Genetic modification of *Bacillus* spp. for enhanced phosphorous and potassium nutrition to plants and vegetative phase transition

Synopsis submitted by

Riddhi Vyas
Ph. D. student
Registration no. : FoS/5/1862
Date of Registration : 14/08/2013

Guide
Prof. G. Naresh Kumar
Department of Biochemistry
Faculty of Science
M. S. University of Baroda
Vadodara-390002
2020

Abbreviations

1.	VHb	*Vitreoscilla* haemoglobin	
2.	GFP	Green fluorescent protein	
3.	*Oah*	Oxaloacetate acetyl hydrolase	
4.	*FpOAR*	*Fomitopsis palustris* Oxalic acid resistance	
5.	TCA	Tricarboxylic acid cycle	
6.	BS	*Bacillus subtilis*	
7.	LB	Lysogeny broth	
8.	LBGM	LB with glycerol and manganese	

Introduction

Plant growth promoting rhizobacteria (PGPR) are known to promote plant growth through variety of mechanisms such as nutrients mobilization, production of plant growth hormones, inducing abiotic stress tolerance, biocontrol of plant pathogens. Recently efforts are also made to isolate rhizobacteria with biocontrol and biofertilizer properties. However, efficacy of plant growth promotion depends on the abundance and functional status in the rhizosphere. *Bacillus* has been one of the dominant plant growth promoting rhizobacteria (PGPR) with efficient biocontrol properties involving secretion of small antimicrobial compounds and enzymes. Since the *Bacillus* spp. possesses good biocontrol abilities and mycorrhizal helper ability, *Bacillus* spp. with phosphate and potassium solubilizing properties could be a very effective PGPR. High amount of oxalic acid secretion is reported in fungi, including *Aspergillus niger, A. fumigatus, Botrytis cinerea, Fomitopsis palustris* and *Penicillium* spp. etc. In fungus oxalic acid synthesis is governed by a cytoplasmic enzyme oxaloacetate acetyl hydrolase (OAH) which breaks down oxaloacetate in oxalic and acetic acid. On the other hand, high amount of oxalate secretion in fungi is mediated by efficient oxalate transporter, which could explain the high amount of oxalate secreted. *Formitopsis palustris* is a wood rotting fungus and degradation of wood is mediated by oxalic acid secretion with the help of an oxalate transporter encoded by *FpOAR* gene. *Bacillus* strains secreting oxalic acid in sufficient amount will be very effective in mineralizing the inorganic phosphate and inorganic potassium.

In addition to this, *Bacillus subtilis* has been considered a model organism to study formation of complex multicellular structures called biofilm. Impaired respiration is one of the environmental signals triggering biofilm formation in *B. subtilis*. Low oxygen concentration impairs aerobic respiration in *B. subtilis* which is sensed by KinA and KinB. KinB - a membrane embedded histidine kinase senses impaired electron transport through respiratory chain via its transmembrane segment 2 and KinA senses decrease in NAD^+ levels through its PAS A domain. Further, transduction of this signal leads to matrix production and consequent colony wrinkling by *B. subtilis*. Similar colony wrinkling is triggered by a combination of glycerol and manganese (GM) in lysogeny broth (LB) medium and biofilm production due to glycerol and manganese is sensed by the extracellular CACHE domain of KinD. Colony wrinkling is an outcome of decreased oxygen concentration that increases the surface to volume ratio facilitating greater access to oxygen. *Vitreoscilla* hemoglobin (VHb) improves aerobic growth and bioproduct synthesis by supplying oxygen to respiratory chain. However, its effect on multicellularity is not clear in *Bacillus* spp. Here, we report that genomic integration of *vgb* in *B. subtilis* DK1042 mitigates complexity of biofilm and associated sporulation under different conditions mainly by improved respiration.

Objectives

1. Developing oxalic acid secretion ability in *Bacillus* spp. by incorporation of oxaloacetate acetyl hydrolase (*oah*) and oxalate transporter (*FpOAR*) genes.
    a. Incorporation of *vgb* and *gfp* genes into *Bacillus* spp.
    b. Incorporation of *oah* and *FpOAR* genes into *Bacillus* spp. using pSW4 shuttle vector.
    c. Developing genomic integrant of *Bacillus* spp. containing *oah, FpOAR, vgb* and *gfp* genes.

2. Biochemical characterization of the MPS and MKS ability of *Bacillus* spp. secreting oxalic acid.
    a. Characterization of MPS ability of *Bacillus* spp.
    b. Characterization of MKS ability of *Bacillus* spp.
    c. Determine the effect of bacterial inoculants on the host plant (*Vigna radiata* (L.)) especially P and K nutrition.

3. Evaluation of effect of VHb on biofilm formation and sporulation in *B. subtilis*

Objective-1: Developing oxalic acid secretion ability in *Bacillus* spp. by incorporation of oxaloacetate acetyl hydrolase (*oah*) and oxalate transporter (*FpOAR*) genes.

Two bicistronic operons *vgb-gfp* and *FpOAR-oah* were constructed under the control of constitutive P43 promoter in amyE based integration vector. Tandem repeats of P43 promoters were incorporated upstream of these two operons in order to increase the expression of target genes in genomic integrants. Three integration vectors pNRM1113, pNRM1114 and pNRM11O containing P43-*lox*71-*kan*-*lox*66-P43-*vgb-gfp* and P43-*lox*71-*kan*-*lox*66–$(P43)_3$-(P43)-*vgb-gfp* and P43-*lox*71-*kan*-*lox*66–$(P43)_3$-(P43)-*vgb-gfp*-P43-*FpOAR-oah* cassettes respectively, were constructed. The integration vectors pNRM1113, pNRM1114 and pNRM11O were transformed in *B. subtilis* via homologous double cross over events to give rise to integrants NRM1113, NRM1114 and NRM11O respectively. Positive integrants were selected on Luria agar plate containing neomycin at 6 μg/ml concentration. Selected integrants were further spot inoculated on starch agar medium to confirm the double cross over events by absence of starch hydrolysis zone on starch agar medium. Integration of all three cassettes in respective integrants was also confirmed by PCR amplification from the genomic DNA by using gene specific PCR.

Objective-2: Biochemical characterization of the MPS and MKS ability of *Bacillus* spp. secreting oxalic acid.

2-A. Promoter Strength Analysis by quantification of GFP fluorescence from *Bacillus subtilis* DK1042 WT, NRM1113 and NRM1114

Effect of multiple P43 promoters was determined by time dependent analysis of GFP fluorescence. Time dependent analysis of GFP Fluorescence/OD_{600} ratio revealed that NRM1114 having five copies of P43 promoter upstream of *vgb-gfp* operon showed 2 fold higher intensity than NRM1113 having 2 copies of P43 promoter in both Luria Bertani and M9 minimal medium.

2-B. Quantification of oxalic acid secretion and solubilization of inorganic phosphorous and potassium by *Bacillus subtilis* DK1042 WT, NRM1113, NRM1114 and NRM11O

Functionality of oxalate operon was checked by HPLC of bacterial culture supernatant. *B. subtilis* DK1042 WT, NRM1113, NRM1114 and NRM11O were grown in M9 minimal medium (Kleijn et al., 2010) having 50 mM Glucose as a sole carbon source and combination of Glucose and Malate (50 mM + 20 mM) at 200 RPM. Growth and pH were monitored at regular time intervals. After 48 hr of incubation, there was no significant difference in growth pattern and pH of the integrants as compared to DK1042 WT in both the broth. Moreover, cells started undergoing sporulation so, after 72 hr culture supernatant from all four DK1042 WT, NRM1113, NRM1114 and NRM11O cultures were taken out and HPLC was carried out to detect the presence of oxalic acid. Oxalic acid was detected in the culture supernatant of NRM11O while integrants NRM1113, NRM1114 and WT did not produce oxalic acid. NRM11O secreted 305 mM and 178 mM oxalic acid in M9 Glucose and M9 Glucose+Malate minimal medium respectively. There was no significant difference among the phosphate solubilization zone and potassium solubilization zone formed by the integrants and the WT. Hence, it is inferred that the amount of oxalic acid secreted by NRM11O is insufficient to increase the mineral solubilization ability of *B. subtilis* DK1042 and therefore microcosm studies to check the effect of integrants on the growth of *Vigna radiata* (L.) were not carried out.

Objective-3: Evaluation of effect of VHb on biofilm formation and sporulation in *B. subtilis*

Incorporation of VHb reduced complexity of colony biofilm in NRM1113 as compared to WT on both LB and LBGM. However there was no significant difference in NRM1114 as compared to WT and therefore, rest all the studies were conducted to compare NRM1113

and WT. Colony diameter of NRM1113 increased 2.83 and 1.95 fold with respect to WT on LB and LBGM respectively ($n = 3$, $P<0.05$). Strikingly, surface spreading by NRM1113 was also observed on LB containing 4% NaCl. In order to determine the reason for this surface spreading, WT and NRM1113 were assayed for growth in LB, LBGM and 6% NaCl LB broth. Growth curve experiments showed that both WT and NRM1113 followed similar growth trajectories but specific growth rate (μ) of NRM1113 was slightly higher than that of WT under all the experimental conditions which may contribute to the increased fitness of the integrant. Doubling time of NRM1113 was 9 min shorter than that of WT under osmostic stress. Biofilm phenotype was also monitored on Msgg medium where WT produced more wrinkled colonies as compared to NRM1113 after 24 h incubation. Morphological difference between these two was reduced with further incubation. Similar trend appeared in pellicles formed in Msgg broth. Morphological difference was less significant between the colony biofilms of WT and NRM1113 grown on Msgg medium in comparison to colonies grown on LB and LBGM possibly because Msgg is a minimal medium containing glycerol and glutamate as the carbon source and glycerol is an energy poor carbon source in comparison to complex LB and LBGM media.

Flagella independent surface spreading on 1.5 % agar occurs due to osmotic pressure gradients generated by EPS secretion and outward pressure of cell growth facilitated by surfactin. To elucidate the role of matrix producing genes in surface spreading in the integrant, comparative gene expression was determined in the integrant. Relative gene expression analysis in comparison to WT showed 64.17% (±11.6) downregulation of *epsE* gene in LBGM grown biofilm of NRM1113 while it was upregulated 3.12 (±0.45) fold in LB grown biofilm of NRM1113 ($n = 3$, $p\leq0.05$). Differential regulation of *epsE* in NRM1113 depending upon different media conditions justifies the extent of colony expansion on LB and LBGM medium. Colony expansion on LB could be due to upregulation of *eps* operon as well as surfactin production while on LBGM it could chiefly be attributed to surfactin mediated sliding motility. *bslA* gene was downregulated 70.02% (±8.0) and 71.65% (±2.7) in LB and LBGM grown biofilm of NRM1113, respectively ($n = 3$, $p\leq0.05$). Expression of *tasA* in NRM1113 was statistically insignificant with respect to WT in both the media conditions. Expression of *spo0A* transcripts from vegetatitve (Spo0Av) as well as sporulation (Spo0As) promoters was also similar in NRM1113 on LB and LBGM indicating that VHb does not alter the transcription of *spo0A* and alterations in biofilm phenotype of integrant could mainly be due to change in the levels of Spo0A~P.

Biofilm associated sporulation was less in NRM1113 than in WT on LB and LBGM agar. The sporulation percentage for WT and NRM1113 was 0.91% and 0%, respectively, on LB at 72 h. On the other hand, the efficiency of sporulation was 87.2% in WT and 17.14% in NRM1113 on LBGM at 72 h. WT and NRM1113 produced similar pellicle morphology in LB and LBGM broth. However, pulcherriminic acid secretion was less in NRM1113 with reference to WT in LBGM broth. Cell differentiation process in *B. subtilis* proceeds through three distinct stages - competence, matrix production and sporulation. Spo0A~P plays a

central role in modulating the complex regulatory network of genes. These findings suggests constitutive expression of VHb could significantly alter entire cell differentiation by improved respiration leading to enhanced energy status to withstand stress under unfavourable conditions. Multicellularity and corresponding signalling pathways are highly conserved in the members of *Bacillus* genus therefore, we hypothesize that incorporation of VHb into other *Bacillus* species could have more or less similar beneficial impact. These findings emphasize on the potential use of genetically modified *Bacillus* species containing VHb as biofertilizers/biocontrol agents for sustainable crop production in future.