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Identification of putative drug targets of *Listeria monocytogenes* F2365 by subtractive genomics approach

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ABSTRACT

The prolonged and uncontrolled use of antibiotics in treatment against many pathogens causes the multiple drug resistance. The drug resistance of *Listeria monocytogenes* F2365 has been evolved, which cause a major disease listeriosis. The drug dose limit against that pathogen was also increased for currently prescribed antibiotics and more often combinational therapy was preferred. Therefore, identification of an extensive novel drug target, unique and essential to the microorganism and subjected to its validation and drug development is imperative. Availability of the total proteome of *L. monocytogenes* F2365 enabled *in silico* identification of putative common drug targets and their subcellular localization by subtractive genomics approach. In the present work subtractive genomics approach is used to identify vaccine and drug targets of *L. monocytogenes* F2365 to speed up the rational drug and vaccine design. It has revealed that out of 2821 reference sequences of the pathogen, 744 represent essential proteins and among them 274 are human non-homolog proteins. Besides, all predicted human non-homologs were then analyzed by subcellular localization servers, in which 46 proteins were identified as surface exposed proteins and can be considered as potential drug and vaccine targets for the pathogen. The 3D structure of two human non-homolog putative drug targets, pantothenate kinase (*Lm*PK) and holliday junction resolvase-like protein (*Lm*HJR) of *L. monocytogenes* F2365 were generated by homology modeling program Easymodeller 4.0; a GUI version of modeller. Generated structures were also validated by several online servers. The overall stereochemical quality of the model was assessed by Ramachandran plot analysis that was provided by PROCHECK. ProQ, ERRAT, Pro-SA web and VERIFY 3D of SAVES programs were also used to compute several validation parameters during the evaluation of the model. This protein structure information is important in structure-based drug and vaccine design. This study provides information about putative drug targets of *L. monocytogenes* F2365 and 3D structures (*Lm*PK and *Lm*HJR), which emphasizes future perspective to design rational drugs and vaccines.

Key words: drug targets, *Listeria monocytogenes*, subtractive genomics approach, *Lm*PK, *Lm*HJR

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Introduction

Listeriosis, the infectious disease caused by *Listeria monocytogenes* causes serious localized and generalized infections in humans especially among pregnant women, the elderly or individuals with a weakened immune system. In serious cases, it can lead to brain infection and even death. Several symptoms are ranged from flu-like illness to severe complications including meningitis, septicemia, spontaneous abortion or listeriosis of the newborn (FAO/WHO, 2002). In the early 1960s, it was demonstrated that *L. monocytogenes* is able to survive and multiply in macrophages. This bacterium has been used in immunological research as a prototype of intracellular parasite (Machesky, 1997). The reference treatment is currently based on a synergistic association of high doses of aminopenicillin (ampicillin or amoxicillin) and gentamicin (Temple & Nahata, 2000). However, some studies have recently reported an increased rate of resistance to one or several clinically relevant antibiotics in environmental isolates (Walsh et al., 2001) and less frequently in clinical strains (Safdar & Armstrong, 2003). Morvan et al. (2010) studied the susceptibility to antibiotics of 4816 clinical *L. monocytogenes* strains isolated since 1926, and the mechanisms of resistance in each strain were also investigated. Where the prevalence of resistant strains was estimated at 1.27% among isolates from humans, the resistance to tetracyclines and fluoroquinolones was more common and has recently emerged. The authors demonstrated the possibility of resistance gene transfers. The description of the first clinical isolate with high-level resistance to trimethoprim, and the recent increase in penicillin minimum inhibitory concentrations (MICs) up to 2 g/ml reinforce the need for microbiological surveillance. Many essential bacterial proteins have been identified as potential drug targets and a number of very good clinically efficacious antibiotics are in use today. Yet, due to the uncontrolled and errant practice, the emergence of multiple drug resistance has become common in pathogenic bacteria and this critical situation necessitates the design of novel antibacterial agents. With advancements in genomics and bioinformatics, it is now possible to search through a bacterial genome to identify potential antibacterial targets. Sophisticated *in silico* approaches has given a tremendous opportunity to pharmaceutical companies to identify new potential drug targets, which in turn affect the success and time of performing clinical trials for discovering new drug targets (Rao & Srinivas, 2011).

In our study, putative common drug targets of *L. monocytogenes* F2365 along with their cellular localization and pathways were identified by employing subtractive genomics approach and other online servers. Pantothenate kinase of *L. monocytogenes* (*Lm*PK) is the key component and essential protein in the biosynthetic pathway of Coenzyme A, which phosphorylates pantothenic acid or vitamin B5 and considered to be a good drug target. Another protein named Holliday junction resolvase-like protein (*Lm*HJR) also considered as a drug target, which has several important functions including DNA repair, DNA recombination and response to DNA damage stimulus, respectively. As there are no available crystal structures of these proteins of *L. monocytogenes* F2365 in PDB database, effort has been taken to generate a reliable model of each, which will facilitate its structure knowledge gap and idea of using as a potential drug target for structure based drug design.

Materials and Methods

In silico drug target identification

The essential proteins of *L. monocytogenes* serotype 4b str. F2365 were identified using subtractive genomics approach and further analyzed for identification of putative potential drug targets. The detailed methodology for identifying of pathogen specific essential proteins using this approach is mentioned as a flowchart in Figure 1.

Identification of putative common drug targets

The complete proteome of *L. monocytogenes* F2365 was retrieved from National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov/). The database of essential genes (DEG) were accessed at http://tubic.tju.edu.cn/deg/ (Zhang & Lin, 2009) for the selection of essential genes/proteomes that are vital for the normal activity of the pathogen. The assumption described by Dutta et al. (2006) was followed in this analysis and the DEG search parameter was set to an expectation value (E-value) cut off of 10^{-10} and bit score not less than 100 (≥100) were selected. Prior to set the DEG search parameter, proteins of less than 100 amino acids and those share >60% sequence identity were excluded from the analysis. Selected sequences form DEG search were BlastP searched against human proteome in the National Center for Biotechnological Information (NCBI) database to identify human non-homologous sequences with threshold E-value of 10^{-3}.

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Subcellular localization prediction and metabolic pathway analysis

Computational prediction of subcellular localization is necessary for genome analysis and annotation in bacterial pathogens since the prediction of proteins on the cell surface is of particular interest due to the potential of such proteins to be primary drugs or vaccine targets. Subcellular localization analysis of the essential proteins has been done by Psortb (Yu et al., 2010) and further confirmed by Gpos-mPloc server (Shen & Chou, 2009). Membrane associated drug targets of *L. monocytogenes* F2365 along with their respective pathway and KEGG entry are enlisted in Table 1. Metabolic pathway analysis of the essential proteins of *Listeria monocytogenes* F2365 was done by KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007). Comparative analysis of the metabolic pathways of the host and pathogen was performed by using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa & Goto, 2000) to trace out essential proteins involved in pathogen specific metabolic pathways for the identification of potential drug targets.

Comparative molecular modeling

After the identification of all putative common drug targets, the model structure of Pantothenate kinase (YP_012842.1) and Holliday junction resolvase-like protein (YP_014119.1) was generated by homology modeling programs EasyModeller 4.0 (Kuntal et al., 2010), a GUI version of the Modeller. Homology modeling of these two proteins was done by using a suitable template structure through BlastP search against PDB database (http://www.pdb.org/pdb/home/home.do) with default parameters. From the best hits, the structure with maximum identity and lowest E-value was selected for model generation. Three sets of models of each protein were generated by using the homology modeling program and out of this three, the model which shows the lowest DOPE score value was selected as a final model.

Model validation

The stereo-chemical quality of each model was evaluated by Procheck and ERRAT plot that gives a measure of structural error at each residue in the protein. The refined model with least number of residues in the disallowed region was further validated by VERIFY 3D (Luethy et al., 1992) of SAVES server (http://nihserver.mbi.ucla.edu/SAVES/) and ProQ (Wallner & Elofsson, 2003). ProQ is a neural network based predictor that based on a number of structural features predicts the quality of a protein model. ProQ is optimized to find correct models in contrast to other methods which are optimized to find native structures. Two quality measures were predicated - LG score and Max-Sub. ProSA-Web (Wiederstein & Sippl, 2007) server (https://prosa.services.came.sbg.ac.at/prosa.php) was also used to evaluate the generated model of protein for potential error. Model visualization and analysis was done with Pymol (Schrödinger, LLC).

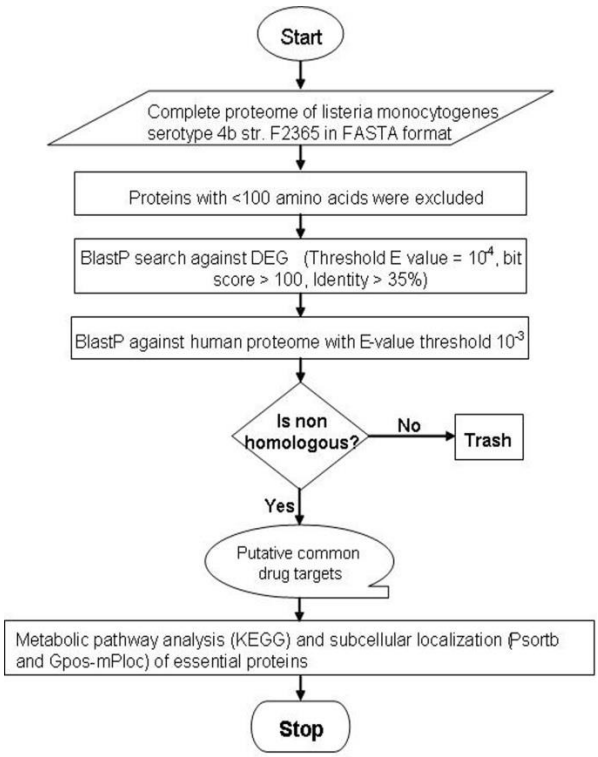

Figure 1. *A flow chart describing the detailed methodology for identification of putative drug targets using subtractive genomics approach.*

Results and Discussion

Putative common drug targets of Listeria monocytogenes F2365

Pathogenic infections still remains a leading cause of global disease burden. The present pharmaceutical scenario is under constant stress of discovering new antimicrobials due to threat of resistance rapidly being developed in target microbes. Identification of microbe-specific proteins for directing drug discovery and to designing new drugs to previously known targets is the two popular means to combat this resistance.

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Table 1. *Membrane targets of Listeria monocytogenes serotype F2365*

Serial no.	Membrane targets of *Listeria monocytogenes* serotype F2365	KEGG entry	Respective pathway/class/module
1	|YP_012642.1| PTS system beta-glucoside-specific transporter subunit IIABC	K02757	ko02060 Phosphotransferase system (PTS)
2	|YP_012766.1| oligopeptide ABC transporter permease	K02034	M00239 Peptides/nickel transport system
3	|YP_012778.1| zinc ABC transporter substrate-binding protein	K09815	ko02010 ABC transporters
4	|YP_012918.1| sensory box histidine kinase	K07652	ko02020 Two-component system
5	|YP_013086.1| transcriptional regulator	na	na
6	|YP_013266.1| fructose-like permease EIIC subunit 2	K11203	ko02060 Phosphotransferase System (PTS)
7	|YP_013456.1| phosphate-starvation-inducible protein PsiE	K13256	Unclassified; Poorly Characterized; Function unknown
8	|YP_013568.1| transporter	K03449	Environmental Information Processing; Membrane Transport; Transporters [BR:ko02000]
9	|YP_013612.1| TerC family membrane protein	na	na
10	|YP_013642.1| histidine kinase	K11617	ko02020 Two-component system
11	|YP_013656.1| PTS system beta-glucoside-specific transporter subunit IIABC	K02777	ko00010 Glycolysis / Gluconeogenesis ko00500 Starch and sucrose metabolism ko00520 Amino sugar and nucleotide sugar metabolism ko02060 Phosphotransferase system (PTS)
12	|YP_013688.1| cell cycle protein FtsW	K03588	ko04112 Cell cycle - Caulobacter
13	|YP_013691.1| techoic acid ABC transporter efflux permease	K09692	ko02010 ABC transporters
14	|YP_013703.1| teichoic acid biosynthesis domain-containing protein	K09809	Unclassified
15	|YP_013704.1| teichoic acid biosynthesis protein B	na	na
16	|YP_013900.1| hypothetical protein	K08591	ko00561 Glycerolipid metabolism ko00564 Glycerophospholipid metabolism
17	|YP_013933.1| membrane-associated zinc metalloprotease	K11749	ko04112 Cell cycle - Caulobacter
18	|YP_013995.1| sensor histidine kinase	na	na
19	|YP_014003.1| FtsK/SpoIIIE family protein	K03466	Genetic Information Processing; Replication and Repair; Chromosome and associated proteins [BR:ko03036]
20	|YP_014059.1| AzlC family protein	na	na
21	|YP_014144.1| secDF gene product	K12257	ko03060 Protein export ko03070 Bacterial secretion system
22	|YP_014243.1| polysaccharide biosynthesis family protein	K03328	Unclassified
23	|YP_014244.1| polysaccharide biosynthesis family protein	K03328	Unclassified
24	|YP_014359.1| amino acid ABC transporter permease	K02029	M00236 Putative polar amino acid transport system
25	|YP_014514.1| penicillin-binding protein	K05366	ko00550 Peptidoglycan biosynthesis
26	|YP_014541.1| hypothetical protein	K06973	Unclassified
27	|YP_014626.1| ABC transporter permease	K02025	M00207 Putative multiple sugar transport system
28	|YP_014664.1| penicillin-binding protein	K08724	ko00550 Peptidoglycan biosynthesis
29	|YP_014745.1| hypothetical protein	na	na
30	|YP_014817.1| oligopeptide ABC transporter permease	K15582	ko02010 ABC transporters
31	|YP_014894.1| PTS system fructose-specific transporter subunit IIABC	K02769	ko00051 Fructose and mannose metabolism ko02060 Phosphotransferase system (PTS)
32	|YP_014906.1| amino acid ABC transporter permease	K02029	M00236 Putative polar amino acid transport system
33	|YP_014942.1| monovalent cation/H+ antiporter subunit C	K05567	Environmental Information Processing; Membrane Transport; Transporters [BR:ko02000]
34	|YP_014987.1| cell cycle protein FtsW	K05837	Genetic Information Processing; Replication and Repair; Chromosome and associated proteins [BR:ko03036]
35	|YP_015058.1| phosohate ABC transporter permease `	K02038	ko02010 ABC transporters
36	|YP_015067.1| ftsX gene product	K09811	ko02010 ABC transporters
37	|YP_015082.1| teichoic acid biosynthesis protein A	K05946	Metabolism; Glycan Biosynthesis and Metabolism; Glycosyltransferases [BR:ko01003]
38	|YP_015094.1| atpF gene product	K02109	ko00190 Oxidative phosphorylation ko00195 Photosynthesis
39	|YP_015096.1| atpB gene product	K02108	ko00190 Oxidative phosphorylation ko00195 Photosynthesis

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40	|YP_015160.1| cobalt ABC transporter permease	K02008	ko02010 ABC transporters
41	|YP_015173.1| secY gene product	K03076	ko03060 Protein export ko03070 Bacterial secretion system
42	|YP_015195.1| cobalt transport protein	K02008	ko02010 ABC transporters
43	|YP_015255.1| cell cycle protein FtsW	na	na
44	|YP_015286.1| cydA gene product	K00425	ko00190 Oxidative phosphorylation ko02020 Two-component system
45	|YP_015350.1| PTS system beta-glucoside-specific transporter subunit IIABC	K02757	ko02060 Phosphotransferase system (PTS)
46	|YP_015375.1| mtlA gene product	K02800	ko00051 Fructose and mannose metabolism ko02060 Phosphotransferase system (PTS)

* na – not available.

If the new drug development efforts are stopped, current trends suggest that some diseases will have no effective therapies within the next ten years (Johnsen et al., 2009). Thus, the development of new drug against bacterial disease is most urgent and continued requirement. Modern tools of computational biology greatly enhance the speed and reliability of antimicrobial discovery. With an objective of identifying proteins potentially useful as drug targets, we have relied on the use of proteomes and a subtractive genomic approach for the identification of putative drug targets. A subtractive genomics approach utilizes the whole proteome of host and pathogen to identify proteins exclusively present in the pathogen by deducting the homologous proteins (Dutta et al., 2006). As for being the drug target, the particular protein should be essential for the survival or be the major component of important metabolic or regulatory pathways of that particular microbe. The principle was well implemented using DEG and the NCBI non redundant database to develop subtractive genomic approach, which has been widely used for fast screening of potential drug targets from the sequenced genomic information of emerging infectious pathogens.

Complete proteomes of *L. Monocytogenes* F2365 was retrieved from the National Centre for Biotechnology Information. The *L. Monocytogenes* F2365 genome size is about 2.91-Mb (chromosome 1) consists of 2934 genes and 2821 reviewed proteins (Figure 2). Similarity search (Threshold E-value=10^{-4}, bit score >100 and sequence identity >35%) for virulence related proteins in DEG led us to identify 744 sequences as essential proteins for *L. Monocytogenes* F2365 of the total proteomes after removing duplicate sequences by CD-HIT and protein sequences with <100 amino acids numbers. Selected essential protein sequences were searched against reference sequences of human in the National Center for Biotechnological Information (NCBI) database to identify human non-homologous sequences by filtering out homologous sequences with threshold E-value of 10^{-3}. A total of 274 protein sequences were identified as non-homolog putative common drug targets from 744 essential protein sequences. Sakharkar et al. (2004) identified 306 essential genes in *Pseudomonas aeruginosa*; Dutta et al. (2006) reported 178 essential genes in *Helicobacter pylori*, and Chong et al. (2006) found 312 essential genes in *Burkholderia pseudomallei* by using the same approach.

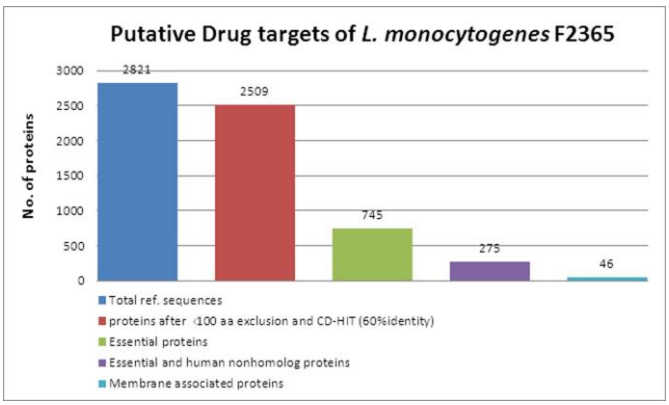

Figure 2. *A chart showing putative drug targets of L. monocytogenes F2365 after several subsequent filtering steps.*

These non-human homologous targets identified were further analyzed for pathway analysis and subcellular localization prediction, which detected 46 membrane associated proteins (Table 1). Membrane localized proteins represent largest group (70%) of effective drug targets in any organism (Lundstrom, 2007) and can also act as potential epitopes for vaccine design.

3D Model generation and validation:

The 3D model of the *Lm*PK and *Lm*HJR was built by Easymodeller 4.0; a GUI version of Modeller, based on template structure 2H3G_X and 1VHX_A having maximum

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identity of 60% and 61%, respectively. The high sequence identity between our target and templates shown in Figure 3 ensure the quality of these homology models. Three models for each protein sequence were generated with different DOPE score and the best model was selected based on its lowest DOPE score. The DOPE score values for three models of *Lm*PK are -29249.673823, -29397.64063 and -28974.04688. Out of this three, the 2nd model with lowest DOPE score was selected as a final model (Figure 4A). Similarly, three models were also generated for *Lm*HJR with different DOPE scores (-13730.51758, -13838.66602, and -13955.43457) among them the 3rd one was selected for further study (Figure 4B). The selected model structure of each protein was then subjected to energy minimization and optimization with steepest descent steps of 80 and 50 conjugate gradient steps. The optimized DOPE score of *Lm*PK and *Lm*HJR was -29433.11328 and -13984.83398, respectively (Table 2). Both models generated by Easymodeller 4.0 were validated by considering several validation parameters using available online servers. Procheck program from SAVES server used to compute ramachandran plot of the 3D models to determine the respective position of each residues, whether it resides on the core, allowed or disallowed region in a particular protein (Figure 5). Besides, the overall quality factor of *Lm*PK and *Lm*HJR computed by ERRAT2 shows 80.08 and 90.00 respectively. Several other validation servers such as, Verify 3D, ProSA web server and ProQ were employed to ensure the protein model qualities are tabulated in Table 2. Pro-SA analysis showed that protein folding energy of the modeled structure is in good agreement favoring the validation of the model (Figure 6).

Table 2. *Validation parameters of generated models computed by several online servers.*

Model name	DOPE score	Computed validation parameters of *Lm*PK and *Lm*HJR model									
		Template	Procheck				ProQ		ERRAT2	Verify 3D (3D-1D score >0.2)	Pro-SA (Z-score)
			Core	Allowed	Generously allowed	Disallowed	LG score	Max-Sub			
***Lm*PK** (YP_012842.1)	-29433.11328	2H3G	96.4%	3.6%	Nil	Nil	5.832	0.534	80.08	90.77%	-8.12
LmHJR (YP_014119.1)	-13984.83398	1ZHX_A	95.1%	4.9%	Nil	Nil	3.617	0.518	90.00	85.61%	-6.66

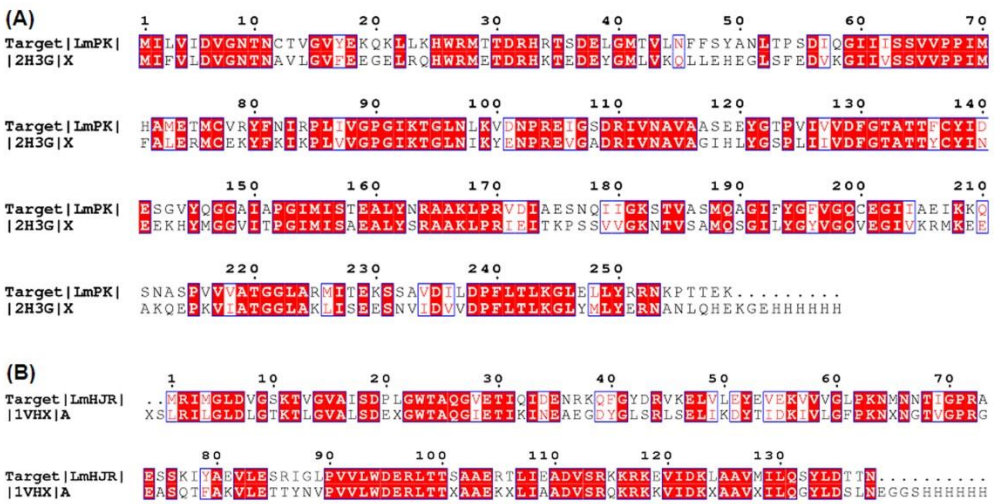

Figure 3. *Sequence alignment of target proteins with their template sequences* ***(A)*** *- LmPK and template structure 2H3G_X* ***(B)*** *- LmHJR and its template structure 1VHX_A.*

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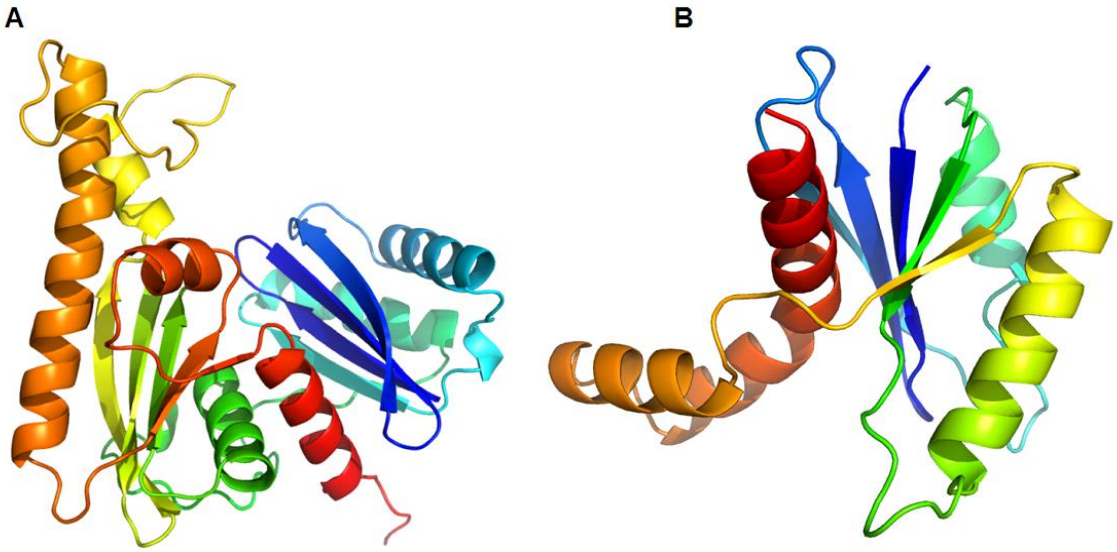

Figure 4. *Generated model structures:* ***A*** *- Pantothenate kinase (LmPK) and* ***B*** *- Holliday junction resolvase like protein (LmHJR) of L. monocytogenes F2365.*

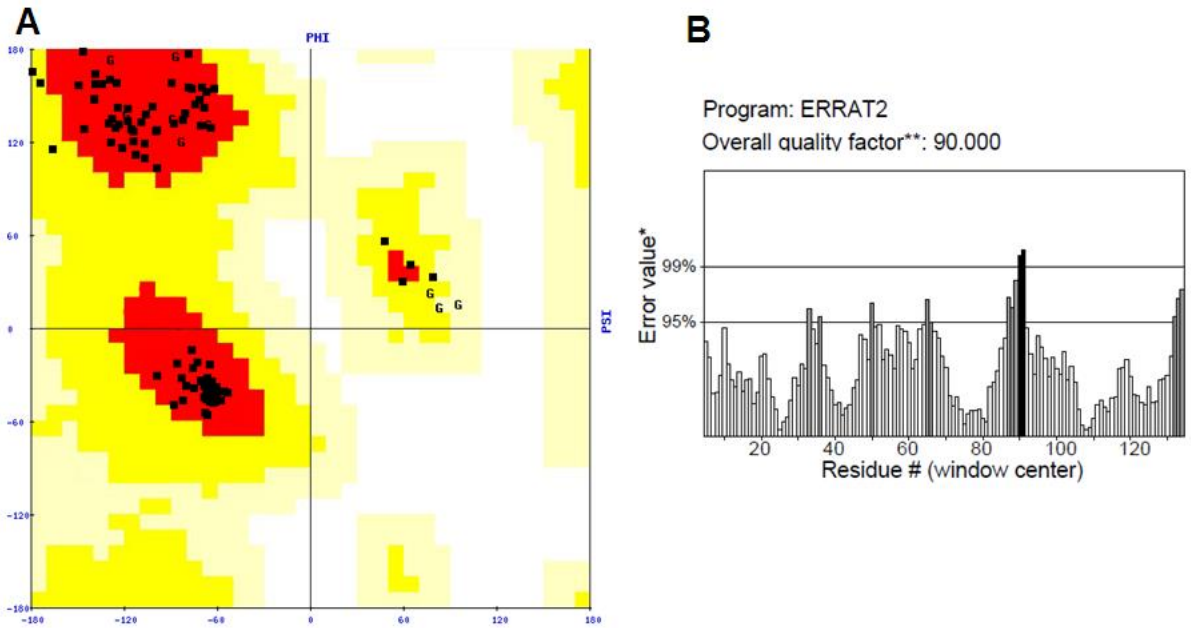

Figure 5. *Ramachandran plot and overall quality factor of LmHJR model computed using Procheck (**A**) and ERRAT2 (**B**) program.*

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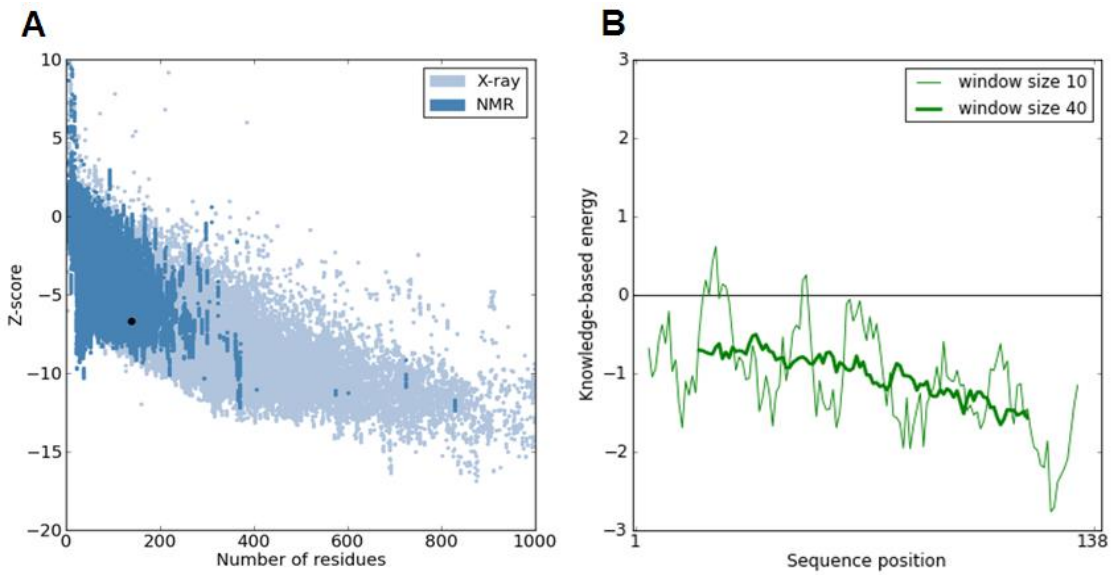

Figure 6. *Structural validation of the generated protein model (LmHJR)* ***A.*** *Pro-SA web z-scores of all protein chains in PDB determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue) with respect to their length. The z-scores of model (LmHJR) highlighted as large black dot.* ***B.*** *Pro-SA energy profile of LmHJR. Thick line indicates average energy over each 40 residue fragment. The thin line indicates same with a smaller window size of 10 residues in the background of the plot.*

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