



Structural and Functional Characterization of a Hypothetical Protein from *Streptococcus mitis* Using *In silico* Approaches

Md. Easin Mia ^a, Tanvir Hossain Emon ^a, Md. Shipul Islam ^a
and Md. Anwar Hossain ^{a*}

^a Department of Biotechnology and Genetic Engineering, Noakhali Science and Technology University, Noakhali-3814, Bangladesh.

Authors' contributions

This work was carried out in collaboration among all authors. Author MEM performed the experiments and wrote the first draft of the manuscript. Author THE analyzed the data. Author MSI performed the experiments and prepared a report. Author MAH designed the study. All authors read and approved the final manuscript.

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ABSTRACT

Streptococcus mitis, a member of the Viridans Group Streptococci (VGS), is commonly found in the normal flora of the human oropharynx, and can cause various infections in humans, including orbital cellulitis, infective endocarditis, and bacteremia in neutropenic individuals. Identifying specific proteins, particularly hypothetical proteins (HPs), is crucial for developing effective treatments. This research aimed to determine the structure as well as the function of a nonannotated hypothetical protein (HP) from *Streptococcus mitis*. Several aspects of the HP (accession no. BCJ11593.1), including physiochemical properties, 3D structures, and functional annotation, were predicted using a variety of computational tools, followed by further validation and quality assessment. The HP was identified as a cytoplasmic and stable protein. NCBI-CD search, and InterProScan, two functional

*Corresponding author: E-mail: anwar.bge@nstu.edu.bd;

annotation tools, predicted that the target HP was the ribosome-associated protein YbcJ, specifically the S4-like RNA-binding protein. Secondary structural investigations found that the alpha helix was prevalent. The study determined its three-dimensional (3D) structure through homology modeling by the SWISS-MODEL server and verified it using quality evaluation tools such as PROCHECK, QMEAN, ERRAT, and ProSA. The findings lay the groundwork for potential antibacterial treatments, highlighting the importance of understanding the structure and function of specific proteins in *Streptococcus mitis*.

Keywords: Functional annotation; hypothetical protein; in silico characterization; *Streptococcus mitis*; three-dimensional structure.

1. INTRODUCTION

Next-generation sequencing (NGS) allows researchers to accumulate enormous volumes of data quickly. However, as more organisms undergo sequencing, assigning genetic functions becomes increasingly challenging [1-2]. The term "hypothetical proteins (HPs)" refers to proteins with unknown molecular functions, constituting over 30% of proteins in many species [3]. *In silico* characterization of HPs facilitates the determination of three-dimensional (3D) structures, unveiling new domains, motifs, pathways, protein networks, and other valuable information [4-6]. Structural and functional annotation of HPs not only establishes a link to potential therapeutic targets and biomarkers but also aids in understanding their roles [7]. To effectively annotate the roles of HPs in various disease-causing microorganisms, several bioinformatics databases and methodologies have been employed [8-11].

Streptococcus mitis, a Gram-positive coccus in the Viridians Group Streptococci (VGS) and mitis groups, functions as a commensal bacterium in the oral cavity by adhering to dental hard tissues and mucous membranes [12-17]. It is widely present in the normal skin, integumentary system, gastrointestinal tract, female vaginal tract, and oropharynx flora [16,18-21]. However, *Streptococcus mitis* is linked to diverse diseases and infections within the human body, functioning as an opportunistic pathogen in

immunocompromised individuals. VGS has the potential to trigger invasive conditions, including bloodstream infections, pneumonia, endocarditis, enteritis, meningitis, dental abscess, septicemia in neutropenic patients, brain abscess, neonatal sepsis, particularly in patients with compromised immune status or other risk factors [18,20,22-24].

Moreover, *Streptococcus mitis* strains cause serious clinical diseases, such as VGS shock syndrome, in cancer patients [22]. Women with preterm premature rupture of membranes (PROM) may develop maternal septicemia and intra-amniotic infection due to *Streptococcus mitis*, possibly associated with periodontitis [25]. There is a significant concern about *Streptococcus mitis*, a type of viridans streptococci, becoming highly resistant to penicillin. This resistance is causing sepsis and meningitis in patients with leukemia, lymphoma, or neutropenia [14,26].

The *in silico* analysis of a potential protein from *Streptococcus mitis* is essential because understanding the genome of this bacterium might help develop useful medications or vaccinations. effective drugs or vaccines. In this study, an HP from *Streptococcus mitis* with accession number BCJ11593.1 was selected and will undergo in-depth structural and functional analysis utilizing a variety of bioinformatics tools. All of the tools and programs used for the functional annotation of *Streptococcus mitis* HPs are listed in Table 1.

Table 1. Tools used for the *In silico* analysis of the hypothetical protein

Function	Tools/Server	URL
Sequence retrieval	NCBI	https://www.ncbi.nlm.nih.gov/
Analysis of Physiochemical properties	ExPASy ProtParam	https://web.expasy.org/protparam/
Subcellular localization	CELLO PSORTb	http://cello.life.nctu.edu.tw/ https://www.psорт.org/psорт/
Functional characterization	Conserved Domain Database	https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

Function	Tools/Server	URL
	InterProScan	http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=iprscan5
Sequence similarity search	BLASTp	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Secondary structure prediction	PSIPRED SOPMA	http://bioinf.cs.ucl.ac.uk/psipred/ https://npsaprabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html
3D Structure prediction	SWISS-MODEL	http://swissmodel.expasy.org/
3D Structure validation	PROCHECK, Verify3D & ERRAT QMEAN4 ProSA-web (Z scores)	https://save.mbi.ucla.edu/ https://swissmodel.expasy.org/qmean/ https://prosa.services.came.sbg.ac.at/prosa.php

2. MATERIALS AND METHODS

2.1 Sequence Retrieval

The amino acid sequence of HP (accession no. BCJ11593.1) from *Streptococcus mitis* was obtained in FASTA format from the protein database of the National Center for Biotechnology Information (NCBI) [27]. In the virtual annotation process, the protein sequence was then submitted to various prediction servers.

The NCBI-provided FASTA format is:
 >BCJ11593.1 hypothetical protein
 SMNM65_20250 [*Streptococcus mitis*]
 MEYKLFEEFITLQALLKELGITHSGGAIKSFLSE
 HSVYFNGELESRRGKKLRIGDKVDIPDMNIDILL
 TQPTSEEQDEYQADKVEKERIAKLVKEMNKGV
 KKDKSKPTSSPKSKQAPRFPGR

2.2 Analysis of Physicochemical Properties

The EXPASy ProtParam tool [28] was utilized to investigate the physicochemical attributes of HP. This analysis encompassed parameters such as molecular weight, theoretical isoelectric point (pI), amino acid composition, total count of positive and negative residues, instability index, aliphatic index (AI), grand average of hydropathicity (GRAVY), molecular formula, and estimated half-life of the protein.

2.3 Subcellular Localization Prediction

Subcellular localization is crucial for understanding the function of proteins and for the study of the genome. Both CELLO [29] and PSORTb3.0 [30] estimated the HP subcellular position.

2.4 Functional Annotation

Proteins, intricate molecules with diverse roles in living organisms, are categorized into various families and superfamilies based on shared sequence characteristics, domains, motifs, and functional attributes [31]. The target protein's potential function and conserved domains were determined using the NCBI Conserved Domain Search Service (CD Search) [32] and InterProScan [33].

2.5 Multiple Sequence Alignment and Phylogenetic Tree Analysis

To identify homologous proteins, a BLASTp search was performed on the NCBI website against the nonredundant database, utilizing the default settings [34]. Then, using CLC Sequence Viewer version 8, a multiple sequence alignment and a tree of phylogeny were produced. This software is widely employed for sequence analysis and visualization, facilitating the comparative analysis of protein sequences and the construction of phylogenetic relationships.

2.6 Secondary Structure Determination

The self-optimized prediction method with alignment, or SOPMA [35], was applied to predict the two-dimensional structure of the HP. PSIPRED [36] was employed as an additional method to confirm the results of SOPMA.

2.7 Homology Modeling

Based on homology modeling, the target protein's three-dimensional (3D) structure was ascertained using the SWISS-MODEL [37]

server. For every protein sequence, the server automatically performs a BLASTp search to find templates. With 96.72% sequence identity, template protein Q8DMX4.1.A was chosen for homology modeling based on the query result. The 3D model structure was viewed with PyMOL v2.0.

2.8 Quality Assessment

The assessment of the anticipated 3D structure's validity was conducted through the SAVES server modules PROCHECK [38] and ERRAT [39]. Additionally, the QMEAN programs [40] on the ExPASy server of the SWISS-MODEL Workspace were employed to determine the QMEAN Z-score and evaluate the model's quality. The ProSA-web server [41] was utilized to estimate Z scores for the obtained model.

3. RESULTS

3.1 Analysis of Physicochemical Properties

The ProtParam program was employed to calculate various physicochemical characteristics of the hypothetical protein (accession no. BCJ11593.1), as detailed in Table 2. The protein was predicted to consist of 122 amino acids, with a grand average of hydropathicity (GRAVY) of -0.807, a molecular weight (M.W.) of 13,910.97, a theoretical isoelectric point (pI) of 9.05, and a total of 20 negatively charged residues (Asp + Glu) and 23 positively charged residues (Arg + Lys). The anticipated half-life (HL) of the target protein was 30 hours, and its instability index (II) was calculated to be 35.04, indicating stability. The aliphatic index (AI) was found to be 76.72, suggesting that the protein is likely stable across a broad range of temperatures.

3.2 Subcellular Localization Prediction

As various cellular compartments correlate to different functions, identifying the hypothetical protein's subcellular location is crucial to understanding its function. This information is valuable for potential drug targets against the identified protein. According to predictions from CELLO, our target protein is anticipated to

be cytoplasmic. Consistently, the PSORTb server also predicted the protein's subcellular location to be in the cytoplasm.

3.3 Protein Family Analysis

To unveil conserved domains and infer the potential function of our target protein, various annotation tools were employed. According to predictions from NCBI-CD Search, the target protein was identified to harbor the S4 superfamily domain and was categorized as a ribosome-associated protein YbcJ, specifically an S4-like RNA-binding protein. The S4 superfamily domain, predicted by the NCBI-CD server, spanned amino acid residues 1-122 with an E-value of 6.57e-32. Additionally, the InterProScan server concurred by predicting the presence of the RNA-binding S4 superfamily domain in the target protein.

3.4 Multiple Sequence Alignment and Phylogenetic Tree Analysis

The BLASTp search against the non-redundant database revealed similarities between our target protein and other known S4 domain-containing proteins, such as YaaA from various bacteria (refer to Table 3). Multiple sequence alignments were conducted on a subset of proteins selected from the BLASTp results to identify conserved and distinctive residues among the homologs (Fig. 1). Using the same dataset, a phylogenetic tree (Fig. 2) was created, showing a common ancestor between the target protein and *Streptococcus* (WP_000455897.1).

3.5 Secondary Structure Determination

The determination of the protein's secondary structure was carried out using PSIPRED and the SOPMA server. Among the identified secondary structures, the alpha helix was the most prominent, alongside the random coil, beta-turn, and extended strand. According to the SOPMA analysis (Fig. 3), the distribution of secondary structures was as follows: random coil accounted for 32.79%, alpha helix for 44.26%, beta-turn for 7.38%, and extended strand, in conjunction with the random coil, constituted 15.57%. This result was supported by a similar finding from the PSIPRED server (Fig. 4).

Table 2. Physicochemical properties of HP estimated by ProtParam tool

Description	Value
Number of amino acids	122
Molecular weight (Da)	13910.97
Theoretical pI	9.05
Number of positively charged residues	23
Number of negatively charged residues	20
Instability index	35.04
Aliphatic index	76.72
Grand average of hydropathicity (GRAVY)	-0.807

Table 3. The BLASTp result shows similarities between proteins

Accession No.	Organism	Protein name	Score	Percent Identity	E-value
WP_000455897.1	<i>Streptococcus</i>	S4 domain-containing protein YaaA	247	100.00%	2e-82
CIY83532.1	<i>Streptococcus pneumoniae</i>	S4 domain-containing protein YaaA	246	99.18%	4e-82
WP_049532442.1	<i>Streptococcus pseudopneumoniae</i>	S4 domain-containing protein YaaA	246	99.18%	4e-82
WP_247942388.1	<i>Streptococcus mitis</i>	S4 domain-containing protein YaaA	245	98.36%	2e-81
CON46485.1	<i>Streptococcus pneumoniae</i>	S4 domain-containing protein YaaA	244	98.36%	2e-81

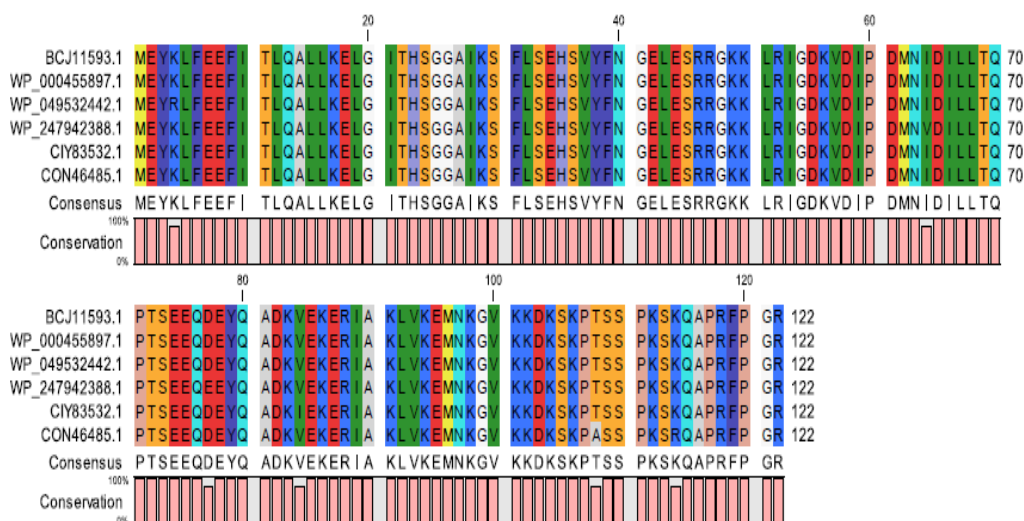


Fig. 1. Multiple sequence alignments among different S4 domain-containing protein YaaA using the CLC sequence viewer

3.6 Tertiary Structure Prediction

The target protein's tertiary structure was determined using template Q8DMX4.1.A from the SWISS-MODEL database, which has 96.72% sequence identity with the target protein. Fig. 5 displays the three-dimensional structure of the protein using PyMOL 2.0.

3.7 Model Quality Assessment

The assessment of the three-dimensional structure models was conducted utilizing PROCHECK, QMEAN, and ERRAT. The PROCHECK analysis revealed that 93.4% of amino acid residues occupied the most preferred zone in the "Ramachandran plot" (Table 4 and Fig. 6). With a commendable quality rating of

97.059, ERRAT indicated that the protein structure exhibited high quality (Fig. 7). The QMEAN tool placed the model within the permissible black zone with a QMEAN4 score of 0.50 (Fig. 8). To ascertain whether the input structure falls within the typical range of scores for native proteins of comparable size, the Z score, representing the overall model quality, was determined. The ProSA web server yielded a Z-score of -5.18 for the model target protein (Fig. 9).

4. DISCUSSION

Numerous ongoing studies focus on elucidating the structures and functions of hypothetical

proteins. Some investigations delve into targeting these proteins for potential applications in various diseases [42]. The characterization of hypothetical proteins contributes to an enhanced understanding of bacterial metabolic pathways, aids in the development of pharmaceutical agents, and informs strategies for managing diseases [43]. Using a range of bioinformatics methods, the HP protein (accession no. BCJ11593.1) of the *Streptococcus mitis* strain was structurally and functionally described in this work. The protein is hydrophilic, or soluble in water, according to a grand average of hydropathicity (GRAVY) of -0.807, a theoretical pI of 9.05 that suggests the protein is acidic (pH > 7), and an instability

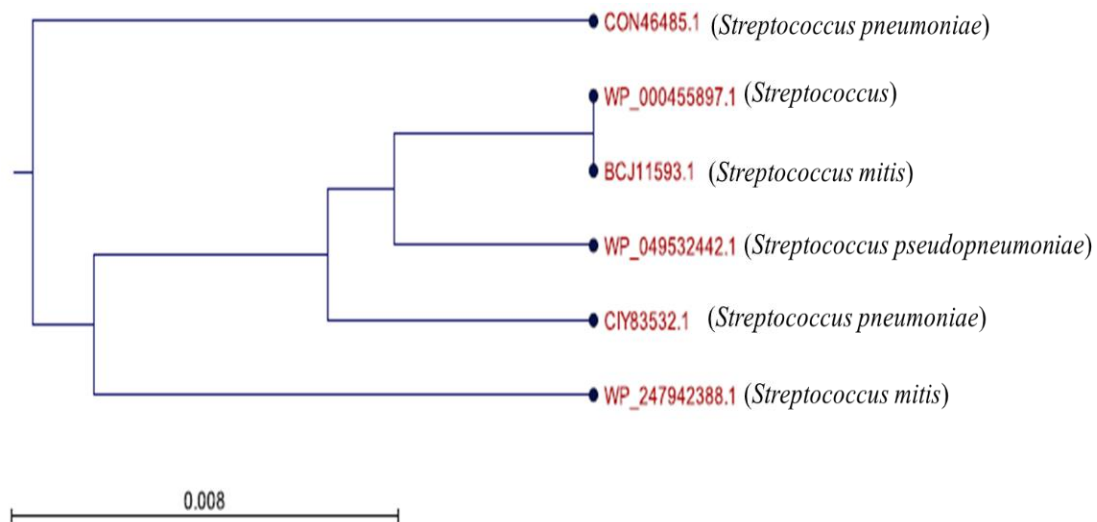


Fig. 2. A phylogenetic tree shows the evolutionary relationship of the target protein with another S4 Domain-Containing Protein, Yaaa

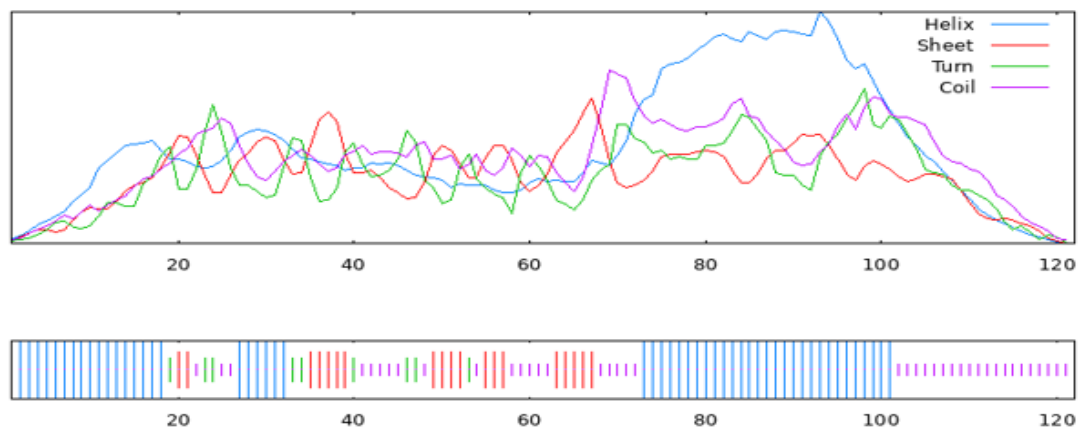


Fig. 3. The SOPMA server predicted the secondary structure

index (II) of 35.04 (Table 2), which shows that the protein is stable. This protein is expected to be located in the cytoplasm, according to the CELLO and PSORTb servers. By using domain and motif analysis, our target hypothetical protein was highly confidently predicted by all annotation techniques to be the ribosome-associated protein YbcJ, an S4-like RNA binding protein. *Escherichia coli* protein YbcJ belongs to the S4-like RNA-binding domains, known for binding structured RNA in tRNA, rRNA, and mRNA pseudoknots [44-46]. YbcJ, a 77-residue protein in a common bacterial family, has structural and sequence characteristics similar to S4 superfamily RNA-binding proteins [47-48]. Its solution structure indicates the presence of an αL motif rich in positively charged residues (arginines and lysines), potentially involved in RNA binding [46,49]. The predominant feature in the protein's secondary structure is the alpha helix, which encompasses other components such as random coil, beta-turn, and extended strand. As indicated by the SOPMA analysis (Fig. 3 and Fig. 4), the distribution of secondary structures was as follows: random coil accounted for 15.57%, alpha helix for 44.26%, beta-turn for 7.38%, and extended strand, in conjunction with random coil, constituted 15.57%. The tertiary structure of the target protein was obtained from the SWISS-MODEL server, utilizing the template Q8DMX4.1.A, which exhibited a sequence

similarity of 96.72% with the target protein (Fig. 5). The three-dimensional (3D) structure created through the SWISS-MODEL server successfully passed all model quality assessment tools, including PROCHECK, QMEAN, and ERRAT. According to PROCHECK data (Table 4 and Fig. 6), 93.4% of amino acid residues fell within the most preferred zone of the "Ramachandran plot." ERRAT predicted a high-quality protein structure with a quality factor of 97.059 (Fig. 7). In comparison to other experimental structures of comparable size, the model also obtained a QMEAN4 score of 0.50, placing it in the desired dark grey zone (Fig. 8). All of these findings point to the high quality of the predicted protein model, which makes it useful for more thorough research and analysis. The Z-score was determined using the ProSA web service, which offers a metric to assess the quality of the predicted protein model. The Z-score for the query model of the target protein was found to be -5.18 (Fig. 9). This score is often used to assess if the input structure is within the score range seen for natural proteins of similar size. This Z-score suggests that the obtained model is reliable, and its overall quality is considered good. However, the research on hypothetical protein annotations helped in the creation of effective medications and vaccines to combat this particular sort of pathogen.

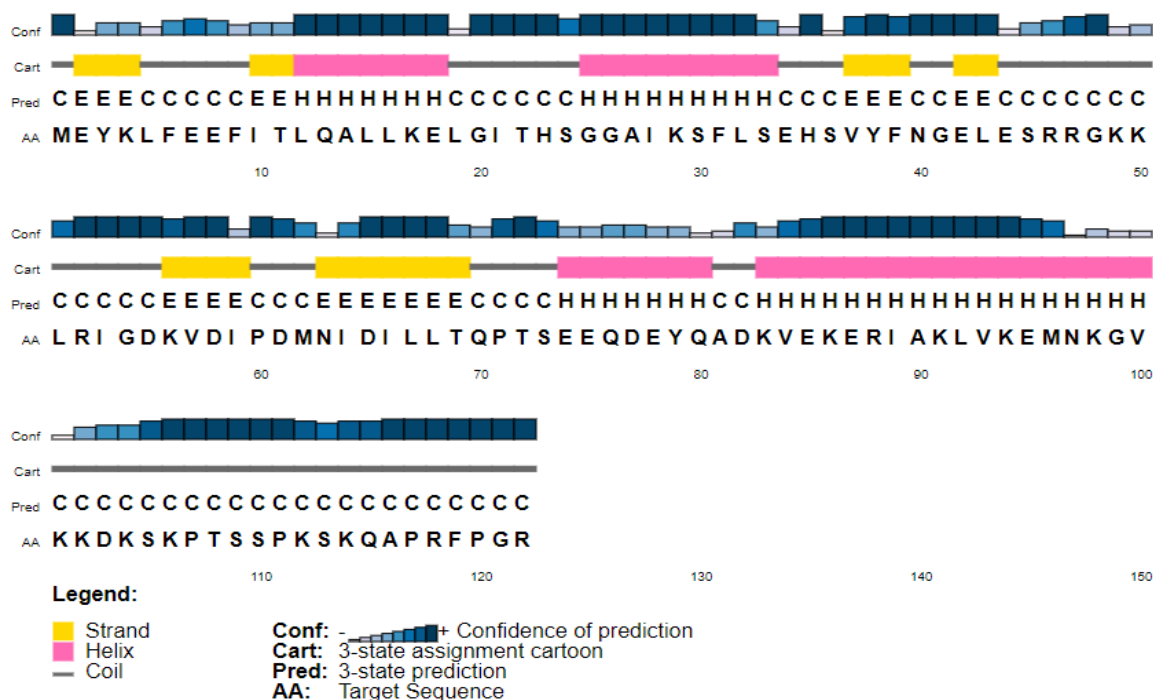


Fig. 4. The PSIPRED predicted the secondary structure of the target protein

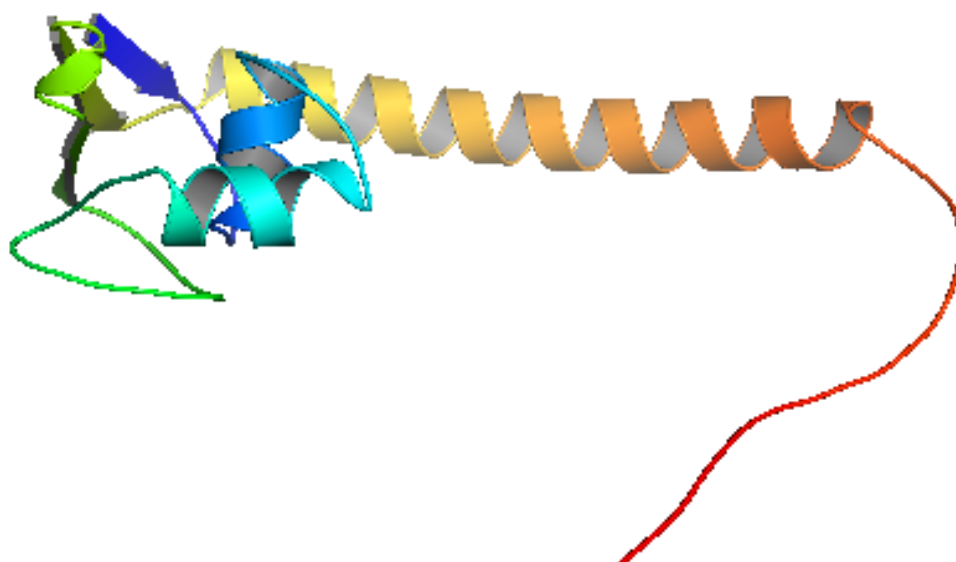


Fig. 5. Generated the target protein's three-dimensional structure using SWISS-MODEL (visualized using PyMOL program)

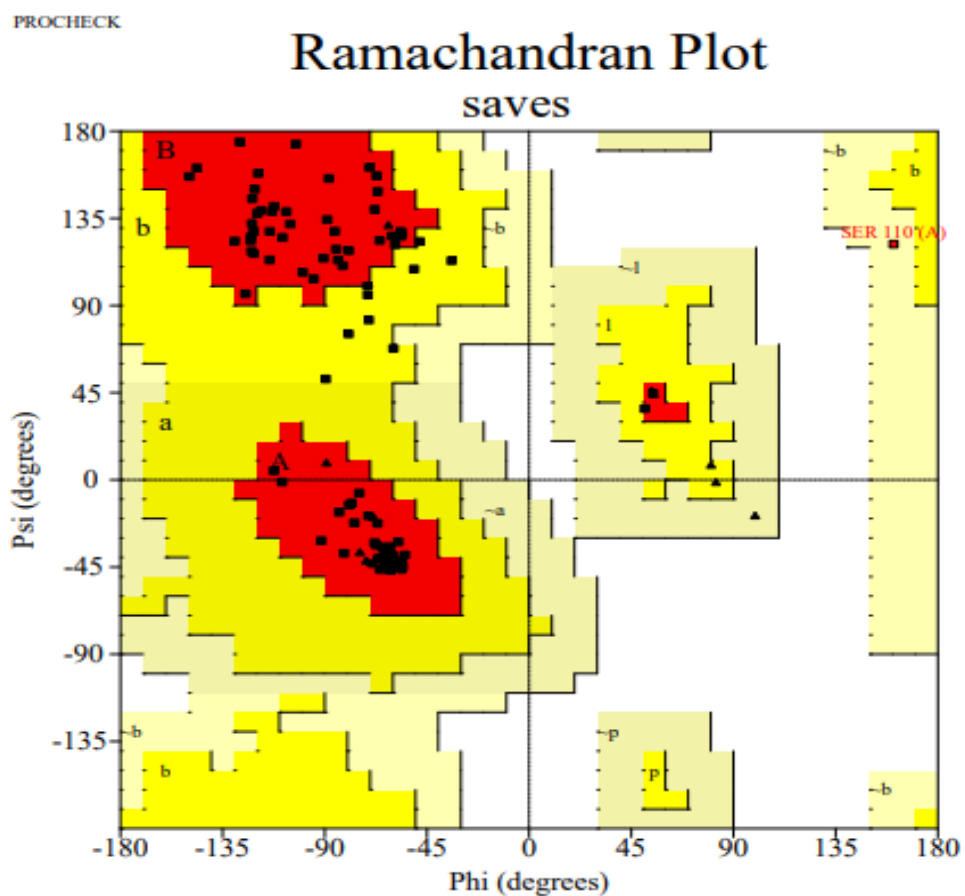


Fig. 6. Ramachandran plot of model structure validated by PROCHECK program

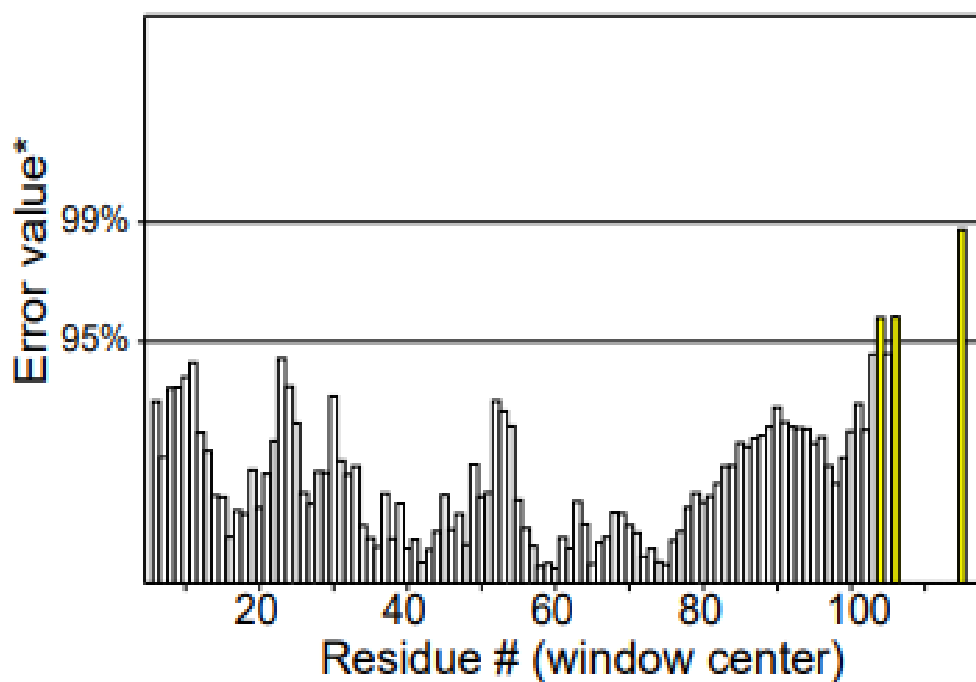


Fig. 7. ERRAT output. Two lines on the error axis represent the confidence required to reject areas that exceed the error value

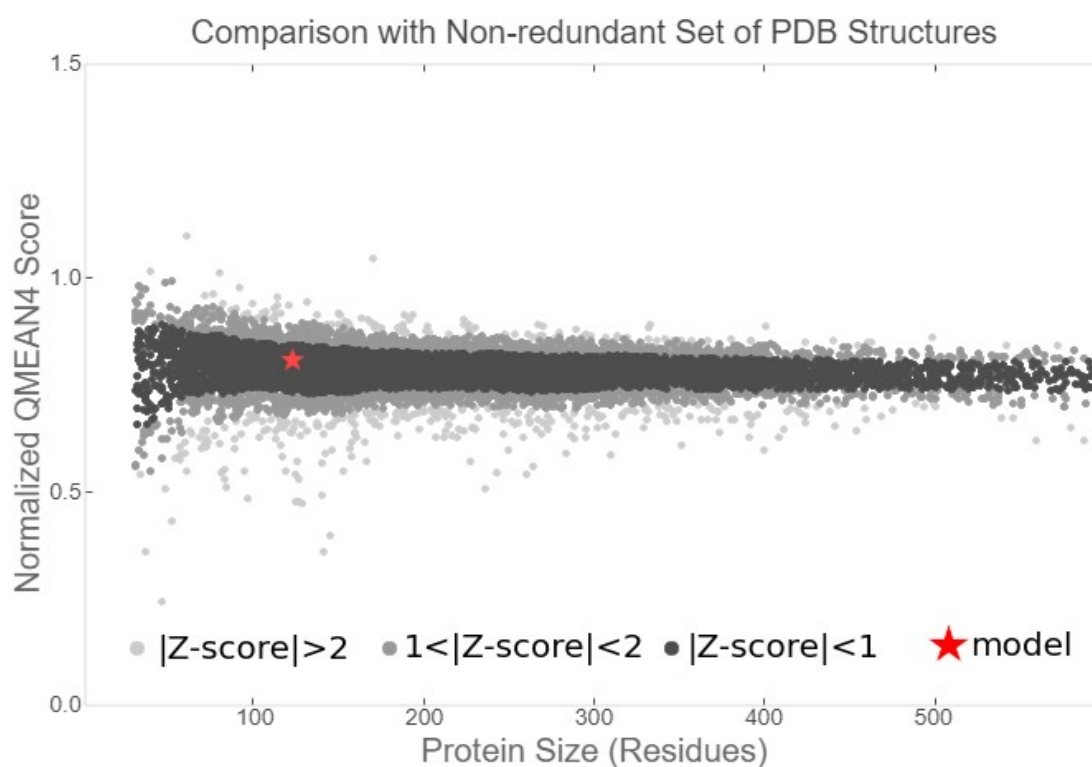


Fig. 8. Graphical representation of the QMEAN result of the model structure

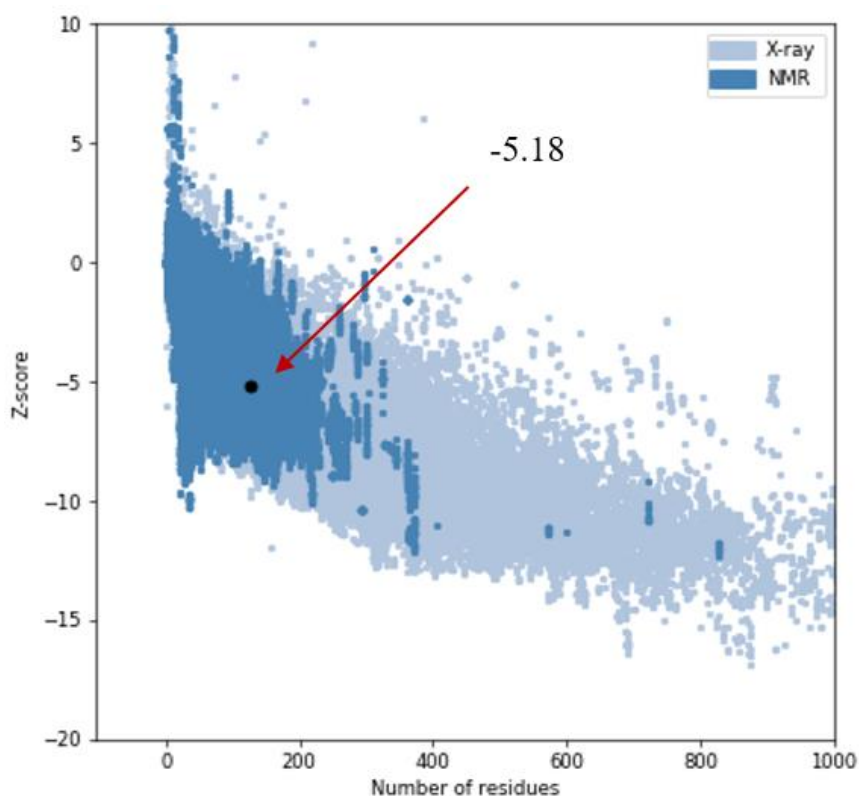


Fig. 9. Z score of the target protein using the ProSA server

Table 4. Statistics of the target protein made by Ramachandran plot

Statistics	Number of AA residue	Percentage (%)
Residues in the most favored regions [A, B, L]	99	93.4%
Residues in the additional allowed regions [a, b, l, p]	6	5.7%
Residues in the generously allowed regions [~a, ~b, ~l, ~p]	1	0.9%
Residues in disallowed regions	0	0.00%
Number of non-glycine and non-proline residues	106	Total= 100%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residue	8	
Number of proline residues	6	
Total number of residues	122	

5. CONCLUSION

This study employed various bioinformatics methods to analyze a hypothetical protein sourced from *Streptococcus mitis*. The implications of our research extend to improving the functionality of the target protein and enhancing resource utilization efficiency. Future research will use structural and functional data to create new ligands for medication development and to experimentally test our findings. To improve future treatment approaches, it is imperative to continue investigating target

proteins and their effectors in *Streptococcus mitis* and other species. This work contributes to understanding the structural and functional aspects of proteins with uncharacterized activities, providing a foundation for further research. The outcomes of this study may serve as a valuable resource for future *in silico* investigations conducted by other researchers.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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