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Structural and Functional Characterization of Biofilm-Related Proteins of *Mycobacterium spp*: An *in silico* Approach

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Abstract: Biofilm-associated infections are characterized by chronicity, recurrence, and the requirement of a prolonged administration of multiple drugs. Several non-pathogenic and pathogenic species of microorganisms including Mycobacteria spp. compose biofilm. Mycobacterial biofilms present a unique composition. Instead of exopolysaccharides in other bacteria, proteins are essential compounds of the biofilm matrix in mycobacteria. To tackle Mycobacterial infections, a detailed understanding of the biofilm-forming mechanisms is crucial. In this present study, all available mycobacterial proteins involved in the biofilm were selected. Their sequences were retrieved and characterized through the determination of their physicochemical properties, secondary structure, 3D structure, subcellular localization, conserved domain, ubiquitination sites, and virulence potentiality. Furthermore, druggability testing was undertaken after excluding proteins with homology to human proteins for identify possible drug targets. The results showed that they possess functionally important domains and families. All of the selected hypothetical proteins were stable. Six of them were classified as soluble and the remaining as transmembrane proteins. A sole protein was found to lack ubiquitination sites. Additionally, three of these were discovered to be virulent. Moreover, host non-homology results indicated eight pathogen-specific proteins that might be potential therapeutic targets. Among them, D-alanyl-D-alanine carboxypeptidase is a druggable target that is inhibited by beta-lactam antibiotics. The remainder of the proteins were categorized as new targets. In conclusion, this study may increase our knowledge of pathogenesis and host adaptation, drug resistance, and identification of drug and vaccine targets against infections caused by Mycobacteria sp.. It can also guide new research.

Keywords: Mycobacterium, Biofilm, Protein Characterization, In Silico analysis

1. Introduction

Mycobacterium species are acid-fast, aerobic, non-motile, non-encapsulated, cylindrical, cord-forming thin bacillus-shaped bacteria. They reproduce slowly and divide in 18-24 hours. Their main difference from other bacteria is their cell wall. The bacterial cell walls do not contain structures such as outer membrane, protein, and lipopolysaccharides found in the cell wall structures of gram-negative or gram-positive bacteria. Lipids (mycolic acid) constitute the majority of the cell wall structures of Mycobacteria sp. [1].

There are pathogens and saprophyte Mycobacteria sp. that cause tuberculosis and cause mycobacteriosis. This genus is split into two groups according to their growth rate. Mycobacterium tuberculosis (human tuberculosis), Mycobacterium bovis (bovine tuberculosis) and Mycobacterium leprae (leprosy) species are slow-growing pathogenic species. Mycobacterium smegmatis is generally a non-pathogenic species. Mycobacterium caprae, Mycobacterium pinnipedi, and Mycobacterium microti rarely could cause disease in humans [2]. Many virulence factors

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affect the pathogenesis of Mycobacteria sp. Mycobacterial lipids, Lipoarabinomannan (LAM), and Lipomannan (LM) are an important virulence factor for the stability of Mycobacteria sp. [3]. Phosphatidylinositol mannosidase (PIM), which is an important component of the bacterial cell envelope, and "trehalose-6,6'-dimicolate (TDM)", known as the “cord factor”, which is the most common in the cell membrane of Mycobacteria sp.. PE proteins (PE-PPE and PE-PGRS transport proteins) are virulence factors that regulate mycobacterial survival in macrophages. “LpqH, LppX, Mpt83, LprG, RpfB, LpqS, LprN, Lprl, and PstS" lipoproteins are involved in the structure of mycobacterial cell membrane and cell envelope and contribute to host-pathogen interaction [4].

Bacterial biofilms are microcolonies formed by exopolysaccharide structures secreted by bacteria following their irreversible attachment to a surface. Because of the biofilm structure, the bacteria escape the host immune response, spread the infection they have created, and show resistance to antibiotics [5,6]. Unlike other bacteria, Mycobacteria sp. do not produce polysaccharides for the biofilm structure. Instead, they produce a biofilm by producing mycolic acid and glycopeptidolipid [7].

Understanding the mechanisms of bacterial virulence and pathogenesis will be effective in the development of effective drugs and vaccine development against infectious diseases [8]. Similar to this research, numerous in silico biofilm-related protein studies have been conducted in bacteria like Pseudomonas aeruginosa, and Acinetobacter baumannii [9,10].

In this paper, in silico analyses of bacterial biofilm proteins of Mycobacterium sp. are presented and discussed. The workflows for the identification of proteins were taken advantage of available online tools and databases, and these identifications are reported here.

2. Computational Method

2.1. Sequence Retrieval and Alignment

The amino acid sequences of eleven different biofilm-related proteins of Mycobacterium spp. were retrieved from the UniProt database (https://www.uniprot.org/) in FASTA format for computational analysis. UniProt database is a comprehensive, high-quality, and traceable protein resource, driven by a high-scale protein annotation [11]. Corresponding gene sequences of the proteins to be investigated were retrieved from the NCBI database (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov). The sequences, accession numbers of the gene sequences along with the proteins were listed in supplementary files. NCBI database was used to conduct multiple sequence alignment and phylogenetic tree. A phylogenetic tree is a branching diagram that helps in the understanding of biological species' evolutionary relationships. The sequence similarity analysis was performed via BLAST [12].

2.2. Analysis of Physicochemical Properties

The ProtParam (http://web.expasy.org/protparam) tool of the ExPASy server was used to determine various physicochemical properties of the proteins such as molecular weight, extinction coefficient, theoretical isoelectric point (pI), the composition of amino acid, total number of positive and negative residues, instability index, aliphatic index (AI) and grand average of hydropathicity (GRAVY), molecular formula, and estimated half-life [13]. The information acquired from the server was found to be necessary for determining the function of the proteins.

2.3. Subcellular Localization

The protein cellular location was determined using the TMHMM server v.2.0 (www.cbs.dtu.dk/services/TMHMM), and the SOSUI program (https://harrier.nagahama-i-bio.ac.jp/sosui/mobile/), for prediction of subcellular localization [14]. SOSUI is a service that uses amino acid sequences to discriminate between the membrane and soluble proteins and predicts transmembrane helices for the former. The aforementioned tool has a good prediction accuracy and can be calculated rapidly [15].

2.4. Secondary Structure Prediction

Protein folding is linked to secondary structure. SOPMA from the Network Protein Sequence Analysis (NPS@) server (https://npsa-prabi.ibcp.fr/cgibin/npsa_automat.pl?page=npsa_sopma.html) was used to predicted secondary structure analysis of the proteins of interest. This included a number of α-helices, extended strand, β-turn, β-sheet, and random coils which were

performed by CYS-REC tool (http://linux1.softberry.com/berry.phtml) was used to predict the possible disulphide bonds between cysteine molecules [16].

2.5. 3D Structure Prediction and Quality Assessment

To predict the 3D structure of all studied proteins. The amino acid sequence of proteins in FASTA format was uploaded to Phyre 2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and the files in pdb formats were created. The model in the specified (.pdb) formats was submitted to by Chimera (version 1.8) (http://www.rbvi.ucsf.edu/chimera), so characterizing and visualizing of the publishable images of the protein model were achieved.

The most crucial component of structure prediction was structure evaluation. Predicted protein models of the studied proteins were evaluated and verified from SAVES server (http://nihserver.mbi.ucla.edu/SAVES) [17].

2.6. Functional Annotation and Protein-Protein Interaction

Pfam (http://pfam.xfam.org/) was used to predict the function of proteins and domains based on sequence similarity. Furthermore, the query sequence was examined to identify the protein's family of origin. Identification of protein-protein functional interaction was performed by STRING v11.0 search [18].

2.7. Virulence Factors Analysis

The virulence feature is important in predicting the therapeutic target. The term "virulence factor" refers to a group of active proteins that play critical functions in pathogen activity and survival. VICMpred (http://crdd.osdd.net/raghava/vicmpred/) was employed for the analysis of proteins virulence nature VICMpred is a tool that categorizes proteins of bacteria as virulence factors, information molecules, cellular processes, and metabolism molecules [19].

2.8. Prediction of Protein Ubiquitination Sites

The ubiquitination regions of proteins are important parts that affect and regulate many cellular mechanisms such as the stimulation of the immune response and autophagy. Ubiquitination study provides information about host-pathogen interactions and thus can contribute to the development of therapeutics that will play a role in the effective treatment of diseases. Putative ubiquitination sites were predicted by using (BDM-PUB) software, which predicts according to Bayesian Discriminant Method (http://bdmpub.biocuckoo.org/prediction.php) [20].

2.9. Human Non-Homology Analysis

Pathogen-specific proteins that are present in the pathogen but absent in the host (human) were defined by using non-homology analysis. Non-homologous proteins were filtered using BLASTp search against the non-redundant Homo sapiens database with an E-value threshold of 0.0001 and bit score cut-off of 100 [21]. Protein sequences that showed no significant similarity to the human proteome were selected for further analysis.

2.10. Analyses of Druggability

After non-homology analysis, host non-homologous proteins were searched using DrugBank, a bioinformatics tool that provides information about drugs and drug targets. A list of molecules that could be drugs was obtained. The absence of drug-like molecules indicates that the protein is new and could be a "novel target" [22].

3. Results and discussion

The role played by bacterial biofilm in Mycobacterium sp. has been emphasized in recent years, particularly in the context of the prolongation of the inflammatory phase of repair. Understanding the mechanisms involved in biofilm and the adaptation by the is central to the development of beneficial interventions. Using traditional experimental methods for this purpose can be costly and time-consuming, and also uncertain since animal models are not always good predictors for bacterial pathogenicity in humans. Rapid high-throughput bioinformatics-based methods are ideal for understanding the proteins which are essential for biofilm.

3.1. Sequences Retrieval

All proteins related to biofilm in Mycobacterium sp. were searched from the UniProt database, a comprehensive, high-quality, and traceable protein resource, driven by a high-scale protein annotation

[23]. The protein of interest and their accession numbers are provided in Table 1.

3.2. Physicochemical Characterization

Physicochemical properties for the proteins are essential for understanding protein function and interaction with other proteins. They involve the molecular mass, amino acid residues, isoelectric point (pI), number of totals positive and negative residues, and extinction coefficient are deduced from the amino acid composition and the grand average of hydropathicity (GRAVY). The GRAVY value of a peptide or protein is defined as the summation of all the amino acid hydropathy values divided by the number of residues in the sequence, which indicate the hydrophobic/hydrophilic nature of the residues [23]. The findings are shown in the supplemental file.

3.3. Cellular Location

Identifying the location of proteins facilitates the classification of them as targets for drug or vaccine targets. Proteins found in the cytoplasm are thought to represent potential therapeutic targets, whereas proteins found in the membrane may be prospective vaccination focus. Six of the proteins were discovered to be soluble cytoplasmic proteins and five as membranous. Membrane parts have been categorized as primary and secondary, and the length of the transmembrane sections has been forecasted. Table 2 contains data on each predicted outcome.

Table 1. Biofilm related protein and their accession numbers

Protein	**Accession Number**
Chaperonin GroEL 1	P9WPE9
Esterase PE11	Q79FR5
Acyltransferase PE	Q2M5K2
Lipoprotein LpqN	O53780
D-alanyl-D-alanine carboxypeptidase	A0R3Y2
Calcium-transporting ATPase CtpE	A0R3Y2
DNA-binding response regulator MtrA	A0QTK2
Lipoprotein LpqB	A0QTK4
Heme uptake protein MmpL11	P9WJT9
Mycolic acid-containing lipids exporter MmpL11	A0QP18
Polyglutamine synthesis accessory protein	P9WM79

Table 2. Protparam result

Protein	**Family**	**Description**	**HMM Length**	**Bit score**	**E-value**
Q79FR5	Cpn60_TCP1	TCP-1/cpn60 chaperonin family	491	284.2	1.9e-84
Q79FR5	PE	PE family	91	76.5	1.6e-21
Q2M5K2	PE-PPE	Acyltransferase PE	227	243.0	3.0e-72
O53780	Lpp-LpqN	Lpp-LpqN	174	198.0	8.6e-59
A0R3Y2	Peptidase_S11	Peptidase_S11	239	120.9	5.9e-35
A0R3Y2	Calcium-transporting ATPase CtpE	Calcium-transporting ATPase CtpE	181	136.7	6.4e-40
A0QTK2	Response reg change name	Response regulator receiver domain	112	102.7	1.3e-29
A0QTK4	Gmad1	Lipoprotein LpqB beta-propeller domain	253	240.4	2.6e-71
P9WJT9	MMPL	MMPL Family	333	128.1	3.7e-37
A0QP18	MMPL change name	MMPL Family	333	145.7	1.7e-42
P9WM79	PGA cap	Bacterial capsule synthesis protein PGA	257	249.2	4.5e-74

3.4. Prediction of Protein Ubiquitination

Ubiquitination in proteins refers to the presence of ubiquitin molecules that are linked to the lysine by covalent bonds. It takes part in many cellular and immunological activities [23]. Many bacterial pathogens have the ability to synthesize and transfer a set of proteins that evade host immune responses via interference with host ubiquitin. This process promotes bacterial pathogenicity as seen in other bacteria such as Escherichia coli [24]. In this study, all the studied proteins were found to possess ubiquitination sites, and that is in the exception of Chaperonin GroEL1 (P9WPE9). Even though Chaperonin lacks ubiquitination, it has a direct effect on the host ubiquitin which promotes the adaptability for existence within the host cell [25].

3.5. Functional Annotation of Biofilm Related-Proteins

3.5.1 Chaperonin GroEL 1 (P9WPE9)

The first protein belongs to the family of chaperonins, which is a subfamily of chaperones. Prokaryotes, chloroplasts, and mitochondria are all rich in this family. They are needed for proper cell development and are triggered by stress, functioning to stabilize or protect deconstructed polypeptides during heat-shock conditions [27].

GroES and GroEL are the bacterial equivalent to the 10kDa (Cpn10) and 60kDa. Both require magnesium ions to be activated. Human Hsp60 is homologous to bacterial GroEL [28]. The main function of chaperonins involves ATP binding and ATP hydrolysis activity. They are characterized by strong immunogenicity. The presence of mammalian chaperonins as well leads to the production of anti-chaperonin antibodies leading to autoimmune disease as found in the literature [29]. The inhibition of chaperonin is suggested to stop the biofilm formation at multiple levels [30].

3.5.2 Esterase PE11(Q79FR5)

The PE family includes a PE motif near the end. This family is varied and classified into various groups. The most common kind of PE protein is the highly recurring PGRS class, which contains a large amount of glycine. The role of these proteins is unclear; however, they have been linked to antigenic diversity in Mycobacterium TB and immunological regulation [31]. M. tuberculosis has a significant number of genes that encode proteins with distinctive motifs at their N-termini, PE, and PEE [32].

3.5.3 Acyltransferase PE (Q2M5K2)

Acyltransferase is involved in the biosynthesis of trehalose polychaetes (TPP). A critical step for glycolipid biosynthesis. The mutation in this protein results in an impaired cell wall [33].

3.5.4 Lipoprotein LpqN (O53780)

LpqN proteins are lipid transport mediators. They interact with other lipid mediators such as MmpL3 and MmpL11, which is critical for M. tuberculosis cell envelope biogenesis. LpqN can also engage with the host ubiquitin ligase Cbl, which suppresses immunity [34].

3.5.5 D-alanyl-D-alanine Carboxypeptidase (I6Y204)

Serine peptidases have a diverse peptidase action. More than 20 serine protease families (denoted S1 - S27) have been reported, and these have been classified into six clans (SA, SB, SC, SE, SF, and SG) based on structural similarities and other functional data [35]. Class C beta-lactamases and D-amino-peptidases are found in families S12 and S13. However, D-Ala-D-Ala peptidases are the only enzymes in family S11 [36]. Enzymes of the S11 family are rendered inactive by beta-lactam antibiotics due to acylation of the active site serine [37]. In this study, one of the proteins of interest is D-Ala-D-Ala Carboxypeptidase which is a membrane bacterial enzyme that operates by transferring the peptidoglycan monomer to an external receptor after removing the C-terminus [38]. It is found that D-Ala-D-Ala carboxypeptidase is required in the maturation of the spore cell wall. A study suggested that the dysfunction of D-Ala-D-Ala carboxypeptidase resulted in increasing the sensitivity to glycopeptide [39].

3.5.6 Calcium-Transporting ATPase CtpE (A0R3Y2)

ATPase or proton pump is a hydrolase, specifically catalyzing the transmembrane movement of substances [40]. Calcium-transporting ATPase CtpE is a P-type ATPase that participates in the selective absorption of calcium. Under calcium inadequacy, it is required for cell proliferation and the preservation of cell mechanical properties. The electrochemical gradients in the plasma membrane are created by ATPase. Proton gradients are employed to drive secondary transport mechanisms in this case. As such, it is required for most metabolite absorption as well as environmental reactions [41]. In an earlier study, it has been shown that the disruption of this type of ATPases significantly impaired biofilm formation in P. aeruginosa [42].

3.5.7 DNA-Binding Response Regulator MtrA (A0QTK2)

Member of the two-component regulatory system MtrA/MtrB controls the expression of a number of genes including dnaA, ripA, fbpB, and itself. It also plays a role in cell division [43]. The mutation in its gene results in antibiotic sensitivity, defects in cell morphology, and biofilm formation [44].

3.5.8 Lipoprotein LpqB (A0QTK4)

This protein modulates the activity of the MtrAB system for mediating cell wall homeostasis division. The disruption in this protein was found to increase antibiotic susceptibility, decrease motility and biofilm formation, alter colony morphology, and increase susceptibility to sodium dodecyl sulfate (SDS) [45].

3.5.9 Heme Uptake Protein MmpL11 (P9WJT9)

This protein is a part of a heme-iron acquisition and transport system. An important contributor to Mycobacterial virulence. It is also

involved in the transport of mycolic acid-containing lipids [45]. This protein is correlated to chronic stages of infection [46].

3.5.10 Mycolic Acid-Containing Lipids Exporter MmpL11 (A0QP18)

A mycolic acid-containing lipids transporter that contributes to wall and biofilm formation. There is evidence that the silencing of the gene coding this protein is found to reduce membrane permeability and altered biofilm formation [47].

3.5.11 Polyglutamine Synthesis Accessory Protein (P9WM79)

This bacterial protein is involved in the wall, and capsule biosynthesis protein found in bacteria. Disruption of this protein mutant affects adaptation to stress. Therefore, it is a potential target for inhibitors specifically blocking its synthesis [48].

3.6 Virulence Factors

Virulence factors enable pathogens to cause harm to the host. They are ideal pharmacological candidates because they allow for the development of new types of therapeutic medications, such as antivirulence interventions. The pathogen becomes avirulent after being treated with an antivirulence drug that targets virulence factors. Antivirulence medications, as hypothesized, will have a far reduced selection for resistance in pathogens than standard antibiotics [49]. In this study, VICMpred depicted one cellular process related to protein, one to information and storage, four to metabolism, and three as virulence factors (Table 3). Three virulence factors were predicted, they include Peptidase_S11, Lipoprotein LpqB, and PGA capsule protein.

3.7 Therapeutic Prospects

A pathogen-specific target is preferable. It must not have near similarity in the human proteome to limit the possibility of reacting with host proteins [50]. In order to discover proteins that are not similar to the human proteins, a host non-homology screening (BLASTp) was conducted. Three proteins showed significant similarity to that of human proteome. The homologous proteins were Chaperonin GroEL 1 (P9WPE9), Calcium-transporting ATPase CtpE (A0R3Y2), and DNA-binding response regulator MtrA (A0QTK2). Targeting those proteins will cause damage to the human as well, therefore, they will not pass to the druggability step. Eight proteins had no substantial matches and were thus classified as non-homologous. As a result, they might be thought of as possible therapeutic targets (Table 4).

Table 3. Prediction of cellular localization

Protein	Region (Transmembrane sequence / Type)
P9WPE9	Soluble
Q79FR5	Soluble
Q2M5K2	Soluble
O53780	Soluble
A0R3Y2	1 Primary
A0R3Y2	2 Primary, 1 Secondary
A0QTK4	1 Primary
A0QTK2	Soluble
P9WJT9	7 Primary, 4 Secondary
A0QP18	7 Primary, 2 Secondary
P9WM79	Soluble

Table 4. VICMpred Result

Function Prediction	No
Cellular process	1
Metabolism molecule	4
Virulence factor	3
Information and storage	3

A druggability analysis was performed to evaluate the druggability of the eight shortlisted candidate proteins. One protein found to be druggable-alanyl-D-alanine carboxypeptidase is a well-known target that is inhibited by beta-lactam antibiotics. A study undertaken by Kumar and his colleagues investigated the potential activity of meropenem against highly drug-resistant M. tuberculosis. Their findings demonstrated that meropenem does, in fact, directly inhibit this enzyme by producing a persistent aggregate at the enzyme active site [51]. D-alanyl-D-alanine carboxypeptidase is also inhibited by the non-specific inhibitor phenylmethylsulphonyl fluoride [52]. An experimental drug in the DrugBank is also found to be interacting with this enzyme [53]. Additionally, seven potential novel targets were proposed (as two drug targets and five vaccine targets). Novel targets include Esterase PE11, Acyltransferase PE, Lipoprotein LpqN, Lipoprotein LpqB, Heme uptake protein MmpL11, Mycolic acid-containing

lipids exporter MmpL11, and Polyglutamine synthesis accessory protein. The results obtained from the analyzes such as the structure and physicochemical properties of proteins in this study may be useful in terms of guiding future in vitro studies on these proteins.

The determination of the three-dimensional structure of protein molecules has proven beneficial in drug design. The most common prediction method is the Homology approach. Phyre2, uses this method 3D building [54]. Visualization of these predictions performed by Chimera software as shown in figure.

Table 5. Host homology and druggability

Protein	**Homology**	**Druggability**
P9WPE9	Yes	Homologous to human
Q79FR5	No	Novel target
Q2M5K2	No	Novel target
O53780	No	Novel target
A0R3Y2	No	Druggable target
A0R3Y2	Yes	Homologous to human
A0QTK2	Yes	Homologous to human
A0QTK4	No	Novel target
P9WJT9	No	Novel target
A0QP18	No	Novel target
P9WM79	No	Novel target

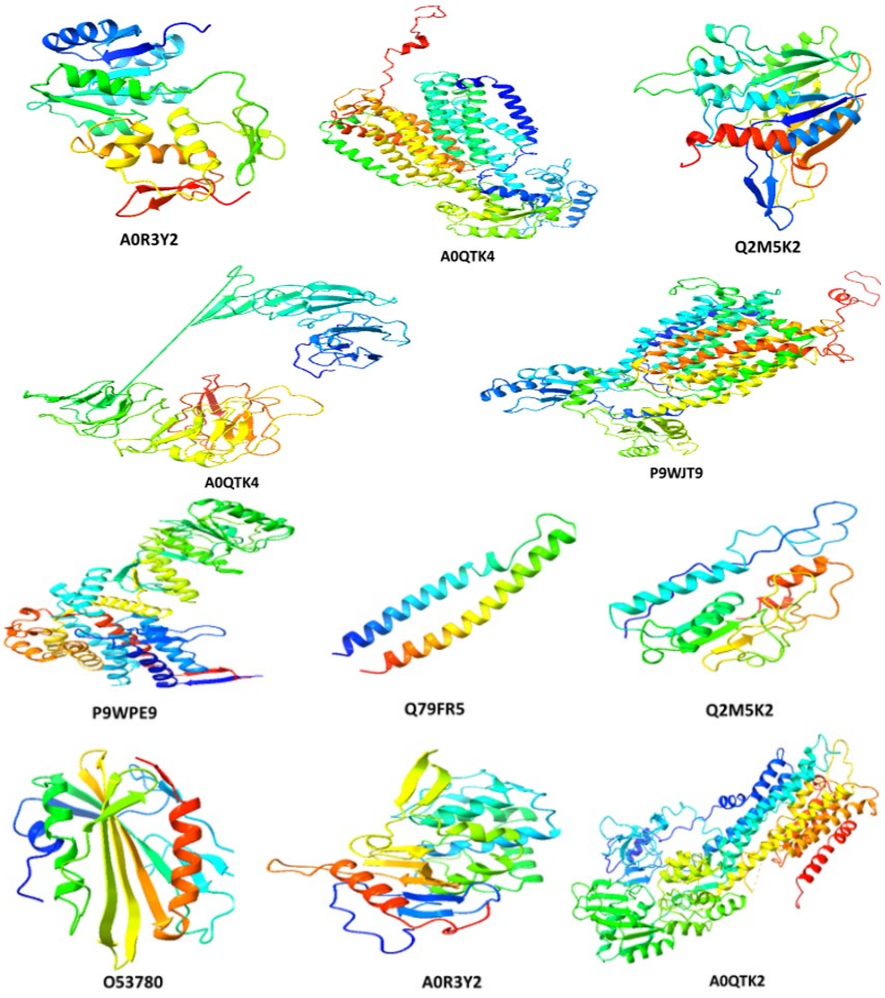

Figure 1. Predicted 3D Structure

4. Conclusions

Addressing biofilm-related proteins is important in both fundamental biology and medicinal research in microbiology. A variety of bioinformatics software and databases were employed in our study to functionally characterize biofilm-related proteins from Mycobacterium spp. The strategy applied predicted the family and function of the proteins of interest. The proteins belong to major functional categories. The subcellular localization of these proteins was predicted using subcellular localization analysis. Furthermore, three were virulence factors. In addition, host similarity testing disclosed that eight proteins are unique to Mycobacterium, and by which can be considered a probable therapeutic candidate. D-alanyl-D-alanine carboxypeptidase is a well-known target that is inhibited by beta-lactam antibiotics. The rest were ‘novel targets' that necessitates experimental validation to design and develop novel therapeutics to cure infections arising by Mycobacterium spp.

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