Synopsis of the thesis on

Genetic Engineering of *Pseudomonas fluorescens* for Enhanced Citric Acid Secretion

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Doctor of Philosophy in Biochemistry

By

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Synopsis 2011: Genetic Engineering of *P. fluorescens* for enhanced citric acid secretion

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3rd October 2011

To,
The Deputy Registrar,
Academic Section,
M. S. University of Baroda,
Baroda, 390 002

Subject: Submission of synopsis of the Ph.D. work titled – Genetic Engineering of *Pseudomonas fluorescens* for enhance citric acid secretion

Dear Sir,

I am submitting 3 copies synopsis of the Ph.D work. My date of registration was 01/08/2008 and registration certificate no. is 354.

Kindly do the needful.

Thanking you,
Sincerely yours,

(Hemanta Adhikary)
Signature of the candidate

(Prof. G. Naresh Kumar)
Guide

Head
Biochemistry Department

Dean
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INRODUCTION

The rational approach of targeted and purposeful alterations of metabolic pathways in an organism by recombinant DNA technology aiming at the directed improvement of cellular properties preventing most of the unfavorable homeostatic responses has been very well documented as "**Metabolic Engineering**". The novelty of the approach as compared to other classical strategies lies in its directionality and emphasis on complete metabolic networks rather than individual reactions.

Escherichia coli is the most commonly explored model system for validating metabolic engineering approaches including perturbations in existing primary or secondary metabolic reactions and incorporation of novel metabolic pathways. The succinic acid production up to theoretical limits from glucose under both aerobic and anaerobic conditions from *E. coli* through series of metabolic engineering operations exemplifies the immense potential of the approach in manipulating even the densely regulated central carbon metabolism. Apart from *E. coli*, other organisms like *Corynebacterium glutamicum*, *Bacillus subtilis*, *Zymomonas mobilis* and *Saccharomyces cerevisiae* have been used as model systems to understand and improve industrially important bioprocesses viz. production of L-lysin and L-glutamate (1), ethanol from glucose and xylose (2, 3), Riboflavin, Shikimic acid.

Fluorescent pseudomonads are gram-negative, rod-shaped, and non-pathogenic bacterium known to inhabit primarily the soil, plants, and water surfaces comprise of a highly diverse group of plant growth promoting rhizobacteria (PGPR) [4]. These bacteria have simple nutritional requirements; can readily thrive in mineral media supplemented with a variety of carbon sources and produce fluorescent pigments under iron-limiting conditions [5, 6]. The catabolic versatility exhibited by these microorganisms coupled with their propensity to survive adverse environments has given a unique status in biotechnological processes. Fluorescent pseudomonads have been utilized in suppressing plant diseases [7], in the production of secondary metabolites [8], in bioremediation processes [9], as biofertilizers [10], and antifreeze agents [11]. Citric acids secreting fluorescent pseudomonads are postulated to be very effective as phosphate biofertilizers. Since fluoresecnt pseudomonads have varied regulatory network of central carbon metabolism, the effects of the genetic manipulations in central carbon metabolic pathways are expected to differ amongst themselves and with the other microorganisms. Present study aims to investigate the effect

heterologous overexpression of genes associated with citric acid synthesis and secretion in *Pseudomonas fluorescens.*

Pseudomonads are obligate aerobes whose glucose metabolism is very distinct from that in *E. coli.* They generally lack the phosphoenolpyruvate dependant phosphotransferase system (PEP-PTS) for glucose uptake and phosphofructokinase (PFK) of the traditional EMP mediated glucose catabolism. Instead, glucose catabolism in pseudomonads principally occurs by Entner and Duodoroff (ED) pathway in which 6-phosphogluconate (6-PG) is the key metabolite. Glucose is converted to 6-PG either by periplasmic direct oxidation pathway or by intracellular phosphorylative oxidation pathway. The direct oxidation of glucose is catalyzed by a membrane-bound pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) to produce gluconate that can be transported into the cell and further transformed by glucokinase to 6-PG. Alternately, glucose can be uptaken by an active transport mechanism in the form of free sugar, which is then converted to glucose-6-phosphate (G-6-P) and 6-PG by the action of glucokinase and glucose-6-phosphate dehydrogenase (G-6-PDH), respectively. Pyruvic acid and glyceraldehyde-3-phosphate are the products of glucose catabolism by ED pathway which are further oxidized completely through tricarboxylic acid (TCA) cycle.

The previous laboratory study dealt with fluorescent *Pseudomonas spp.* as the model organism for investigating the flexibility/rigidity of the central glucose metabolism in response to the targeted genetic modifications at the anaplerotic node. Overexpression of phosphoenolpyruvate carboxylase (*ppc*) and citrate synthase (*cs*) genes, respectively, demonstrated a direct correlation of increased CS activity with citrate accumulation and enhanced phosphate solubilization. However, the level of citric acid secreted was not sufficient for solubilizing soil phosphates.

Hence the objectives of the present study were defined as follows-

1. Effect of constitutive heterologous overexpression of *E. coli* NADH insensitive citrate synthase in *Pseudomonas fluorescens* Pfo-1

2. Metabolic characterization of engineered *Pseudomonas fluorescens* Pfo1coexpressing *E. coli* NADH insensitive *cs* and *Salmonella typhimurium* sodium citrate transporter or *Bacillus subtilis* magnesium dependent citrate transporter.

3. Evaluation of the effect of overexpression *csYF-citC* operon on P-solubilizing ability of plant growth promoting rhizospheric fluorescent pseudomonads.

4. Plant growth promotion and P solubilisation abilities of *csYF-citC* operon genome integrants of fluorescent pseudomonads.

1. Effect of constitutive heterologous overexpression of *E. coli* NADH insensitive *cs* gene on physiology and glucose metabolism of *P. fluorescens* Pfo-1

pUC18 plasmid carrying three NADH insensitive *cs* gene viz.Y145F, R163L, K167A under natural promoter,generously gifted by Pro Harry Duckworth, University of Manitoba, Canada, were used as a source of subcloning into broad host range vector. Genes were isolated from the vector by PCR using gene specific primer and cloned into T vector pTZ5R/T and subcloned into pseudomonas stable vector under *lac* promoter having kanamycin resistance pUCPM18 using EcoRI and SalI site. The resultant plasmids were designated as pYF, pRL and pKA and could functionally complement *E. coli* W620 CS mutant suggesting expression of a functional CS protein. These NADH insensitive *E. coli cs* genes were constitutively overexpressed in *P. fluorescens* Pfo-1. Metabolic alterations were monitored by measuring the critical enzyme activities during growth and organic acids at the stationary phase. A 4 fold increase in CS activity was found in *P. fluorescens* Pfo-1 harboring pYF compared to wild type and vector control strain when grown on M9 minimal medium with excess glucose. Interestingly, the intracellular citrate level was found to increase upto 50 mM with an extracellular increase upto 3 mM. Increased CS activity and citrate level did not alter the specific growth rate, specific total glucose utilization rate and total amount of glucose utilized as compared to the control; however the biomass yield was found to increase with a concomitant reduction in glucose uptake. No significant alterations in GDH activity was found but PYC activities increased in all the recombinants as compared to the wild type and vector control which accounted for the enhanced glucose consumption. Additionally, G-6-PDH, ICL and ICDH activities remained unaltered. *P. fluorescens* Pfo-1 transformants

secreted pyruvic acid more than acetic acid in M9 minimal medium as compared to the control strain which correlated with enzyme activities. Thus, *P. fluorescens* Pfo-1 harbourig pYF had better citric acid secretion ability.

2. **Metabolic characterization of engineered *P. fluorescens* Pfo-1 coexpressing *E. coli* NADH insensitive *cs* and *S. typhimurium* sodium citrate transporter or *B. subtilis* magnesium dependent citrate transporter**

P. fluorescens Pfo1 harbouring pYF secreted low levels citrate even though more than 10 fold higher citric acid was found intracellularly. This suggested that the H+ dependent citrate transporter of *P. fluorescens* Pfo1 is not effective in the efflux of citrate. In order to determine the efficient citrate efflux system, Na^+ dependent *S. typhimurium* (CitC) and Mg^{2+} dependent *B. subtilis* (CitM) citrate transporter were chosen for citric acid secretion. *Salmonella typhimurim* sewage isolate and confirmed by 16s rRNA sequence (Gene Bank accession number). *CitC* gene from the sewage isolate was cloned into pBluescript KS. *B. subtilis* 168 *citM* gene was cloned using gene specific primers into TA vector pTZ5R/T. Both these citrate transporter genes separately cloned into pUCPM18 vector having gentamycin resistance. The functionality of the constructs was checked by the growth of *E. coli* DH5α transformants on Kosers citrate broth in the presence of 0.1mM IPTG. Artificial pYC operon (*csYF-citC*) was constructed in pUCPM18Gm vector.

To determine the effect overexpression of these genes on the citric acid secretion in a variety of fluorescent pseudomonads, the pYC operon ws transformed in *P. fluorescens* ATCC13525, *P. fluorescens* PF-5, *P. fluorescens* CHAO-1, *P. fluorescens* P109, *P. fluorescens* Fp315 and *P aeruginosa* P4. All these recombinant strains had secreted citric acid upto 7.68±1.28 mM compared to the control strain. Growth, organic acid secretion and enzyme activities of the transformants, except in a few, were similar.

3. **Evaluation of the effect of overexpression *csYF-citC* operon on P-solubilizing ability of plant growth promoting rhizospheric fluorescent pseudomonads.**

Mineral phosphate solubilizing ability of the strains was monitored on Pikovaskya's (PVK) agar and 100mM Tris buffer rock phosphate media (TRP) containing 75mM glucose.

On Pikovaskya's agar plates, pYC transformants of non- and weak- P solubilizing strains like *P. fluorescens* CHAO-1, PfO-1, ATCC13525, P109 and PF-5, showed enhanced P-solubilization upto varying extents while acidification of the inherently efficient P-solubilzers Fp315 and *P. aeruginosa* P4 was further improved. On Tris buffered-rock phosphate (RP) minimal agar, weak P solubilizing *P. fluorescens* pYC transformants except CHAO1, showed improved zone of acidification compared to control strain. Fp315 and P4 *pYC* transformants required lesser time for acidification on agar plates as compared to their controls. Hence, overexpression of pYC operon in fluorescent pseudomonads had consistent improvement its P-solubilizing ability.

4. **Plant growth promotion and P solubilisation abilities of *csYF-citC* operon genome integrants of fluorescent pseudomonads.**

Cloning of YC operon (2.6Kb) is being carried out in Mini Tn7 delivery plasmid AKN66 in SmaI site. The genome integrants will be monitored for their plant growth promotion and P solubilisation abilities.

Conclusion

Present study demonstrates the effect of overexpression of NADH insensitive *E. coli* citrate synthase in metabolically distinct *P. fluorescens Pfo-1* and other fluorescent pseudomonads. CS activity was correlated with citrate production. Amongst the NADH insensitive *cs* strains YF had the higher citric acid synthesis whereas CitC transporter had good efflux ability. MPS ability on agar plates correlated with the levels of secreted citric acid. Genetic modifications amongst different natural fluorescent pseudomonads, with the exception of CHAO1 strain, showed similar effects. Thus, citric acid secretion by genetic modifications appears to have the good potential in developing efficient phosphate solubilizing fluorescent pseudomonads.

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Abstract Published

Hemanta Adhikary, Naresh Kumar G[1], Silvia R. Macwan. Effect of NADH insensitive *E. coli* citrate synthase on citric acid secretion of fluorescent pseudomonads. Abstract Book, First Asian PGPR Congress, Hyderabad, India

Poster presented

Presented a poster entitled **Effect of NADH insensitive E. coli citrate synthase on citric acid secretion and phosphate solubilization of fluorescens pseudomonad** at First Asian PGPR Congress for sustainable Agriculture held at Acharya N.G. Ranga Agricultural University, Hyderabad, India during 21-24 June, 2009.

Manuscript under preparation

A metabolic study of engineered fluorescence pseudomonads strains for high citric acid Secretion. **Hemanta Adhikary**, Paulomi Sanghvi, Silvia R. Macwan, Archana Gayatri, G. Naresh Kumar: June, 2011

International Conference attended in PhD duration

1. Participated in the 3 day First Asian PGPR congress held at Hyderabad India jointly organized by ANGRAU, Hyderabad and Auburn University USA.
2. Participated in 3 day international conference on Mass spectrometry in Life sciences held at national center for Biological Sciences (NCBS) Bangalore India.

Date: **Signature of the candidate**

(Prof. G. Naresh Kumar)
Guide

Head **Dean**
Biochemistry Department **Faculty of science**