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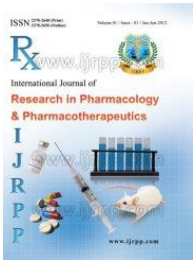

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Purification, immunogenicity and insilico functional characterisation of a putative metallopeptidase from *Staphylococcus aureus*

Dileep Francis and Surekha Kuyyalil

Department of Biotechnology and Microbiology, Kannur University, Thalassery Campus, Palayad P.O., Kannur- 670661, Kerala, India.

***Corresponding author: Dileep Francis**
Email: dilipfrans@gmail.com

ABSTRACT

Staphylococcus aureus has been a serious healthcare concern due to the wide range of diseases caused and the development of antibiotic resistance to almost all antibiotics developed so far. Methicillin resistant *Staphylococcus aureus* has been particularly damaging due to its enhanced virulence and emergence of community acquired MRSA alongside the hospital acquired MRSA. A preventive immunotherapeutic alternative in the form of vaccines is being pursued by pharmaceutical companies and academic researchers across the globe. Multiple clinical trials to develop a vaccine have not been successful. Recent research data suggest that antigens with the potential to activate a $CD4^+$ T cell mediated immune response rather than a B-cell mediated response would be the key to the development of a vaccine. Here we describe the purification, immunogenicity and insilico functional characterization of a putative metallopeptidase identified to be a $CD4^+$ T cell antigen from *S.aureus*. The gene coding for the putative peptidase designated as MP1 was isolated using PCR, recombined with pET28a, and transformed into *E.Coli* BEL21 (DE3). The protein was purified and intraperitoneally administered to BALB/c mice. Protein specific antibodies in mice serum was measured using indirect ELISA. The protein sequence was analyzed for the presence of transmembrane helices, domains and signatures using bioinformatics tools. The protein structure was modeled using Swiss modeler workspace. Our results show that the protein induced significantly higher ($p<0.05$) antibody responses in immunized mice compared to control mice. The titers of IgG, IgG1 and IgG2a on day 35 after initial immunization were 51200, 25600 and 12800 respectively. Insilico studies revealed that the protein belongs to MEROPS peptidase family M4 and contains the PepSY domain.

Keywords: Staphylococcus aureus, Vaccine, Peptidase, CD4+ T cell antigen, Indirect ELISA, Modeling.

INTRODUCTION

Staphylococcus aureus is a gram positive opportunistic human pathogen of serious health concern due to the wide range of diseases caused and the emergence of multiple antibiotic resistant strains [1]. The organism which is capable of inflicting diseases ranging from minor skin infections to life-threatening infections such as bacteremia,

endocarditis, and toxic shock syndrome has evolved with time to produce antibiotic- resistant strains that are resistant to almost all antibiotics available [2, 3, 4]. Drug resistant strains like methicillin resistant *Staphylocccus aureus* (MRSA) once confined to the hospital settings has now spread into the community settings further aggravating the health concern [5]. For these reasons there has been an active search for a vaccine against Staphylococcal infections [6, 7]. Attempts to develop a vaccine against *Staphylococcus aureus* so far has been focused on antigens that trigger humoral immunity mediated by B cells. Unfortunately none of these attempts translated into a clinically viable vaccine [8, 9].

Recent research data suggests that the key to success with a *Staphylococcus aureus* vaccine lies in the identification of antigens that can activate cell mediated immunity along with humoral immunity [10]. Particularly, CD4+ T cell antigens are envisaged as vaccine candidates as they are critical in resolving a bacterial infection owing to their ability to produce cytokines which can enhance the bactericidal activity of neutrophils and macrophages. Adoptive transfer of activated CD4+ T cells protected mice from lethal dose of *Staphylococcus aureus* but neither B-cells nor antibodies were protective [11]. Identification and evaluation of immunogenicity of CD4+ T cell antigens from *staphylococcus aureus* becomes important in this context. Lawrence et al [12] identified a set of CD4+ T cell antigens from *Staphylococcus aureus* following immunization of outbred cattle with heat killed bacteria. Among the antigens identified in this study was a putative protein named as NWMN_0364.

In this paper we describe the analysis of immunogenicity of NWMN_0364 in mice models. The protein was cloned and expressed in *Escherichia coli* and purified using Immobilized Metal Ion Affinity Chromatography (IMAC). BALB/c mice were immunized with the purified protein and the antibody responses generated by the protein were analyzed. The protein was functionally characterized using bio-informatics tools and its structure was modeled. The bioinformatics analysis showed that the protein is probably a metallopeptidase and hence the protein was designated as MP1. The protein was purified in its native form and was shown to induce robust antibody responses.

MATERIALS & METHODS

Bacterial Strains, Plasmids and Culture conditions

Staphylococcus aureus strain NCTC3750 (MTCC No: 3160) was purchased from the Microbial Type Culture Center and Gene bank (MTCC), Chandigarh, India. The bacterium was routinely cultured in Tryptone Soya broth (Himedia) or agar at 37^0 C. *E.coli* DH5α and BL21 (DE3) were routinely grown on Luria Bertani (LB) Broth (Miller) or agar at 37^0C. The vectors pJET1.2 (Fermentas) and pET-28a (+) (Novagen) were used for cloning. After transformation with pJET1.2, DH5 α cells were grown in LB media supplemented with 100 µg/ml of Ampicillin and BL21 (DE3) cells after transformation with pET-28a were grown in LB media supplemented with 35 µg/ml of Kanamycin.

Genomic DNA Isolation

Genomic DNA was isolated from of NCTC3750 using GeneiUltra pure bacterial genomic DNA isolation kit (Bangalore genei, Bangalore, India) as per the instructions of the manufacturer.

Isolation of gene of interest, cloning into pJET vector and sequence confirmation

The nucleotide sequence information of NWMN_0364 of *Staphylococcus aureus* strain Newman was obtained from NCBI (Accession Number: YP_001331398). This sequence was used to design the primers as shown in Table 1. The primers were designed such that the amplification product contains restriction sites for NcoI and XhoI at the 5' and 3' ends, respectively. The primers were used in a polymerase chain reaction containing genomic DNA isolated from *Staphylococcus aureus* NCTC 3750 as template to isolate and amplify the gene for MP1 in a thermo cycler (Eppendorf, Germany). The PCR product was analyzed on a 1.5% agarose gel. The amplified product was then recombined with the pJET1.2 vector using T4 DNA ligase. The recombinant vector was then transformed into competent DH5α cells using standard procedures. The screening for positively transformed clones was performed using colony PCR. The gene insert in a positively transformed colony was sequenced using automated Sangers sequencing and compared to the expected gene sequence.

Table 1: Primers used to isolate *mp1*

Primer Designation	Primer Sequence
***mp1*Forward**	GCCAGTCCATGGGCATGAAATTAAAATCATTA
***mp1*Reverse**	GCATATCTCGAGGTGATCTTGCTCACTCTTTA

Sub-cloning of *mp1* in expression host

After conformation of the sequence, the gene insert was sub cloned into pET28a vector and transformed into *E.Coli* BL21 (DE3) for expression of the protein. Briefly, pJET vector containing the gene insert and the pET28a vector were double restriction digested with NcoI/XhoI. The gene insert was purified and recombined with pET28a in the presence of T4 DNA ligase. The recombinant vector was then transformed into *E.coli* DH5α. Positively transformed colonies were screened using colony PCR. The vector containing the gene of interest was isolated from the positive colonies and was used to transform *E.Coli* BL21DE3. The transformed cells were grown on LB –kanamycin plates. The plates were incubated at 37^0C for 16 hours.

Expression and Purification of rMP1

The concentration of IPTG for induction, growth stage (OD600) for induction and the duration of induction were determined empirically. 250 ml LB broth was inoculated with 1% of cells grown overnight from a single transformed colony. The expression of rmp1 gene was induced by the addition of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 37 0C for 4 hours with vigorous shaking. After 4 hours of growth the induced cells were sedimented by centrifugation (4000 ×g, 4^0C, 20 min). The cell pellet was mixed thoroughly in a solution containing 50 mM $NaHPO_4$, 300 mM NaCl and 10 mM imidazole (pH 8). The cells were then lysed by sonication. The cell lysate was centrifuged (6000×g, 4^0C, 30 min) to separate soluble and insoluble fractions. Both the fractions were analyzed on SDS- PAGE. The protein was purified from the soluble fraction under native conditions using IMAC. Briefly, the soluble fraction of the lysate was loaded on a Nickel-iminodiacetic acid (Ni-IDA) resin equilibrated with as solution containing 50 mM $NaHPO_4$, 300 mM NaCl and 10 mM imidazole (pH 8). A solution containing 50 mM $NaHPO_4$, 300 mM NaCl and 20 mM imidazole (pH 8) was used to wash the column. The bound protein was eluted from the column using a solution containing 50 mM $NaHPO_4$, 300 mM NaCl and 250 mM imidazole (pH 8). The protein was dialysed in Phosphate buffered saline (pH 7.4) for 48 hrs with buffer changes at every 12 h interval. The purity of the protein was determined using SDS- PAGE. The amount of protein obtained was determined using an automated biuret test.

Immunization of mice with rMP1

Twelve 6 week old female BALB/c mice were divided into two groups containing 6 mice each. One of the groups (test group) received 50 µg of recombinant MP1emulsified in 200 µl of complete Freunds adjuvant on day zero by intraperitoneally. The other group (control group) received equal volume of Phosphate buffered saline. On day 21 the test group received a booster dose containing 50 µg of rMP1 emulsified in Freunds incomplete adjuvant. Control group was again injected with PBS. Blood samples were collected from both the the test and control groups by tail vein bleeding on days 21, 35 and 42 after immunization. Serum was separated from the blood samples by centrifugation and stored at -20^0C for further analysis.

Determination of antibody titers

Antibody titers in the mice sera collected from the test group and control group mice were determined using indirect ELISA. Briefly, 1µg of rMP1 suspended in 200 µl of coating buffer (Carbonate-bicarbonate buffer, 0.1M, pH 9.3) was coated in 96-well micro titer plates and incubated overnight at 4^0C. The plate was washed with PBS containing 0.05% Tween 20 (PBS-T) to remove the unbound protein. Unbound spaces in the plate was blocked using 400 µL of blocking buffer containing 5% BSA in PBS (pH 7.4) for one hour at room temperature followed by washing with PBS-T. Serially diluted mice serum was added to the plate and incubated at room temperature for one hour. The serum was poured off and the plates were washed with PBS –T. Bound immunoglobulin was detected by incubating the plate with rabbit anti mouse IgG conjugated with HRP (Invitrogen) diluted 1:2000 in PBS-T, for 1 hour at room temperature. After washing the plates three times with PBS-T, 200 µl of TMB/H202 was added

to each well and incubated in dark for 20 minutes. The reaction was arrested by adding 50 µl of 2N H_2SO_4 to each well. The absorbance at OD 450 was read in an ELISA reader.

The antibody isotypes IgG1 and IgG 2a were also detected using the same procedure as described above with some modifications. To detect IgG1 the plate was incubated with rat anti-mouse IgG1 conjugated with HRP (Invitrogen) diluted 1:1000 in PBS-T. To detect IgG2a the plate was incubated with rabbit anti-mouse IgG2a conjugated with HRP (Invitrogen) diluted 1:1000 in PBS-T

In-silico functional characterization of MP-1

Theoretical pI and molecular weight was determined using the ExPASy (Expert Protein Analysis System) proteomics server [13]. The transmembrane regions were predicted using the web server HMMTOP [14]. The signal peptides present were predicted by SignalP 4.1 server [15]. A sequence similarity search was done using pBLAST [16]. The multiple sequence alignment between the homologous sequences obtained from pBLAST was done using the CLUSTAL Omega programme [17]. The structural motifs found in functionally important regions of the protein structure were obtained from Interpro server [18]. The fingerprints, which are a group of conserved domains used to characterize a protein, were obtained from pfam server [19]. The Swiss - model workspace [20] was used for the homology modeling of the protein using structurally homologous proteins from Protein Data Bank (PDB)

Statistical Analysis

Statistical analysis of the results, wherever applicable was performed using two sample independent t test. P values < 0.05 were considered significant. All the graphing and statistical analysis was performed using Origin Pro 8 software.

Ethics Statement

The animal experiments described in this study were approved by the Institutional Animal Ethical committee (IAEC) with approval number (KULS/IAEC/2013/11). All the experiments were carried out according to the provisions of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

RESULTS

Recombinant *mp1* was expressed and purified

The *mp1* gene was amplified from *Staphylococcus aureus* NCTC3750 genomic DNA by PCR (Figure 1A). The sequence of the gene was determined using Sangers sequencing and was deposited in the GenBank database under the accession number KT884617. The figure 1 shows the major steps in the cloning and expression of *mp1* gene. The expression construct designed for MP1 turned out to be a moderately inducible construct. The protein induction level remained moderate despite changes in the expression condition. The presence of a C-terminal hexa-histidine tag enabled easy purification of the protein using a Ni-IDA column. Significant amount of the recombinant protein was present in the soluble fraction after sonication. This enabled the purification of the protein under non-denaturing conditions.

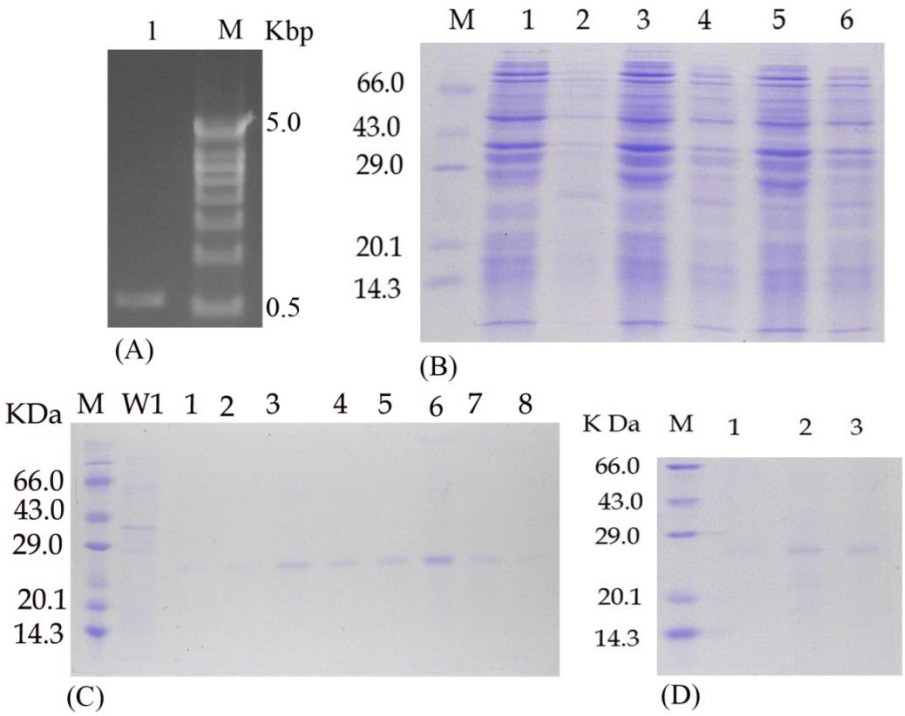

Figure 1: Cloning, expression and purification of mp1.

The *mp1* gene was recombined with pET 28a (+) vector, cloned and expressed in *E.coli* BL21DE3, and purified by IMAC. (A) Amplification of *mp1* gene by PCR. Lane M, DNA marker; Lane 1, amplified *mp1*. (B) Analysis of Expression of MP1 in *E.Coli* on SDS-PAGE. Lane 1,3 & 5, supernatant from non-induced bacteria; Lane 2, 4& 6, Supernatant from bacteria induced for expression of MP1 by addition of IPTG; Lane M, Protein marker. (C) Purifiaction of MP1. Lane W 1, wash from column; Lane 1-8, Eluted protein Fractions; Lane M, Protein Marker (D)MP1 after IMAC purification. Lane 1, 2 & 3, Purified MP1; Lane M, Protein marker.

Recombinant MP1 induces robust antibody responses in Immunized mice

The antibody responses generated by MP1 in BALB/c mice was determined from the sera obtained after immunization experiments in the test and control groups. Indirect ELISA was used to determine the antibody titers. As is evident from the Figure 2A mice immunized with rMP1 produced significantly higher IgG ($p<0.001$) as compared to mice immunized with PBS. In order to analyze the type of antibody response involved we determined the amounts of antibody subclasses IgG1 and IgG2a in immunized sera. As shown in Figure 2B and 2C immunized mice produced significantly higher amounts of IgG1 ($p<0.001$) and IgG2a ($p<0.002$) when compared to the control group. The antibody titers of IgG, IgG1 and IgG2a on days 21 and 35 after immunization are shown in the Table 2 and represented graphically in Figure 2D. It can be seen that IgG1 titers are higher than IgG2a titers on both days.

Table 2. Antibody Titers induced by rMP1

Days After Immunization	Antibody Titer		
	IgG	**IgG1**	**IgG2a**
Day 21	12800	3200	1600
Day 35	51200	25600	12800

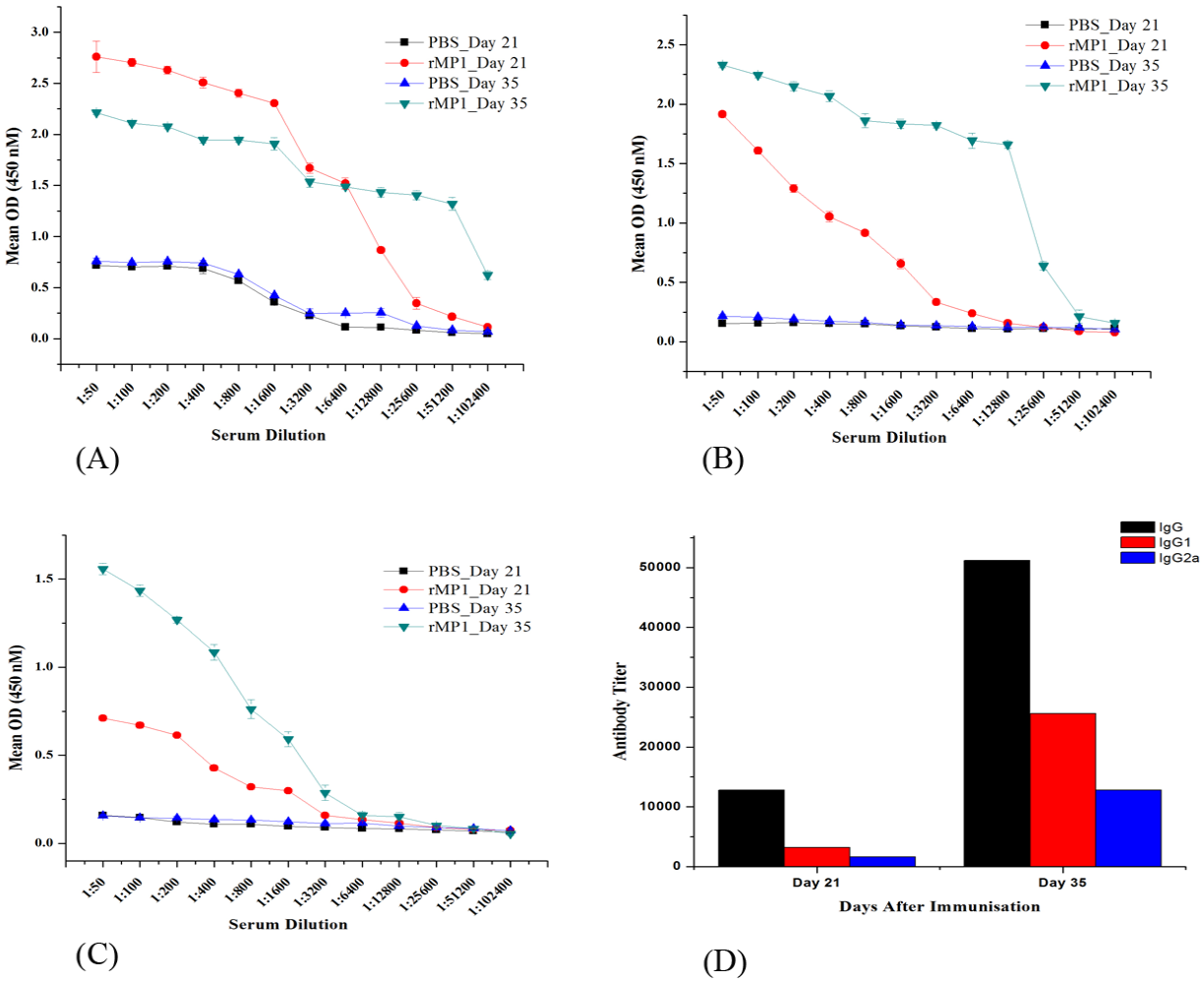

Figure 2: Immunogenecity of rMP1 in murine models.

The levels of IgG, IgG1 and IgG2a in serum collected from the test group mice were compared with the control. (A) Levels of IgG on day 21 and 35 (B) Levels of IgG1 (C) Levels IgG2a (D) Antibody titers* of IgG subtypes on Day 21 and 35. Note: The error bars in each case were generated from 6 mice.

*The cutoff OD at 450 nM for titer determination was calculated as the mean of the control values + 3 × Standard deviation of control. The antibody titers were calculated as the reciprocal of the highest serum dilution that gives an OD greater than the cutoff.

MP1 is probably a metallopeptidase with a PepsY doamian

An attempt was made to derive clues regarding the functional role of MP1 in *Staphylococcus aureus* using in-silico approaches. This would help in assessing the role of the protein in pathogenesis and infection. The MP1 protein contains 190 amino acids with a theoretical mass of ~ 22 KDa and isoelectric pH of 5.70. The protein does not anchor transmembrane helices. The SignalP4.1 server predicted the presence of a signal peptide in MP1 with the sequence "MKLKSLAVLSMSAVVLTAC" and showed that the cleavage occurs between the 20th glycine and 21st asparagine residues. The Interpro server detected two structural motifs in functionally important regions of the protein along with 8 unintegrated signatures (Table 3). The server predicted the presence of two PepSY domains spanning amino acid positions 51-100 and 129-187. The Pfam server also predicted the presence of the PepSY domains in the same regions of the proteins.

The pfam servers also showed that MP1 may be occupied with conserved domain characteristics of Peptidase propeptide and YPEB domain. PepSY domain comes under the protein family called MEROPS peptidase family M4 and is likely to have protease inhibitor function. The most probable structure of the protein was modeled using Swiss modeler workspace (Figure 3) and the model was evaluated using the QMEAN4 scoring function. The QMEAN Z score for the model shown in Figure 3 is -0. 95 which mean that the modeled structure may vary significantly from the actual crystal structure of MP1 (21). The template selected for modeling based on homology was of a putative lipoprotein (CD1622) from *Clostridium difficile (PDB ID: 4exr).* The identity to the sequence was only 26.24 %.

Table 3: Domains, repeats and Signatures predicted for MP1

Domains and repeats:	**Unintegrated signatures:**
PepSY domain (54-100)	Non-cytoplasmic domain (21-190)
PepSY domain (129-187)	Prokaryotic membrane lipoprotein (1-19)
	Signal peptide (1-20)
	Signal peptide C region (17-20)
	Signal peptide H region (6-16)
	Signal peptide N region (1-5)
	Signal-TM (1-20)
	Signal-noTM (1-20)

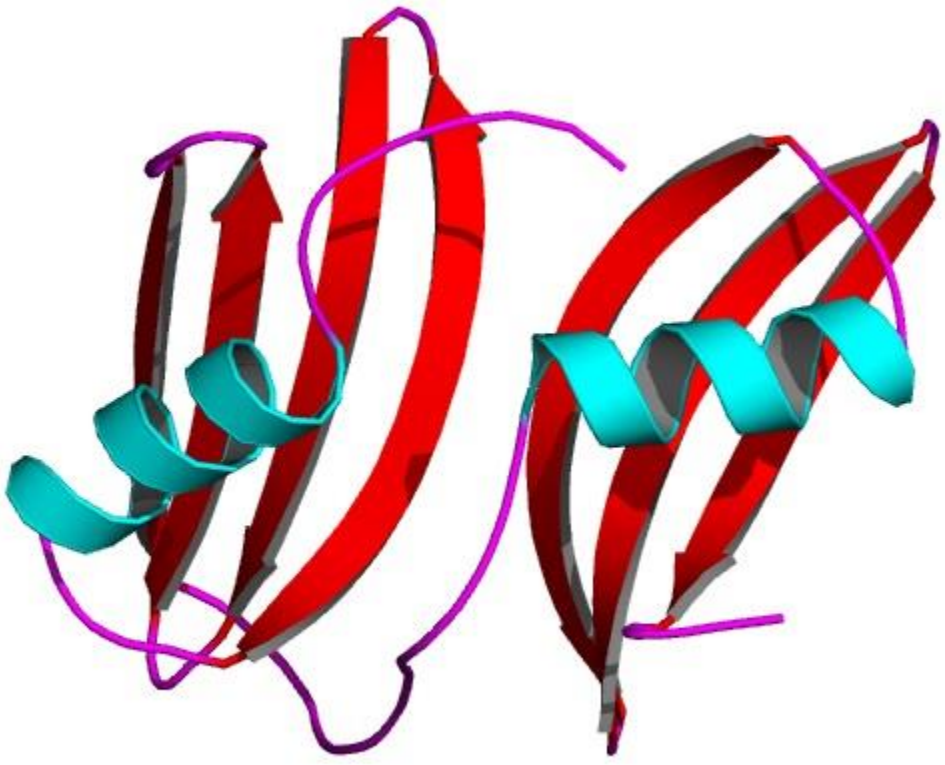

Figure 3: Structure of MP1 modeled using SWISS-MODEL workspace:

The model was developed using the protein with PDB ID 4exrA as the template. The model encompasses residues form position 47 to 182. The model shows 2α helices from positions 55-65 and 131- 141. β sheets were shown to span residues from position 70-113 and 145-176.

DISCUSSION

The propensity to develop resistance against almost all antibiotics developed has made *Staphylococcus aureus* the organism least vulnerable to antibiotic therapy [4]. A vaccine has been envisaged as a therapeutic alternative to chemotherapy to check the alarming spread of *Staphylococcus aureus* in community and healthcare settings. Based on cues obtained from antigens used vaccines against other gram positive bacteria many antigens of staphylococcus aureus including polysaccharides were tried as vaccine candidates but none translated into a clinically viable vaccine [22]. Based on research data the focus has now shifted to antigens that can activate CD4+ T cells along with humoral responses. In this study we analyzed the immunogenicity of MP1, a protein identified earlier in as a CD4+ T antigen, in mice models. The protein obtained in recombinant form was used to immunize BALB/c mice. The antibody responses were measured using indirect ELISA. We found that rMP1 induces strong antibody responses in mice system. The antibody subtypes IgG1 and IgG2a were also measured. Both the subtypes were induced but the titer of IgG1 was higher compared to IgG2a. In mice serum IgG1 is related to a Th2 type response while IgG2a corresponds to a Th1 type response. A well balanced Th1 and Th2 response is believed to be a requisite for the clearance of an infection in humans [24].

An insilico assessment of the probable function of MP1 in *Staphylococcus aureus* showed that the protein anchors two PepsY domains characteristic of metallopeptidases. Eubacterial metallopeptidases are involved in a range of functions from nutrient production to pathogenicity. It was also reported that PepSY domain acts as a regulator of peptidase activity in the microbial local environment and protects the cell from lysis [25]. One major reason for the success of *Staphylococcus aureus* as a human pathogen is its ability to evade the innate responses of the immune system including the ability to neutralize anti-microbial defensin peptides [26]. Based on the structural prediction it can be assumed that MP1 is involved in such peptidase activity and hence may be critically associated with the survival of *Staphylococcus aureus* inside the host. The strong immunogenicity in mice model and probable role as a metallopeptidase makes MP1 a promising vaccine antigen against *Staphylococcus aureus.* Results obtained in the presented work is preliminary in nature and warrants advanced experiments to further elucidate the efficacy of the immune response produce by MP1, nevertheless, it gives a clear indication that MP1 would be a promising vaccine candidate against *Staphylococcus aureus.*

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REFERENCES

[1]. Raygada JL, Levine DP. Methicillin-Resistant *Staphylococcus aureus*: A Growing Risk in the Hospital and in the Community. *Am Health Drug Benefits*. 2, 2009, 86–95.

[2]. Daum RS. Clinical practice. Skin and soft-tissue infections caused by methicillin-resistant *Staphylococcus aureus*. *N Engl J Med*. 357, 2007, 380–90.

[3]. Petti CA, Fowler VG. *Staphylococcus aureus* bacteremia and endocarditis. *Cardiol Clin.* 21, 2003, 219–33.

[4]. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol.* 7, 2009, 629–41.

[5]. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerging Infect Dis.* 9, 2003, 978–84.

[6]. Foster TJ. Potential for vaccination against infections caused by *Staphylococcus aureus*. *Vaccine.* 9, 1991, 221–7.

[7]. Proctor RA. Is there a future for a *Staphylococcus aureus* vaccine? *Vaccine.* 30, 2012, 2921–7.

[8]. Schaffer AC, Lee JC. Vaccination and passive immunisation against Staphylococcus aureus. *Int J Antimicrob Agents*.32(1), 2008, S71–8.
[9]. Salgado-Pabón W, Schlievert PM. Models matter: the search for an effective Staphylococcus aureus vaccine. *Nat Rev Micro*.12, 2014, 585–91.
[10]. Fowler VG, Proctor RA. Where does a *Staphylococcus aureus* vaccine stand? *Clinical Microbiology and Infection.* 20, 2014, 66–75.
[11]. Joshi A, Pancari G, Cope L, Bowman E, Cua D, McNeely T. Th17 stimulation and IL17A play critical roles in the Staphylococcus aureus iron regulated surface determinant B (IsdB) mediated protection in a disseminated challenge model. *The Journal of Immunology.* 186, 2011, 99.11.
[12]. Lawrence PK, Rokbi B, Arnaud-Barbe N, Sutten EL, Norimine J, Lahmers KK, et al. CD4 T cell antigens from *Staphylococcus aureus* Newman strain identified following immunization with heat-killed bacteria. *Clin Vaccine Immunol.* 19, 2012, 477–89.
[13]. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 31, 2003, 3784–8.
[14]. Tusnády GE, Simon I. Principles governing amino acid composition of integral membrane proteins: application to topology prediction. *J Mol Biol.* 283, 1998, 489–506.
[15]. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods.* 8, 2011, 785–6.
[16]. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 1997, 3389–402.
[17]. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 7, 2011, 539.
[18]. Mitchell A, Chang H-Y, Daugherty L, Fraser M, Hunter S, Lopez R, et al. The InterPro protein families database: the classification resource after 15 years. *Nucleic Acids Res.* 43, 2015, D213–21.
[19]. Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, et al. The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res.* 44, 2016, D279–85.
[20]. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics.* 22, 2006, 195–201.
[21]. Benkert P, Biasini M, Schwede T. Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics.* 27, 2011, 343–50.
[22]. Jansen KU, Girgenti DQ, Scully IL, Anderson AS. Vaccine review: "Staphyloccocus aureus vaccines: Problems and prospects." *Vaccine.* 31, 2013, 2723–30.
[23]. Jansen WT, Hogenboom S, Thijssen MJ, Kamerling JP, Vliegenthart JF, Verhoef J, et al. Synthetic 6B di-, tri-, and tetrasaccharide-protein conjugates contain pneumococcal type 6A and 6B common and 6B-specific epitopes that elicit protective antibodies in mice. *Infect Immun.* 69, 2001, 787–93.
[24]. Berger A. Th1 and Th2 responses: what are they? *BMJ.* 321, 2000, 424.
[25]. Yeats C, Rawlings ND, Bateman A. The PepSY domain: a regulator of peptidase activity in the microbial environment? *Trends in Biochemical Sciences.* 29, 2004, 169–72.
[26]. Foster TJ. Immune evasion by staphylococci. *Nat Rev Micro.* 3, 2005, 948–58.