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Isolation, cloning and characterization of *phl*B gene from an Indian strain of Gram negative soil bacteria *Pseudomonas fluorescens*

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Phloroglucinol (PHL) or 2,4-diacetylphloroglucinol (DAPG) is a polyketide compound that exhibits broad spectrum anti-bacterial and antifungal properties against soil-borne plant pathogens. It is produced by gram negative bacterium *Pseudomonas* (fluorescent *Pseudomonad*) which renders them a potential biological control agent. The process of PHL production is accomplished by a well coordinated activity of four (*phlABCD)* genes arranged in an operon. All four genes are essential and indispensable for biosynthesis of DAPG from precursor malonyl CoA. Here, we report cloning and analysis of *phlB* gene from an Indian strain of *Pseudomonas fluorescens*. Further, we predicted the structure of PHLB protein and analyzed its intrinsic details. Stereo-chemical properties were analyzed by Ramachandran plot and secondary structure was retrieved by PDB sum. Our study provides insight into the structure of PHLB protein and revealed that though *Pseudomonas* PHLB lacks conserved motifs and domains it is indispensable for monoacetyl phloroglucinol (MAPG) synthesis.

Keywords: Antibacterial, Antifungal, DAPG, MAPG, Operon, PGPR, *phlABCD*, Phloroglucinol

Plant growth promoting rhizobacteria (PGPR) are plant associated bacteria that colonize and persist in the rhizosphere[1]. They promote growth by directly stimulating the production of plant hormones like auxin, inhibiting plant pathogens, and/or inducing host defense mechanisms against pathogens[2]. Some of the most effective PGPR controlling root and seedling diseases include strains of *Pseudomonas fluorescens*, which produce polyketide antibiotic 2,4-diacetyl phloroglucinol (2,4-DAPG). Different strains of *Pseudomonas* show specificity towards different soil borne pathogen, such as (i)*P. fluorescens* strain CHA0 suppresses black root rot of tobacco[3], (ii) *P. fluorescens* strain J2 antagonises bacterial wilt of tomato[4], *Pythium* damping-off of cucumber[5], and take-all disease of wheat[6]; (iii) *P. fluorescens* strain F113 suppresses *Pythium* damping-off of sugar beet[7] and cystnematode[8]and soft rot of potato[9]; and (iv) *P. fluorescens* strain Q2-87 and Q8r1-96 suppress take-all disease of wheat[10,11].

Till date *Pseudomonas* spp. are the only source of natural phloroglucinol production and 2,4-DAPG producing strains of *Pseudomonas* are of worldwide distribution[12]. The active compound in phloroglucinol is a phenolic polyketide (DAPG) that exhibits antifungal activity and is synthesized from monoacetyl phloroglucinol (MAPG), by an acetyl transferase activity capable of converting MAPG to 2,4-DAPG[13]. Besides genetic regulation, DAPG production is also regulated by external stimuli, such as glucose[14], sucrose or ethanol[7,14,15], zinc sulfate and ammonium molybedate[14] that have stimulatory growth while inorganic phosphate has an inhibitory effect[14].

Cellular level DAPG synthesis is accomplished by tandem action of six genes *viz*., such as *phlA*, *phlB*, *phlC*, *phlD*, *phlE* and *phlF* present in *phl* operon. All six genes in coordination carry out regulation, synthesis, and export of DAPG in and out of cell[16]. It is reported that the genes for DAPG biosynthesis are conserved among *Pseudomonads* isolated from multiple geographical locations[12,17,18]. Function of all six genes of *phl* operon has been elucidated at molecular level. The *phlD* gene product catalyses the condensation of three molecules of malonyl CoA and converts it to monoacetyl phloroglucinol (MAPG) by claisen condensation[19]. The tandem acetyl transferase activity of *phlABC* further converts MAPG to 2,4-diacetyl-

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pholroglucinol (DAPG)[19]. The *phlABCD* genes are flanked by *phlF* and *phlE* on either side with *phlF* having a regulatory activity while *phlE* is an efflux protein that facilitates transport of DAPG in/out of cell[16].

Unlike other PHL proteins, PHLB is a unique enzyme that does not show similarity to other five enzymes of *phl* operon. However, its activity is mandatory for conversion of MAPG to DAPG making it a cardinal gene in *phl* operon. The fact that PHLB lacks conserved domains/motifs and there is lack of elucidation of holomeric three dimensional structure of this protein. In this study, we tried to isolate and clone *phlB* gene from an Indian strain of *Pseudomonas* to get insight into its 3D structure and function.

Material and Methods

Genomic DNA isolation

Genomic DNA of *Pseudomonas fluorescens* was isolated from overnight grown culture by the method described earlier[20]. Quality and quantity of isolated genomic DNA were assessed by agarose gel electrophoresis and spectrophotometrically by estimating absorbance at 260/280 and230/260.

Amplification and cloning of *phl*B gene

phlB gene was PCR amplified from the isolated genomic DNA and was cloned into pBluescript (SK+) vector. Following set of primers, *phlB*FP: AAGGGATCCTACCCGACCATGTCCCTTTAC and *phlB*RP: AGCACTAGTTTATTTCGCCAATACGAA were designed using the nucleotide sequence of *P. fluorescens* available in EMBL database using PRIMER 3 tool[21, 22]. Restriction sites for enzyme *Bam*HI and *Spe*I were incorporated at 5′ end of forward and reverse primer, respectively. The PCR reaction mixture consisted of 10 pmol of gene specific primer, 50ng of template DNA and 5 units of *Taq* DNA polymerase in a 50 µL volume. A gradient PCR (47-60°C) was carried out to optimize the annealing temperature for maximum amplification of *phlB* gene. The thermal cycling was performed after initial denaturing cycle of 5 min at 95°C. Then 30 cycles of PCR were carried out using the following temperature profiles: (i) denaturation at 95°C for 1 min; (ii) annealing at 49°C for 30 s and (iii)extension at 72°C for 1 min. PCR amplified gene product and control pBluescript (SK+) DNA were double digested with *Bam*HI / *Spe*I for 3 h at 37°C. Restriction digested PCR product and linearized control vector were resolved on 1% agarose gel and eluted for ligation. Ligation was carried out for overnight (4°C) and ligated mix was used for transformation of chemically competent *E.coli* XL-1Blue cells by heat thaw method. The transformed colonies (white in colour) obtained after overnight incubation at 37°C were streak purified on LA-carbenicillin plates.

DNA sequencing

Recombinant white colonies were screened for *phlB* gene by colony PCR with gene specific primers (*phlB*FP: AAGGGATCCTACCCGACCATGTCCCTTTAC and *phlB*RP: AGCACTAGTTTAT TTCGCCAATACGAA) and clones harbouring *phlB* gene were confirmed by plasmid DNA isolation and restriction with *Bam*HI/SpeI enzymes. The positive clones were sequenced by sanger di-deoxy sequencing with vector based M13 universal primers and gene specific *phlB* primers (*phlB*FP: AAGGGATCCTACCCGACCATG TCCCTTTAC and *phlB*RP: AGCACTAGTTTAT TTC GCCAATACGAA). The deduced nucleotide sequence of *phlB* was submitted to GenBank with accession no. #KU641633.

Molecular structure prediction

Model of the PHLB protein was constructed using I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)[23]. I-TASSER (Iterative Threading ASSEmbly Refinement) is a hierarchical approach to protein structure and function prediction. Structural templates are first identified from the PDB by multiple threading approach; full length atomic models are then constructed by iterative template fragment assembly simulations. The generated model was refined using ModRefiner (http://zhanglab.ccmb.med. umich.edu/ModRefiner/) that uses the algorithm for high- resolution protein structure refinement. Further discrete molecular dynamic simulation of refined structure was performed to remove steric clashes and reduced van der waals repulsion forces.

Ramachandran plot analysis and validation of modelled structure

The stereochemical properties of PHLB protein were assessed by Ramachandran plot using RAMPAGE[24]. The residues in disallowed region were further refined using Modloop (https://modbase.compbio.ucsf.edu/modloop/)[25]. The validation of the modelled structure was performed using SAVES server, PDBsum[26] and PROCHECK[27]. Structure visualization was performed using Pymol. The predicted model of protein was submitted to Protein Model DataBase[28] and was assigned identifier #PM0080922.

Results

phlB gene from an Indian strain of *Pseudomonas* was isolated and cloned by PCR amplification using gene specific primers. A gradient PCR between the temperature range of 47°C and 60°C was carried out to optimize (Fig. 1A) the annealing temperature. Our standardization revealed that 49°C was the optimum temperature for precise amplification of *phlB* gene (Fig. 1B). Subsequently, a ~451 bp fragment of *phlB* gene was PCR amplified from the genome of *Pseudomonas* spp. (Fig. 2). The gene specific primers

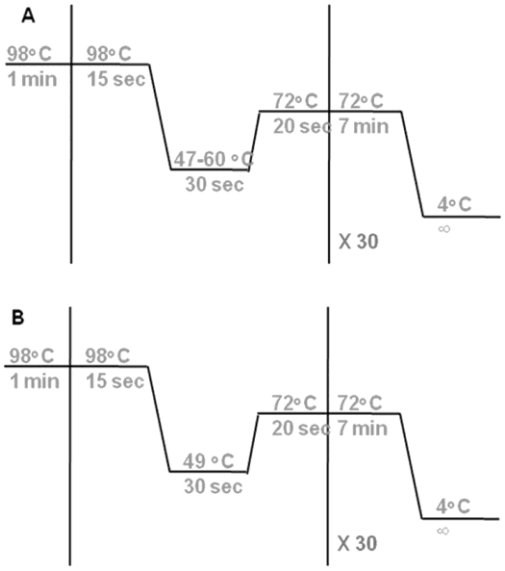

Fig. 1 — Standardization of optimum annealing temperature for maximum amplification of *phlB* gene. (A) Gradient PCR (47-60°C) carried out to optimize annealing temperature for maximum amplification of *phlB* gene; and (B) The optimum annealing temperature was 49°C at which *phlB* gene was amplified.

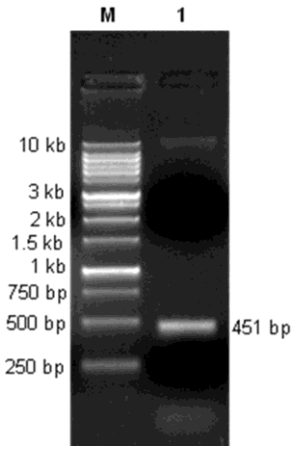

Fig. 2 — PCR amplification of *phl*B gene from genomic DNA of *Pseudomonas fluorescens.* [Lane M: 1 kb DNA ladder (Fermentas); Lane 1: 451 bp amplicon of *phl*B gene]

contained specific recognition sites for restriction enzyme *Bam*HI and *Spe*I for directional cloning. After successful PCR amplification, the 451 bp fragment was restricted with *Bam*HI and *Spe*I. Similarly, the vector DNA of pBluescript was restricted with *Bam*HI and *Spe*I enzyme that resulted in 3 kb linearized pBluescript (SK+) vector with sticky ends of *Bam*HI and *Spe*I (Fig. 3). *phlb* gene was directionally cloned by ligating PCR amplified gene with linearized pBluescript vector and ligated mix was transformed into chemically competent *E. coli* cells.

Random screening of five colonies for the presence of *phlB* gene by colony PCR with gene specific primers resulted in an amplicon of 451 bp (Fig. 4A). Further, the isolated plasmid DNA from five of the clones were restriction digested with *Bam*HI/*Spe*I enzymes that released the expected fragment of ~451 bp (Fig.4B). DNA sequencing of the positive clone confirmed that the 451 base pair fragment is obtained from *phl*B, with the longest ORF of 441 bp. Our result was consistent with the reported sequence of *phlB* gene from *P. fluorescens* strain Q2-87[16] (Fig. 5). Based on the blast results, the *phlB* gene was found to contain complete coding sequence (CDS) of 146 amino acids.

To get insight into PHLB protein, its three dimensional structure was generated (Fig. 6) using i-TASSER server. The homology model was generated based on the available crystal structure of DUF35 family protein from *Sulfobolus solfataricus*

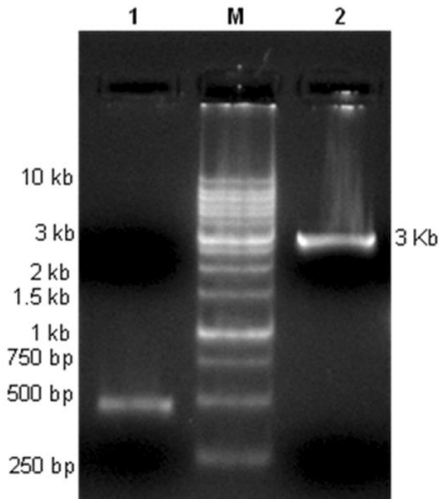

Fig. 3 — Restriction profile of double digested PCR product and control pBluescript vector. [Lane M: 1 kb DNA ladder (Fermentas). Lane 1: 451 bp fragment of purified PCR product digested with *Bam*HI/*Spe*I. Lane 2: ~ 3 kb fragment of vector (pbluescript) DNA digested with *Bam*HI/*Spe*I]

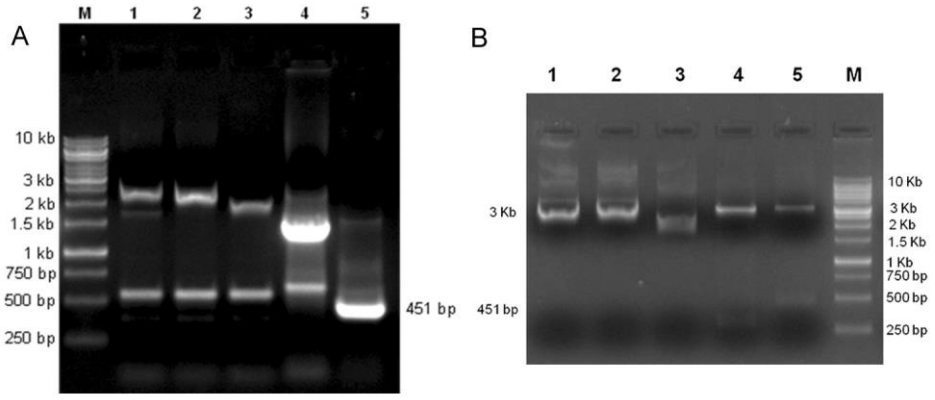

Fig. 4 — Confirmation of *phl*B positive clone. (A) Screening of putative *phlB* positive clone by colony PCR with gene specific primers. [Lane M: 1 kb DNA ladder (Fermentas); Lane 1-5: PCR amplified product. Colony 5 yielded an expected amplicon of 451 bp of *phl*B]; and (B) Screening *phlB* positive clone by restriction digestion. [Lane M: 1 kb DNA ladder (Fermentas); Lane 5: Restriction digestion with *Bam*HI/*Spe*I released a fragment of 451 bp of *phl*B gene and ~3 kb fragment of pBluescript (SK+) vector backbone]

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                1        10        20        30        40        50        60        70        80        90       100
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PHLBour         MSLYPEQIHRMTTASMLREWREHGGKYRLEGSRCQDCEEIFFPRRTVCGACNSLNVEPYRCARTGTIQVSAHAENPILAAMGYGETVPRYMAMVRLDDGL
PHLBQ2-87       MSLYPEQIHRMTTASMLREWREHGGKYRLEGSRCQDCEEIFFPRRTVCGACNSLNVEPYRCARTGTIQVSAHAENPILAAMGYGETVPRFMAMVRLDDGL
Consensus       MSLYPEQIHRMTTASMLREWREHGGKYRLEGSRCQDCEEIFFPRRTVCGACNSLNVEPYRCARTGTIQVSAHAENPILAAMGYGETVPR%MAMVRLDDGL

                101     110       120       130       140   146
                |-------+---------+---------+---------+-----|
PHLBour         VIASEIVDVIDPRQVVSGAPVRMVTRKHVRESNLAWQYAYKFVLAK
PHLBQ2-87       VIASEIVDVVDPRQVVTGAPVRMVIRKHVRESNLAWQYAYKFVLAK
Consensus       VIASEIVDV!DPRQVVsGAPVRMViRKHVRESNLAWQYAYKFVLAK
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Fig. 5 — Pairwise sequence alignment of deduced amino acid sequence of PHLB protein with reported DAPG producing PHLB protein from *Pseudomonas fluorescence* Q2-87 generated using Multalin program revealed >95% similarity.

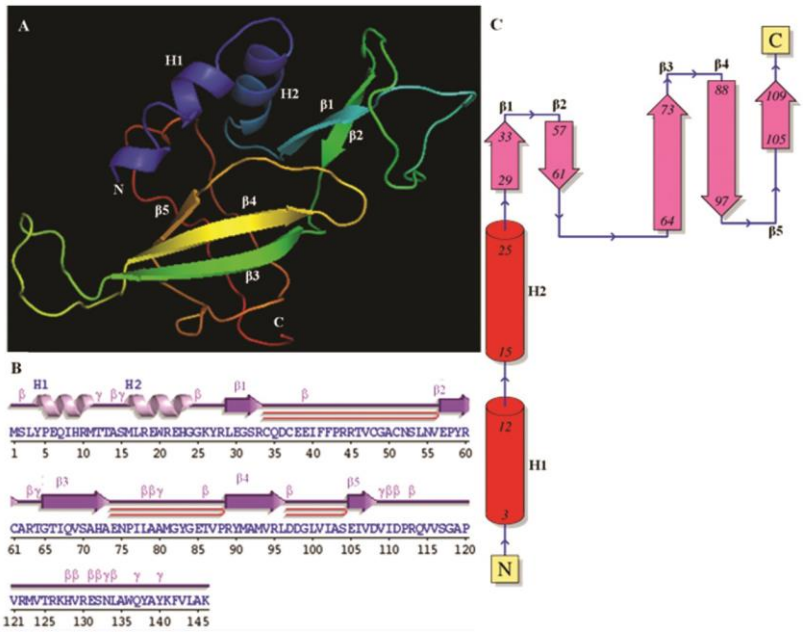

Fig. 6 — Structure of PHLB protein of *Pseudomonas* spp. (A) Stereo ribbon diagram of the PHLB monomer (chain *A*) color-coded from the N-terminus (blue) to the C-terminus (red). Helices (H1–H2) and β-strands (β1–β5) are indicated; (B) Diagram showing the secondary structure elements of PHLB superimposed on its primary sequence. The labeling of secondary-structure elements is in accordance with *PDBsum* (http://www.ebi.ac.uk/pdbsum): α-helices are labeled H1 and H2, the β-strands are labeled β1–β5, β-turns and γ-turns are designated by their respective Greek letters (β, γ) and red loops indicate β-hairpins; and (C) Topology of PHLB protein showing the orientation of α-helices and β-strands

(PDB entry 3irb)[29] as template. Generated model with the highest C-score of–0.65 was selected for further refinement using discrete molecular dynamic simulation. The refined model with reduced number of clashes 33 (Initial model = 166), Van der waals repulsion energy of 20.5397 kcal/mol (Initial model = 167.405 kcal/mol) and clash ratio of 0.0155722 (Initial model = 0.0762316) was generated for further study. The quality of final refined model was evaluated through SAVES server using PROCHECK, Verify-3D, ERRAT program that focused on backbone conformation and its geometric properties, nature of residual interaction, etc. While PROCHECK evaluated the reliability of torsion angle Φ, Ψ and quantified the number of allocated residues, the refined model showed 91% residues in the favoured region and 9% of residues in allowed region with no residues in the disallowed/outlier region of the plot (Fig. 7). The quality of refined model was further validated by the ERRAT score. The ERRAT score 70, of the refined protein, indicate reliable protein environment. Additionally, the verify-3D evaluation reflected 90% of the amino acids with an average 3D-1D score of >0.2 indicate robustness of the proposed structure of the PHLB protein.

The three dimensional structure predicted by homology modelling was in accordance with the secondary structure predicted by PDBsum. PHLB structure revealed the presence of two β-sheets and two α-helices. The protein enclosed two β-sheets and composed of five β- strands. While, β-sheet A contained two anti-parallel β-strands with topology 1, β-sheet B contains three β-strands in opposite orientation with topology 1 1[30]. The protein also contains two α-helices such as, helix 1 (Tyr4-Met11) containing eight residues and helix2 (Met6-Gly24) containing nine residues. Three β-hairpins were distributed between β-sheets A and B belonging to class 23:23, 15:15 and 12:12, which denotes the number of residues in the defined loop[31]. In general there are nine classes of β-turns[32]. The 17 β-turns identified in PHLB protein belonged to four classes of β-turns, such as class I, II, IV and VIII. While class I and II contained one β-turn each i.e., (Met1-Tyr4) and (Leu96-Gly99),respectively, class IV contained 11 β-turns (Thr13-Met16), (Gly24-Tyr27), (Ala62-Gly65), (Ile77-Ala80), (Leu78-Met81), (Ile110-Arg113), (Pro112-Val115), (Lys127-Arg130),(His128-Glu131), (Arg130-Asn133), (Glu131-Leu134). Class VIII contained four β-turns (Glu38-phe41), (Glu85-Pro88), (Val109-Pro112) and (Asn133-Trp136). Out of eight γ turns identified in PHLB protein, seven were inverse type and only one was classic type.

Discussion

The gram negative bacteria of genus *Pseudomonas* promote plant growth and development by producing

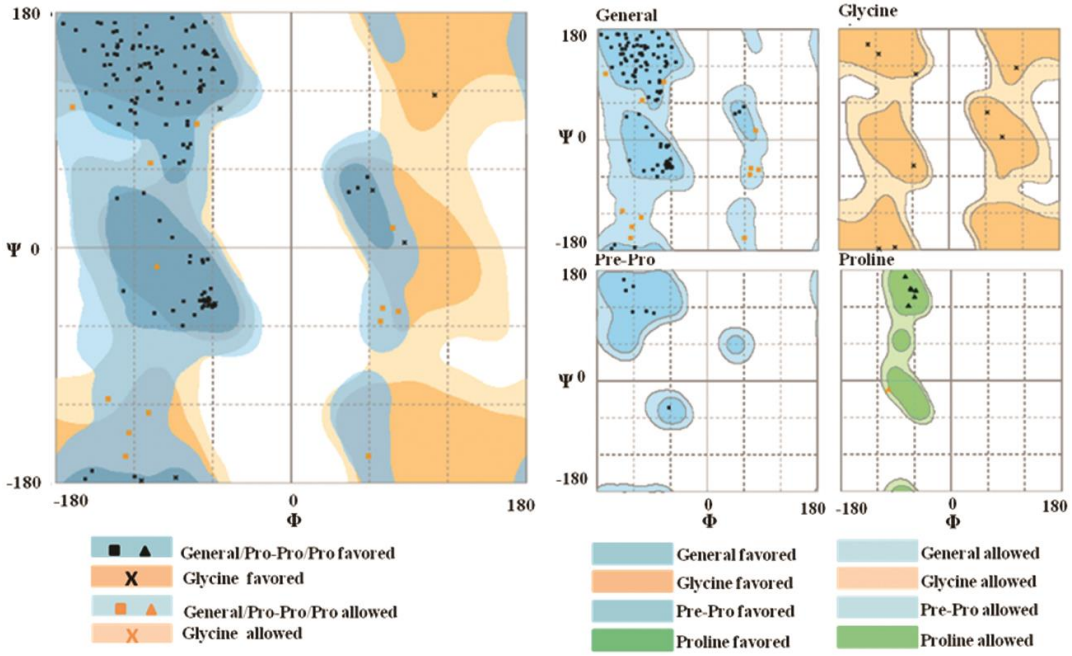

Fig. 7— Analysis of stereochemical structure stability of PHLB protein by Ramachandran plot analysis using RAMPAGE. Refined structure of PHLB had 91% residues in favoured region while 9% fell in the allowed region and none in outlier region

several anti-bacterial and antifungal compounds such as phloroglucinol or 2,4-DAPG. Apart from promoting growth of plant, *Pseudomonads* are also reported to be involved in pigment production[33] and absorption of heavy metals[34,35]. The 2,4-DAPG or phloroglucinol is a phenolic compound and exhibits anti-bacterial and antifungal properties[16] and promote plant growth *in vitro*[36,37]. DAPG production in *Pseudomonas* is not a single gene product. Nevertheless, this compound is produced as a result of well regulated complex chemical reactions by coordinated action of six genes. These six genes *viz*., *phl*A, *phl*B, *phl*C, *phl*D, *phl*E and *phl*F are arranged in an operon on a genomic fragment of ~6.5 kb. *phlB* works in tandem with *phl*A and *phl*C for conversion of MAPG to DAPG.

In the present study, 441 bp *phlB* gene was successfully cloned from an Indian strain of *Pseudomonas* spp. (Fig. 4). The deduced amino acid sequence was 146 amino acids and is consistent with the reported sequence of cloned *phlB* gene[16]. The sequence identity of the cloned *phl*B gene is different from previously cloned *phl*B genes from other strains of *Pseudomonas*, such as strain CM1'A2, strain 2P24, Q2-12, P12 and Q37-87. However, the deduced amino acid sequence (~16 kda) from Indian strain showed significant similarity to PHLB protein from *Pseudomonas fluorescens* strain Q2-87[16] (Fig. 5).

The predicted 3-D structure of PHLB is based on the available crystal structure of DUF35 family protein from *Sulfobolus solfataricus*. PHLB is a DUF domain protein[38] and is orthologous to SSO2064, which is the first structural representative of Pfam protein family PF01796 (DUF35)[29]. Genome-context analysis (http://string.embl.de) have revealed that the members of DUF35 family have a strong gene-neighborhood association with members of the thiolase superfamily (EC 2.3.1.9) that are involved in condensation of acyl-CoA moieties in the formation of longer chain aliphatic and cyclic compounds[29]. PhlB, like other members of DUF35 family, lacks a conservation pattern suggestive of an enzymatic role; it most likely acts as the acyl-CoA carrier protein in the reaction[29]. Though PHLB protein lacks designated motifs to carry out chemical catalysis but is indispensable for DAPG biosynthesis made us inquisitive to study its structural properties. The holomeric structure of PHLB protein predicted by I-TASSER server provided a deeper insight into its structural moieties (Fig. 6). The reliability of the predicted structure was confirmed by examining the C-score value of the deduced protein. Usually, the C-score of predicted proteins range from –5 to 2 and the higher C value indicates robust model. Our deduced structure of PHLB exhibited a C-score value of –0.65 indicating a good protein model. Further, topology of the model was ratified by TM score. While, TM-score of >0.5 indicates a model of correct topology, TM-score <0.17 means a random similarity. The deduced structure of PHLB protein exhibited TM score of 0.63±0.14 and suggested it to be of correct topology. Additionally, occurrence of >90% amino acid residues (91% residues) in favoured region of Ramachandran plot authenticated the refined model to be of good quality (Fig. 7).

The three dimensional structure is consistent with the predicted secondary structure as confirmed by PDBsum (Fig. 6). PHLB protein contained two β-sheets and five β-strands. One anti-parallel classic type beta bulge was also present. Two α-helices were identified interacting in CN manner with one residue from helix 1 and three residues from helix 2 as interacting. Three β-hairpins, 17 β- turns and eight γ turns were also present.

Conclusion

Through this investigation, we identified and cloned *phlB* gene from an Indian strain of *Pseudomonas* spp. Our results confirmed that PHLB protein lacks specific motifs. Further, site directed mutagenesis of *phlB* (knockdown/gene silencing) may provide insight into its cardinal role in DAPG production.

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Conflicts of interest

Authors declares no conflict of interests.

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