

## Isolation and Detection of Genes Responsible for Pyoverdines Biosynthesis in *Pseudomonas putida* KNUK9

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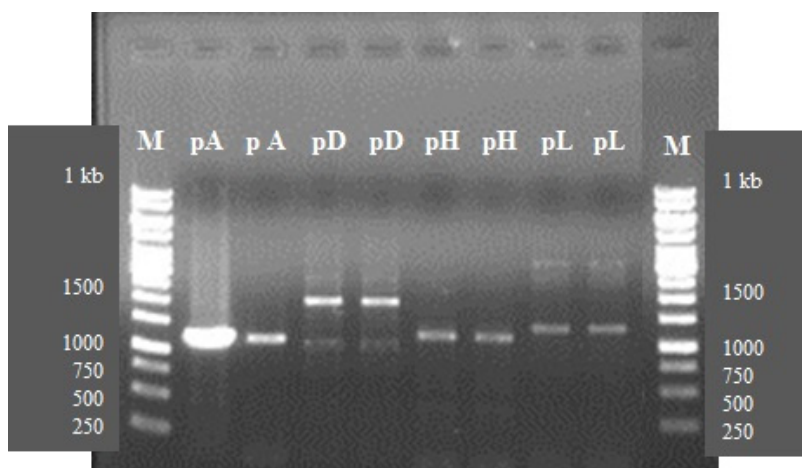
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**Pyoverdines (PVDs) are organic compounds produced by the fluorescent Pseudomonads under iron starvation conditions. Among the isolated rhizosphere pseudomonads strains, *P. putida* KNUK9 showed the highest production of PVDs and its production reached to 62.81% siderophores units. DNA isolation, ligation, PCR amplification, and transformation using *E. coli* DH5 $\alpha$  cells were carried out for preparing the strong pyoverdine producer strains. We detected seven genes playing the fundamental roles in the pyoverdine metabolism in Pseudomonads. According to data and analysis obtained from the study, we deduced that the strain *P. putida* KNUK9 contains the essential genes required for pyoverdine biosynthesis.**

**Key words:** *Pseudomonas putida*, Pyoverdines genes, Biosynthesis, Detection



Agarose gel image of the pyoverdine genes pA; PvdA, pD; PvdD, pH; PvdH, pL; PvdL solated from *P. putida* KNUK9.

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## Introduction

In nature iron is abundant; however, in aerobic environments and under general physiological conditions, iron typically exists in the insoluble ferric ( $\text{Fe}^{3+}$ ) form, thus rendering acquisition by organisms difficult. Iron is an important co-factor for redox-dependent enzymes and an essential element for almost all living entities. Most aerobic and facultative anaerobic microorganisms produce low molecular weight (<10,000Da) iron chelating compounds called siderophores to combat low iron stress (Lamont et al., 2002). Ochsner et al. (2002) showed that siderophores excretion by rhizosphere bacteria activated plant growth by enhancing Fe nutrition of the plants or by suppressing the growth of plant pathogens (Beare et al., 2003). Pyoverdines (PVDs) are organic compounds produced by the fluorescent *Pseudomonads*. The importance of these high-affinity siderophores for rhizosphere colonization by *P. putida* has been previously reported (Nelson et al., 2002; Spencer et al., 2003). However, little is known about the molecular mechanisms of pyoverdines secretion and regulation in plant growth promoting bacteria. Genes responsible for the biosynthesis, excretion, uptake and regulation of these complex compounds are located either at a single region or at different separated regions in the genomes pseudomonads (Zhang et al., 1997). Regenhardt et al. (2002) reported that *Pseudomonas putida* strain KT2440 is the best characterized saprophytic *Pseudomonad* that has retained its ability to survive and function in the environment. In addition to the metabolic potential of this bacterium, the ability of *P. putida* KT2440 to colonize the rhizosphere of crop plants (Espinosa-Urgel et al., 2002) may facilitate the development of biopesticides and plant growth promoters.

Here, we present the isolation and detection results of genes involved in pyoverdine-mediated iron uptake by the plant growth-promoting bacterium *P. putida* KNUK9.

## Materials and methods

**Sampling and bacterial isolation** Rhizosphere soil samples of the Korean ginseng plant (*Panax ginseng*) were collected from different locations in a farm near to Kangwon National University, Chuncheon city, Kangwon province. Samples were carefully taken in zipper bags and stored at 4°C. Five grams of each sample was suspended in 45 ml of aseptic water. After appropriate dilutions, samples were spreaded to beef extract peptone agar medium (beef extract 0.3%, peptone 1%, NaCl 0.5%, agar 1.5%, w/v, pH 7.4–7.6). *Pseudomonas* strains were grown at 30°C in King's medium B (KB) (King et al., 1954). Plates were incubated at 31°C for 48 hours. Bacterial isolates were obtained from agar plates presenting between 30 and 100 colonies. Stock cultures were prepared in aseptic water supplemented with glycerol and kept frozen at -80°C

prior to study.

**CAS assay for siderophores production** CAS-liquid medium (MM9, Tris buffer, casamino acids 0.3%, sucrose 0.2%, thiamine-HCl 0.0002%, and succinate 0.2%, w/v) was prepared to screen siderophore-producing bacteria according to Alexander and Zuberer (1991). The ability of the bacterial isolates to produce siderophores was detected by CAS liquid medium and CAS agar plates (Schwyn and Neilands, 1987). The siderophores production was measured as siderophore units (%) in liquid medium (Machuca and Milagres, 2003). Distilled water was used to prepare all media and reagents. Glasswares were washed three times by 6 M HCl and then washed well with distilled-deionized water several times to remove any iron traces.

**Biochemical and physiological tests** The biochemical tests were conducted by the API 20E strip (bioMerieux Inc., Durham, N.C.) to study the characteristic biochemical activities such as,  $\beta$ -galactosidase,  $\text{H}_2\text{S}$  production, Urease, and Indole production by bacteria strain *Pseudomonas* KNUK9. Color change observations were recorded after 24 h incubation. Universal primer 16F27 (forward) (5'-AGAGTTTGATCCTGGCTCAG-3') and (reverse) (5'-AAGGAGGTGATCCAGCCGCA-3') was used for the amplification of 16S r-RNA of the bacterial isolate *Pseudomonas* KNUK9 according to Brosies et al. (1978).

**DNA isolation and PCR amplification** The bacterium was grown in "Luria-Bertani" medium (LB) broth on a rotary shaker (150 rpm at 37°C for overnight). Wizard® Genomic DNA Purification Kit for Isolation of Genomic DNA from Gram Negative Bacteria was used to isolate DNA as advised by the manufacturer. PCR reactions were conducted in a final volume of 50  $\mu\text{l}$  with 2  $\mu\text{L}$  of each primer, 1.5  $\mu\text{L}$  DNA, 4  $\mu\text{L}$  dNTPs, 5  $\mu\text{L}$  10 x buffers, 0.1  $\mu\text{L}$  Taq polymerase enzyme. Primers sequences used in amplification and PCR of pyoverdines genes were shown in Table 1. All mixed in PCR buffer (TaKaRa Biotechnology (Dalian) Co., Ltd.), DNA was amplified over DNA was amplified over 35 cycles of denaturation for 3 min at 94°C annealing at 55°C for 0.5 min and extension at 72°C for 2 min. After the last cycle, DNA was extended at 72°C for 7 min. Amplification was confirmed by analyzing 5  $\mu\text{L}$  of PCR products mixture on 1% agarose gel (Promega).

**Agarose gel electrophoresis** Agarose gel electrophoresis: 0.7% (w/v) agarose (Sigma-Aldrich CAS NUMBER: 9012-36-6) was dissolved in 1x TAE buffer (40.0 mM Tris Base, 0.1% (v/v) glacial acetic acid, 1.3 mM EDTA, at pH 8). Ethidium bromide of 5  $\mu\text{L}$ / 100 ml gel was added well before solidification to stain DNA. Gels were run to verify the success of the plasmid extractions and to analyze PCR results.

**Table 1. Primers sequences used in amplification.**

Gene	Size bp	Primer/Direction sequence
1 FpvA	441	F- 5' TGGAACGTTACTATCGTGAG 3' R- 5' GCTTCATGGCATTGACGA 3'
2 PvdA	1188	F- 5' AGCCCTCACAGCAGGAAA 3' R- 5' TGGCTGTTTTTCGCAGAATC 3'
3 PvdD	1373	F- 5' AGCCAACGGATGTAGTCACC 3' R- 5' CAGGCACTGCAACATGAACT 3'
4 PvdE	1450	F- 5' GTGACGGTTTCGATCATCCT 3' R- 5' GGCAGCAGCTCTGTGTAGAA 3'
5 PvdF	1530	F- GTCTGCCAGGTTTCTTCTGC R- TTCGGTGACTACATCCGTTG
6 PvdG	1373	F- 5' AGCCAACGGATGTAGTCACC 3' R- 5' CAGGCACTGCAACATGAACT 3'
7 PvdH	1305	F- 5' AACCCCTGTACGAATTCACC 3' R- 5' CAACCTCGTCGATCTGCTC 3'
8 PvdI	1426	F- 5' TCACCTTGCACCACATCATT 3' R- 5' AGTTCGTGTTGGTTCAATGC 3'
9 PvdJ	1402	F- 5' TGTCGAACAGGTCTTCATCG 3' R- 5' GATACCCCTTTGCAGCAGTT 3'
10 PvdL	1485	F- 5' TCGTTGACCTGGGTGTGATA 3' R- 5' TATTGGCGAGCTGTATGTCG 3'
11 PvdM	1319	F- 5' TGAGACCGAAATGACGACAG 3' R- 5' CCCCATAGCTTGGAATATC 3'

8  $\mu$ L of each prepared sample were loaded onto the gel along with 1  $\mu$ L of GeneRuler™ DNA Ladder (Fermentas; Cat. No. SM0311) mixed with 1  $\mu$ L 6x DNA loading dye and 4  $\mu$ L deionized water. Gels were run at 100 V for 15 minutes in 1x TAE buffer. The gel was then imaged instantly and documented using MultiDoc-It™ Imaging System, UVP® under an ultraviolet lamp.

**Cleaning of PCR products** Thick agarose gel (0.7% (w/v) agarose (Sigma-Aldrich CAS NUMBER: 9012-36-6)) was prepared, was dissolved in 1x TAE buffer (40.0 mM Tris Base, 0.1% (v/v) glacial acetic acid, 1.3 mM EDTA, at pH 8). Ethidium bromide of 5  $\mu$ L/ 100 ml gel was added well before solidification to stain DNA. The PCR products were thawed on ice, 10–15  $\mu$ L was loaded with appropriate amount of loading dye.

**Ligation** PCR clean-up procedures were carried out with MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology, Inc. Cat. No.17286). After electrophoresis, the interesting DNA fragment was cut out with a sharp scalpel. Three times volumes of BNL Buffer were added to 1 volume of gel (300  $\mu$ L per 100 mg of agarose gel). The mixture was vortexed and incubated at 55°C for 10 minutes to dissolve gel slice completely. The dissolved gel mixture was transferred to the MEGAquick-spin™ column assembly and

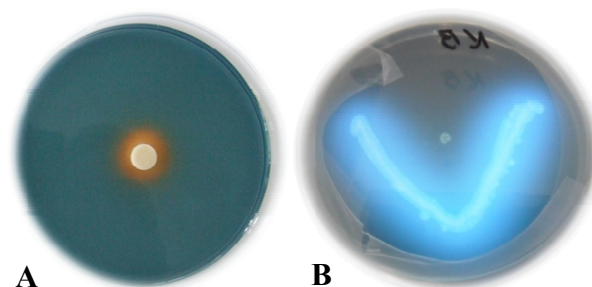
centrifuged for 1 min. The flow-through was discarded. 700  $\mu$ L of washing buffer was added to column and centrifuged at 13,000 rpm for 1 min. The Spin membrane was dried by centrifugation for 1 min at 13,000 rpm. 30–100  $\mu$ L of the elution buffer was applied directly to the center of the column and incubated at room temperature for 1 minute then centrifuged for 1 min at 13,000 rpm. The microcentrifuge tube containing the eluted DNA was incubated at -20°C.

**Transformation of *E. coli* DH5 $\alpha$  cells** 5  $\mu$ L of ligation solution were added to 30  $\mu$ L of chemically competent *E. coli* DH5 $\alpha$  cells. The mixture was incubated on ice for 30 minutes, followed by incubation at 4°C for overnight. The transformation mix was then plated out in varying quantities onto agar plates containing ampicillin and where necessary IPTG and X-gal. The plates were incubated overnight at 37°C. Individual white colonies were resuspended in either 100  $\mu$ L of sterile water for colony PCR and 5 ml of media to harvest the plasmids.

**Plasmid extraction** The *P. putida* KNUK9 plasmid was extracted from 3–5ml of overnight cultures of *E. coli* DH5 $\alpha$  with the DNA-spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Inc.) according to the manufacturer's instructions.

## Results and discussion

In previous study (Hussein and Joo, 2014) several strains were selectively isolated from the rhizosphere of ginseng plant, *Panax ginseng*, and evaluated quantitatively to produce siderophores. Among these strains, *P. putida* KNUK9 showed extensive siderophores production (Fig. 1) and its production reached to 62.81% siderophores units (Hussein and Joo, 2014). The spectra analysis using Sephadex LH-20 was adopted to purify siderophores which were isolated from *Pseudomonas* KNUK9. The analysis indicated the presence of catecholate fluorescent peptidic siderophore (pyoverdine), pyoverdine (PVD) which was well-known as strong iron scavengers. As for all siderophores, PVDs are produced in response to



**Fig. 1. The ferric uptake ability of siderophores via filter paper disc impregnated with culture extract of *P. putida* KNUK9 (A). Effect of (KB) medium on fluorescent pigment production under ultraviolet light (312nm) (B).**

**Table 2. Genes detected in *P. putida* KNUK9 in this study and their specific functions referring to other literatures.**

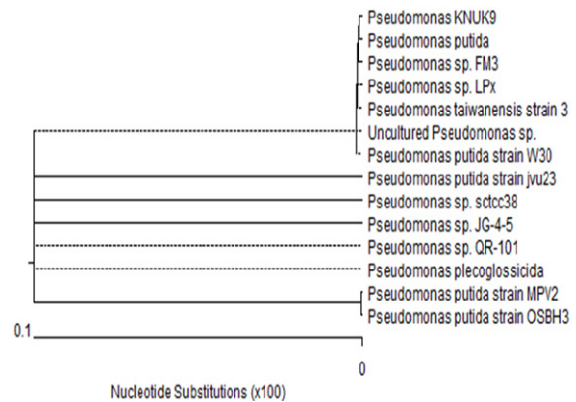
Gene	Primer	Strain	Function	Reference
PvdA	F- 5' AGCCCTCACAGCAGGAAA 3' R- 5' TGGCTGTTTTTCGCAGAATC 3'	<i>Pseudomonas putida</i>	Formation of L-Ornithine. Produce ornithine hydroxylase.	Visca et al. (1994)
PvdD	F- 5' AGCCAACGGATGTAGTCACC 3' R- 5' CAGGCACTGCAACATGAACT 3'	<i>Pseudomonas putida</i>	Pyoverdine peptide synthetase. responsible for incorporating peptide bonds among amino acids.	Merriman et al. (1995)
PvdH	F- 5' AACCCCTGTACGAATTCACC 3' R- 5' CAACCTCGTCGATCTGCTC 3'	<i>Pseudomonas putida</i>	Aminotransferase.	Vandenende et al. (2004)
PvdL	F- 5' TCGTTGACCTGGGTGTGATA 3' R- 5' TATTGGCGAGCTGTATGTCG 3'	<i>Pseudomonas putida</i>	Chromophore biosynthesis.	Mootz and Marahiel (1997)

nutritional iron deficiency. This is inferred by the evidence that pyoverdine synthesis is repressed in suspension cultures grown in chemically-defined laboratory media containing more than 5  $\mu\text{M}$   $\text{FeCl}_3$  (Meyer and Abdallah 1978). Iron-dependent transcriptional repression is primarily dependent on the binding of the Fur-Fe (II) holorepressor complex to the promoters of iron-regulated genes. The Fur repressor together with Fe (II) as a corepressor (Beare et al., 2003) controls the expression of the *pvdS* and *fpvI* genes (Redly and Poole 2003). Table 2 summarize the functions of the detect genes in this study, moreover, *pvd* genes that have not yet been assigned for biochemical functions may catalyze reactions involved in chromophore maturation (Baysse et al., 2002).

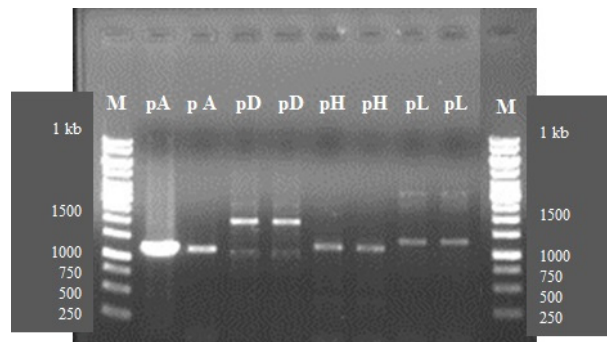
**Biochemical and Phenotypic Identification** Biochemical reactions of isolate *Pseudomonas* KNUK9 was investigated by the API 20E strip (bioMe'rieux Inc., Durham, N.C.). Strain *Pseudomonas* KNUK9 was very closed to *P. putida* based on 16S r-RNA analysis. The strain was positive in the reactions of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, gelatinase, citrate utilization, and urea hydrolysis, and was negative in the reactions of beta-galactosidase,  $\text{H}_2\text{S}$  production, deaminase, indole production, and acetoin production. Moreover, the strain was Gram negative short rods, and catalase positive. The apparent results was confirmed to be very close to *Pseudomonas* sp. Furthermore The 16S r-RNA analysis revealed that isolate *Pseudomonas* KNUK9 had 99% homology with *P. putida*.

**Phylogenetic analysis** 16S rRNA DNA sequence was submitted to the database of National Center for Biotechnology Information (NCBI) and the sequences were compared to other available ribosomal RNA sequences using an automatic alignment tool (Blast). Using the online program [www.phylogeny.fr](http://www.phylogeny.fr), the construction of the phylogenetic tree was generated by PhyML and the visualization of the tree by TreeDyn (Fig. 2). The ribosomal RNA sequences of *Pseudomonas* KNUK9 were deposited in the NCBI Gene Bankit nucleotide sequence database under accession number KC549673).

**The detected pyoverdine genes** Out of 15 different genes responsible for the pyoverdine biosynthesis, we detected seven genes playing the fundamental roles in the pyoverdine metabolism.



**Fig. 2. Phylogenetic tree designed by neighbor-joining analysis of 16S rRNA gene sequence to show the position of *Pseudomonas* KNUK9 the siderophore producer isolate among the genus *Pseudomonas*. *Pseudomonas* sp.(JN253502), *P. putida* (GQ303714), *P. plecoglossicida* (AB642174), *P. putida* (GU329915), *P. taiwanensis* (JX417431), *Pseudomonas* sp. (JF825993), *Pseudomonas* sp. (HM488993), Uncultured *Pseudomonas* sp. (HM152684), *P. putida* (JX237836), *P. putida* (JQ701740), *Pseudomonas* sp. (JN381542), *Pseudomonas* sp. (HQ622342), *P. putida* (AB681214).**



**Fig. 3. Agarose gel image of the pyoverdine genes pA; PvdA, pD; PvdD, pH; PvdH, pL; PvdL solated from *P. putida* KNUK9.**

From all above data and analysis we concluded that the *Pseudomonas* strain *P. putida* KNUK9 contains the essential genes required for pyoverdine biosynthesis (Fig. 3). One of the first pyoverdine synthesis genes to be characterized was *pvdD* (Merriman et al., 1995; Ackerley et al., 2003) with a mutation in this gene preventing pyoverdine synthesis. DNA sequencing showed that this gene encodes an enzyme that is part of a large family of enzymes, the non-ribosomal peptide synthetases (NRPSs). Enzymes in this class catalyze the formation of peptide bonds with substrate of amino acids, and are responsible for the synthesis of a very vast range of peptides and peptide-like secondary metabolites including siderophores (Finking and Marahiel 2004). Different substrate specificities of pyoverdine synthesizing NRPSs in different strains and species results in the production of different peptides present in each of different pyoverdines (Ravel and Cornelis 2003). *PvdI* and *PvdJ* were predicted to direct the incorporation of d-Ser, l-arginine (l-Arg), d-Ser, and l-N5-formyl-N5-hydroxyornithine and the incorporation of l-lysine (l-Lys) and the second l-N5-formyl-N5-hydroxyornithine residue into the pyoverdine peptide, respectively (Ravel and Cornelis 2003). *PvdL* is another NRPS that is also required for pyoverdine synthesis and is involved in synthesis of the chromophore group (Mossialos et al., 2002). The *PvdL* gene is located in the pyoverdine gene cluster though it is not adjacent to the other NRPS genes. The *PvdH* gene was identified through bioinformatics and gene-expression approaches (Ochsner et al., 2002; Lamont and Martin 2003). Its sequence analysis suggested that *PvdH* was an aminotransferase. The *PvdN* and *PvdO* genes tend more to form operons (Lamont and Martin 2003; Ochsner et al., 2002). Their mutations prevent pyoverdine synthesis. Among characterized proteins, *PvdN* has highest sequence similarity to an isopenicillin N epimerase from *Streptomyces clavuligerus* (Kovacevic et al., 1990). However, the exact role of *PvdN* in pyoverdine synthesis has yet to be determined. *PvdO* is suggested to be an Fe<sup>3+</sup> reductase (Ochsner et al., 2002) and its exact role in pyoverdine synthesis is also unclear. Homologues of *PvdP* protein are restricted to the fluorescent pseudomonads, however, the exact function of this protein in pyoverdine synthesis is still unknown.

## Conclusion

The iron scavengers' pyoverdines are produced in response to nutritional iron deficiency. The process is entirely regulated by many *Pvds* genes with variable role for each. For example, *PvdD* gene comes in the first grade of importance during pyoverdine biosynthesis which controls the chromophore biosynthesis. However, the exact role of some genes of pyoverdine production by bacteria is still unclear. We detected seven genes playing the fundamental roles in the pyoverdine

metabolism in Pseudomonads. According to our investigation we can conclude that the *Pseudomonas* strain of *P. putida* KNUK9 possesses all the essential genes required for pyoverdine biosynthesis process.

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