column operated at room temperature using a mobile phase of 0.01 M H₂SO₄ at a flow rate of 1.0 ml

min⁻¹ and the column effluents were monitored using a UV detector at 210 nm (Buch *et al.*, 2008). For citric acid estimation, the same column was operated at room temperature using a mobile phase of 20 mM Na₂HPO₄ with 2.5 % (v/v) acetonitrile at a flow rate of 1.0 ml min⁻¹. Organic acid yields were expressed as g organic acid formed per g glucose depleted per g dry cell weight (dcw). The phosphate liberated from rock phosphate in the TRP medium was measured by the Ames method (Ames, 1966).

Physiological parameters (growth rate, specific glucose depletion rate and biomass yield) were calculated as described earlier (Buch *et al.*, 2008). The total amount of glucose depleted in the medium was obtained by deducting the value of residual glucose concentration (at the end point) from the initial glucose concentration supplied in the medium. The difference between the total glucose depleted and gluconic acid produced was considered as glucose consumed. The statistical analysis of all the parameters was done using Graph Pad Prism (version 3.0) software.

Preparation of cells/cell-free extracts and enzyme assays. M9-glucose-grown cells were harvested in the appropriate growth phase from 30 ml of cell culture by centrifugation at 9200 g for 2 min at 4 °C. CS, isocitrate lyase (ICL) and ICDH were assayed in the stationary phase; other enzymes were assayed using mid- to late-exponential-phase cultures. The whole-cell preparation for GDH assay and preparation of cell-free cytosolic extracts by sonication treatment for assay of remaining enzymes were carried out as described earlier (Buch *et al.*, 2008).

CS (2.3.3.1) activity was estimated by following the absorbance of 5,5'-dithiobis(2-nitrobenzoic acid) at 412 nm, which would change due to its reaction with the thiol group of CoA (Serre, 1969). The assay mixture contained the following in 1.0 ml: Tris/HCl (pH 8.0), 93 mM; acetyl-CoA, 0.16 mM; oxaloacetate, 0.2 mM; 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM and cell lysate. The reaction was started by addition of oxaloacetate. The molar absorption coefficient was taken as 13.6 mM⁻¹ cm⁻¹ at 412 nm. PPC (EC 4.1.1.31) and PYC (EC 6.4.1.1) activities were monitored by following NADH

oxidation at 340 nm in an assay combined with malate dehydrogenase; G-6-PDH (EC 1.1.1.49) and ICDH (1.1.1.42) activities were determined by following the reduction of NADP at 340 nm; ICL (4.1.3.1) activity was monitored by measuring glyoxylate formation at 324 nm with the aid of phenylhydrazine.HCl; and GDH (EC 1.1.5.2) was assayed by following the coupled reduction of 2,6-dichlorophenolindophenol at 600 nm, as described earlier (Buch *et al.*, 2008).

All the enzyme activities were determined at 30 ± 2 °C, against appropriate controls lacking the substrate or the enzyme source in the reaction mixture. One unit of specific enzyme activity was defined as the amount of protein required to convert 1 nmol substrate per min per mg total protein, unless specified otherwise. Total protein concentration of crude extracts and whole-cell suspensions was measured by a modified Lowry method (Peterson, 1979) using BSA as standard, with corrections made for Tris buffer.

Enzymic measurement of organic acids. Cell extracts prepared from stationary-phase cultures of *P. fluorescens* transformants grown on M9-glucose (procedures being the same as for the enzyme assays) were filtered through 0.2 µm nitrocellulose membrane (continuously stored in an ice-bath or frozen until further analysis) and were used to estimate the amount of intracellular citric acid. Supernatants of the same cultures were also filtered through 0.2 µm nitrocellulose membrane and were used to measure extracellular citric and pyruvic acids.

Intracellular citric acid was assayed spectrophotometrically according to the method described by Petrarulo *et al.* (1995) with minor modifications. The assay system contained 50 mM phosphate buffer mix, 1.0 ml; 246 mM phenylhydrazine, 0.02 ml; citrate lyase, 0.27 U (0.02 ml of 13.3 U ml⁻¹ stock); and citric acid standard or cell extract. Buffer mix contained 50 mM phosphate buffer (pH 6.5), 0.1 mM ZnSO₄·7H₂O and 0.2 g sodium azide l⁻¹. Citric acid standards of 5 µM, 7.5 µM, 10 µM, 15 µM and 20 µM were used to generate a standard curve. The difference in A₃₃₀ 3 min after addition of citrate lyase was used for calculating the intracellular citrate concentration (in mM), for which cellular volume was assumed to be 1.63 µl per mg dcw (Emmerling *et al.*, 1999). Extracellular citric acid was analysed using the method involving citrate-lyase-mediated cleavage of citrate to oxaloacetate, which is subsequently utilized in a malate-dehydrogenase-catalysed reaction requiring NADH. Change in NADH absorbance would be proportional to citrate concentration (Petrarulo *et al.*, 1995; Boehringer Mannheim/R-Biopharm Enzymic BioAnalysis/Food Analysis Manual of Citric acid Determination kit, Cat. no. 10 139 076 035). The assay system per cuvette contained 50 mM phosphate buffer mix, 1.0 ml; citrate lyase, 0.27 U (0.02 ml of 13.3 U ml⁻¹ stock); citric acid standard or test sample; 5 U malate dehydrogenase; and 0.1 mM NADH. Citric acid standards of 5 µM, 10 µM, 15 µM and 20 µM were used to generate a standard curve. The difference in A₃₄₀ 4 min after addition of citrate lyase was used for the calculations.

Pyruvic acid was estimated with lactate dehydrogenase, following the rate of NADH utilization, as described by Coccagn-Bousquet *et al.* (1996) with several modifications. The assay mixture contained the following in 1 ml: Tris/HCl (pH 7.5), 200 mM; NADH, 0.12 mM; lactate dehydrogenase, 5 U; and pyruvate (variable). A standard curve prepared using known concentrations of sodium pyruvate was used to estimate pyruvate levels in the culture supernatant.

RESULTS

Overexpression of *E. coli gltA* and its effect on growth, biomass and glucose utilization of *P. fluorescens* ATCC 13525

The pAB7 plasmid containing the *E. coli gltA* gene under *lac* promoter control was constructed using the plasmid

backbone of a broad-host-range plasmid, pUCPM18. The functional expression of *gltA* from pAB7 was confirmed by its ability to relieve the glutamate auxotrophy of the CS mutant *E. coli* W620 when induced with 0.1 mM IPTG, unlike the control pAB8 plasmid which was unable to complement the mutant phenotype (unpublished results). *P. fluorescens* ATCC 13525 harbouring pAB7 [strain *Pf*(pAB7)] showed 107.30 ± 9.7 U of CS activity on M9 minimal medium in the presence of 100 mM glucose, about twofold higher than that in the control *Pf*(pAB8), which showed 51.41 ± 3.07 U CS activity. The kinetic properties and nature of the allosteric regulation of CS in *E. coli* and *Pseudomonas* are known to be similar (Donald *et al.*, 1989; Mitchell *et al.*, 1995)

Both *Pf*(pAB7) and the control *Pf*(pAB8) had similar growth profiles on excess glucose and could acidify the extracellular medium within 30 h (Table 2; Fig. 1). Glucose consumption by *Pf*(pAB7) was reduced moderately but significantly, by ~1.3-fold as compared to *Pf*(pAB8), while growth rate, specific glucose depletion rate, biomass yield and total amount of glucose depleted after 30 h remained unaffected (Table 2).

Effect of *E. coli gltA* overexpression on secretion of citric acid and other organic acids by *P. fluorescens* ATCC 13525

In order to determine the effect of increased CS activity on citric acid secretion, extracellular citric acid levels were monitored. Since *E. coli icd* mutants accumulated citric acid in the stationary phase, stationary-phase culture supernatants of *Pf*(pAB7) and *Pf*(pAB8) were used. Interestingly, the citric acid levels ($\sim 1.25 \pm 0.15$ mM) and yield [0.031 g (g glucose)⁻¹ (g dcw)⁻¹] in the extracellular medium of *Pf*(pAB7) increased by about 19- and 26-fold, respectively, as compared to the control *Pf*(pAB8), which secreted negligible levels of citric acid (Fig. 2a, b). Concomitantly, the intracellular citric acid levels and yields increased about 2- and 2.3-fold in *Pf*(pAB7), respectively, as compared to *Pf*(pAB8).

Culture supernatants of *Pf*(pAB7) and *Pf*(pAB8) also contained gluconic, pyruvic and acetic acids. At the end of 30 h, *Pf*(pAB7) showed ~2.7- and ~2.5-fold higher levels and yield, respectively, of gluconic acid as compared to the control *Pf*(pAB8) (Fig. 2c, d). On the other hand, pyruvic acid levels and yield in the extracellular medium decreased significantly, by about 2.2- and 2.8-fold respectively, while acetic acid yields increased by ~1.8-fold in the *gltA*-overexpressing strain (Fig. 2c, d). Additionally, *Pf*(pAB7) demonstrated improved phosphate-solubilizing ability as compared to its control *Pf*(pAB8), as evident from an enhanced zone of clearance on Pikovskaya's agar (Fig. 3). Under buffered conditions, *Pf*(pAB7) could release 0.92 ± 0.15 µg phosphate (µg dcw)⁻¹ from rock phosphate after 72 h, which was nearly 2-fold higher than the 0.50 ± 0.06 µg phosphate (µg dcw)⁻¹ released by *Pf*(pAB8) ($n=3$; $P=0.031$).

Table 2. Physiological variables and metabolic data for *P. fluorescens* ATCC 13525 transformants grown on M9 minimal medium with 100 mM glucose

The results are expressed as the mean $\pm$ SEM of readings from six independent observations.

Bacterial strain	Growth rate, μ (h ⁻¹)*	Total glucose depleted (mM)†	Glucose consumed (mM)‡	Biomass yield $Y_{dcw/Glc}$ * (g g ⁻¹)	Sp. glucose depletion rate Q_{Glc} * [g (g dcw) ⁻¹ h ⁻¹]
<i>Pf</i> (pAB8)	0.69 $\pm$ 0.04	66.88 $\pm$ 3.84	60.02 $\pm$ 2.27	0.15 $\pm$ 0.02	7.10 $\pm$ 0.74
<i>Pf</i> (pAB7)	0.75 $\pm$ 0.04 ^{ns}	66.94 $\pm$ 2.99 ^{ns}	50.62 $\pm$ 1.31‡	0.17 $\pm$ 0.05 ^{ns}	8.55 $\pm$ 1.56 ^{ns}
<i>Pf</i> (pAB48)	0.68 $\pm$ 0.04	61.58 $\pm$ 3.61	47.45 $\pm$ 3.93	0.14 $\pm$ 0.02	8.30 $\pm$ 1.57
<i>Pf</i> (pAB37)	0.69 $\pm$ 0.03 ^{ns}	63.88 $\pm$ 2.56 ^{ns}	52.67 $\pm$ 3.05 ^{ns}	0.13 $\pm$ 0.02 ^{ns}	8.32 $\pm$ 1.32 ^{ns}

*Determined from mid-exponential phase of each experiment.

†Determined at the time of pH drop (30 h).

‡ $P < 0.01$, ns, non-significant (as compared to respective controls).

Effect of *E. coli gltA* overexpression on enzymes of glucose catabolism

The nature of the metabolic response observed in *gltA*-overexpressing *P. fluorescens* was investigated by assessing the specific activities of key enzymes of glucose catabolism: GDH of the periplasmic direct oxidation pathway; G-6-PDH, representing the intracellular phosphorylative pathway; enzymes at the anaplerotic node including PPC and PYC; the TCA cycle enzyme ICDH; and the glyoxylate shunt enzyme ICL. Since citric acid accumulation was monitored in stationary phase, the activities of CS, ICDH and ICL, directly involved in citrate biosynthesis and catabolism, were also determined in the stationary phase. In *Pf*(pAB7), the periplasmic GDH activity increased by ~ 1.6 -fold while G-6-PDH remained unaltered as compared to the control *Pf*(pAB8) (Fig. 4). Interestingly, PYC activity in *Pf*(pAB7) increased significantly by ~ 1.4 -fold as compared to *Pf*(pAB8) while ICDH activity was unaltered. PPC and

ICL activities were negligible in *Pf*(pAB8) and were not altered in response to increased CS activity (Fig. 4).

Co-expression of *ppc* and *gltA* genes in *P. fluorescens* ATCC 13525 and its effect on physiological and biochemical parameters

Increase in PYC activity in the citric-acid-accumulating *Pf*(pAB7) suggested increased cellular demands for the precursor, OAA. Hence, the *S. elongatus* PCC 6301 *ppc* gene expressed under control of the *lac* promoter of pUCPM18, encoding tetracycline resistance (plasmid pAB3), was introduced into *P. fluorescens* ATCC 13525 along with *E. coli gltA*. This cyanobacterial PPC is devoid of all allosteric regulation; when expressed in *P. fluorescens* ATCC 13525 it led to ~ 12 -fold increase in PPC activity, and this improved the biomass yield without altering the growth or glucose depletion rates (unpublished results).

Fig. 1. Extracellular pH (Δ , $\square$; top scale) and growth profile (Δ , $\blacksquare$; bottom scale) of *P. fluorescens* ATCC 13525 expressing *E. coli gltA*. Δ , $\blacksquare$, *Pf*(pAB8); $\square$, $\blacksquare$, *Pf*(pAB7). OD₆₀₀ and pH values at each time point are represented as the mean $\pm$ SD of four to six independent observations.

Fig. 2. Effect of *E. coli gltA* overexpression on organic acid secretion by *P. fluorescens* ATCC 13525. (a, b) Intracellular and extracellular citric acid levels and yield ($Y_{C/GInt}$, $Y_{C/GExt}$), respectively. (c, d) Gluconic, pyruvic and acetic acids levels and yields ($Y_{G/G}$, $Y_{P/G}$ and $Y_{A/G}$), respectively. Yields are expressed as g citric or other organic acid (g glucose)⁻¹ (g dcw)⁻¹. Grey bars, *Pf(pAB48)*; black bars, *Pf(pAB7)*. The results are the mean $\pm$ SEM of four to seven independent observations. *, $P < 0.05$; ***, $P < 0.001$.

The *ppc-gltA* co-expressing strain, *Pf(pAB37)*, demonstrated 12.79 ± 0.86 U PPC and 97.69 ± 3.23 U CS activities, which were ~ 5.2 - and ~ 1.4 -fold higher than the corresponding control *Pf(pAB48)* (Fig. 5). Like the single transformants, *Pf(pAB37)* and the control *Pf(pAB48)* had similar growth profiles on excess glucose and could acidify the extracellular medium within 30 h (data not shown). The growth rate, specific glucose depletion rate, biomass yield, total amount of glucose depleted and glucose consumed after 30 h in *Pf(pAB37)* remained unaffected as compared to the control *Pf(pAB48)* (Table 2). Gluconic, pyruvic, acetic and citric acid levels and yield in the extracellular medium of *Pf(pAB37)* were unaltered as compared to *Pf(pAB48)* (Supplementary Fig. S1). Specific activities of GDH, G-6-PDH, PYC, ICDH and ICL remained unaltered in *Pf(pAB37)* as compared to *Pf(pAB48)* (Fig. 5). Again, ICL contributed negligibly to glucose catabolism.

DISCUSSION

P. fluorescens ATCC 13525 exhibited enhanced citric acid biosynthesis to overcome aluminium toxicity when grown on malate as the carbon source, by employing a unique metabolic shift involving decreased activities of enzymes utilizing oxaloacetate and citrate (phosphoenolpyruvate carboxykinase, aconitase and ICDH) with a parallel increase in activities of enzymes involved in oxaloacetate and citrate biosynthesis: malic enzyme, malate dehydrogenase and CS (Mailloux *et al.*, 2008). On the other hand, phosphate-solubilization requires ~ 5 – 10 mM citric acid to be secreted by fluorescent pseudomonads utilizing sugars. The present study describes the metabolic changes in response to increased CS activity in *P. fluorescens* ATCC 13525 when grown on glucose.

The ~ 2 -fold increase in CS activity in *Pf(pAB7)* as a result of *E. coli gltA* expression from the *lac* promoter in the

Fig. 3. Phosphate solubilization by *gltA* transformants of *P. fluorescens* ATCC 13525: zone of clearance formed by *Pf*(pAB7) and *Pf*(pAB8) on Pikovskaya's agar with 12.5 μ g kanamycin ml^{-1} , after incubation for 48 h (top) and 120 h (bottom), at 30 °C

absence of IPTG was in accordance with the reports that the *lac* promoter is constitutively expressed in pseudomonads (Labes *et al.*, 1990). Walsh & Koshland (1985) reported a similar increase in CS activity, up to 2-fold, by overexpression of the homologous *gltA* gene of *E. coli* under control of the *tac* promoter. The effect of enhanced CS activity on growth and glucose depletion rates in *Pf*(pAB7) was similar to the results of moderate (~2-fold) overexpression of CS in *E. coli* (Walsh & Koshland, 1985). Unlike in *Pf*(pAB7), in which biomass yield was unaltered, *gltA* overexpression in *E. coli* increased the maximum cell dry weight by 23 % (De Maeseneire *et al.*, 2006). The

growth pattern suggested that glucose utilization was not subject to organic-acid-mediated catabolite repression, known to occur in pseudomonads, despite various organic acids being secreted under the experimental conditions (Basu *et al.*, 2006). However, glucose distribution between the direct oxidative and phosphorylative pathways was altered. Reduced glucose consumption in *Pf*(pAB7) was counterbalanced by increased direct oxidative pathway activity, as evident from increased periplasmic GDH activity resulting in increased gluconic acid levels.

Remarkably, only ~2-fold increase in CS activity in *Pf*(pAB7) elevated extracellular and intracellular citric acid levels by about 15- and 2-fold, respectively, which suggested that CS activity was probably limiting for citrate accumulation in *P. fluorescens* ATCC 13525 utilizing glucose. In contrast, *icd* mutation was the major genetic modification, accompanied by resultant ~2.5- and 3.8-fold increase in CS activity, that led to citrate accumulation in *E. coli* K and B strains (Aoshima *et al.*, 2003; Kabir & Shimizu, 2004). Altered intracellular citric acid levels in *Pf*(pAB7) were accompanied by altered metabolite balance at the anaplerotic node, since intracellular pyruvate was diverted towards increased OAA biosynthesis to meet the increased CS activity. This was evident from increased PYC activity in *Pf*(pAB7) concomitant with reduced extracellular pyruvic acid secretion. Similar enhancement in biosynthetic reactions was also observed in *E. coli* K and B *icd* mutants in the form of increased glyoxylate pathway activity (Aoshima *et al.*, 2003; Kabir & Shimizu, 2004). However, the glyoxylate shunt did not cater for the increased biosynthetic requirements in *Pf*(pAB7), as evident from the very low and unaltered ICL activity detected in *Pf*(pAB8) and *Pf*(pAB7). Low ICL activity was consistent with earlier reports on *Pseudomonas indigofera*, in which ICL contributed negligibly to glucose metabolism (Howes & McFadden, 1962; Diaz-Perez *et al.*, 2007). A parallel increase in acetic acid secretion by *Pf*(pAB7) could be the result of increased conversion of pyruvate to acetyl-

Fig. 4. Activities of key carbon utilization enzymes in *P. fluorescens* ATCC 13525 overexpressing *E. coli gltA*. Grey bars, *Pf*(pAB8); black bars, *Pf*(pAB7). The results are the mean $\pm$ SEM of six independent observations. ***, $P < 0.001$; **, $P < 0.01$; ns, non-significant.

Fig. 5. Activities of key enzymes of carbon utilization in *P. fluorescens* 13525 co-expressing the *ppc* and *gltA* genes. Grey bars, *Pf*(pAB48); black bars, *Pf*(pAB37). The results are the mean $\pm$ SEM of four to six independent observations. ns, non-significant.

CoA; the CO₂ generated would be utilized as a co-substrate by PYC for increased OAA biosynthesis (Papagianni, 2007). In contrast, CS overexpression in *E. coli* reduced acetate secretion (De Maeseneire *et al.*, 2006).

The presence of extracellular citrate in the spent medium of *Pf*(pAB7) was consistent with the presence of an inherent citrate transporter. However, considering the intracellular citrate levels in *Pf*(pAB7), relatively low extracellular citrate levels could be attributed to inefficient citrate transport; *P. fluorescens* are generally known to possess H⁺-dependent citrate transporters, high citrate efflux through which could be thermodynamically unfavourable (Stover *et al.*, 2000; Nelson *et al.*, 2002). The active transport system for citrate excretion appears to be the main rate-determining factor in citrate overproduction by yeasts (Anastassiadis & Rehm, 2005). However, citric acid secretion by an *icd* mutant of *E. coli* BL21(DE3) on 2YT medium is unexplainable as it lacks a citrate transporter (van der Rest *et al.*, 1992; Aoshima *et al.*, 2003).

Co-expression of the *ppc* and *gltA* genes in *Pf*(pAB37), although it increased the PPC and CS activities by about 5 and 1.3-fold respectively, did not alter the citrate levels as compared to *Pf*(pAB48), as evident from the unaltered metabolic profile. However, the increase in PYC activity observed in *Pf*(pAB7) was no longer found in *Pf*(pAB37). The increase in PPC and CS activities in *Pf*(pAB37) was relatively lower as compared to *Pf*(pAB3) and *Pf*(pAB7) independently overexpressing the respective genes, which could be attributed to the reduced copy numbers of the two plasmids in the double transformant *Pf*(pAB37) (data not shown). Inability of *ppc* overexpression to increase the biomass yield in *Pf*(pAB37) as observed in *Pf*(pAB3) could be due to the channelling of the increased biosynthetic precursors to maintaining two different stably replicating plasmids.

In conclusion, CS appears to be the 'bottleneck' enzyme for TCA cycle flux in *P. fluorescens* grown on glucose. The

present study demonstrates a direct correlation of increased CS activity and citrate accumulation, which is contrary to earlier reports from the known citric-acid-producing bacteria, in which increase in CS activity is either the result of a TCA block in the form of *icd* mutation as in *E. coli* or is in response to aluminium toxicity. Increasing CS activity in *P. fluorescens* for citric acid overproduction from glucose is a better strategy than *icd* mutation in *E. coli*, which reduces biomass and growth (Lakshmi & Helling, 1976; Aoshima *et al.*, 2003). The amount of citric acid produced by *P. fluorescens* overexpressing *E. coli gltA* was similar to that secreted by the PSB *Bacillus coagulans* and *C. koseri* on glucose (Gyaneshwar *et al.*, 1998). Enhanced phosphate solubilization by *Pf*(pAB7) suggested that CS overexpression could be an interesting strategy in developing efficient phosphate-solubilizing *P. fluorescens*. Nevertheless, further studies are required to enhance the citric acid levels in the medium up to ~10 mM, for developing effective phosphate-solubilizing fluorescent pseudomonads.

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