Phylogenetic and *in silico* Proteomic Analysis of Fructose 1, 6 Biphosphate Aldolase-II in Community Acquired-Methicillin Resistant *Staphylococcus aureus* (CA-MRSA)

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ABSTRACT

In 1990s, a new strain of methicillin resistant *Staphylococcus aureus* (MRSA) emerged in the community setting occurring among young healthy individuals with no exposure to the healthcare setting. The infections caused by these strains are called community-acquired MRSA (CA-MRSA). Skin and soft-tissue infections (SSTIs) are the most common type of CA-MRSA infection including furuncles, abscesses, folliculitis, impetigo, cellulitis, and more rarely, in cases of severe sepsis, necrotizing fascitis, and necrotizing pneumonia. Recently, fructose 1, 6 biphosphate aldolase-II (FBA) enzyme has been identified as potential drug target for CA-MRSA through metabolic pathways analysis. In the present work, phylogenetic analysis and computational proteomic analysis of FBA for CA-MRSA was carried out. The phylogenetic analysis results of FBA in CA-MRSA reveal that apart from various *S. aureus* strains, it is closely related to other pathogenic non-aureus staphylococcal species as well. In addition, FBA is evolutionarily conserved in other pathogenic species of *Bacillus*. Therefore, FBA might be exploited as potential therapeutic drug target for various aureus and non-aureus species of Staphylococci. The proteomic analysis of FBA reveals that this protein belongs to Aldolase class-II family, which is absolutely distinct from the mammals. The physico-chemical properties data suggest that the FBA protein is stable in nature.

Keywords: Community-acquired MRSA, Fructose 1, 6 biphosphate aldolase, drug target, phylogenetic analysis, proteomic analysis

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most notorious strains of *S. aureus* which have become resistant to most â-lactam antibiotics. It is responsible for several difficult to treat infections. Any strain of *S. aureus* which develops resistance to beta-lactam antibiotics, which include the penicillins and the cephalosporins are called as MRSA (1, 2 and 3). These strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections (4). The original MRSA infections associated with exposure in the health care setting, particularly in hospitals are referred to as hospital-acquired MRSA (HA-MRSA) (5). In 1990s, a new strain of MRSA emerged in the community setting occurring among young healthy individuals with no exposure to the healthcare setting. The infections caused by these strains are called community-acquired MRSA (CA-MRSA) [6, 7]. The CA-MRSA strains have been involved in skin and soft tissue infections including furuncles, abscesses, folliculitis, impetigo, cellulitis, and more rarely, in cases of severe sepsis, necrotizing fascitis, and necrotizing pneumonia (8). The emergence and increasing prevalence of CA-MRSA strain that is resistant to available antibiotics demands the discovery of new therapeutic approaches. Recently, fructose 1, 6 biphosphate aldolase-II (FBA) enzyme has been identified as potential drug target for CA-MRSA through metabolic pathways analysis (9, 10). The fructose-bisphosphate aldolase (FBA) enzyme is encoded by fbaA gene, which has been found essential for the survival of CA-MRSA. These FBAs are involved in second reversible step of the glycolytic pathway, which supplies glyceraldehyde 3-phosphate for downstream enzymes in the pathway and fructose 1,6-bisphosphate (FBP) for gluconeogenesis. Together, the substrates and products of the FBA reaction are crucial for the supply of these precursor molecules to other biochemical pathways essential for the survival of pathogenic bacteria. This enzyme is also involved in other metabolic pathways of CA-MRSA such as pentose phosphate pathway, fructose and mannose metabolism, and methane metabolism (9). In the present work, phylogenetic analysis and computational proteomic analysis of fructose 1, 6 biphosphate aldolase-II (FBA) for CA-MRSA was carried out.

MATERIALS AND METHODS

Phylogenetic analysis of Fructose-bisphosphate aldolase

In order to establish the evolutionary relationship of fructose biphosphate aldolase (FBA) of CA-MRSA with other bacteria, the similar sequences were identified by database searching against non-redundant databases (NRDB) using the BLASTp program (11). This program searches protein databases using the protein query, which returned 100 similar sequences on first page. Since the FBA sequences of almost all species of *Staphylococcus aureus* were 100% identical to the CA-MRSA, they were excluded for the phylogenetic analysis. Only non-aureus staphylococcal species were selected apart from the other bacterial species for the multiple sequence alignment (MSA) and phylogenetic analysis. The similar sequences which were having more than 75% identity were selected for the MSA. Total 39 sequences were chosen for the MSA and phylogeny consequently, to establish the evolutionary relationship between

FBA of CA-MRSA and other bacterial species. Using the COBALT program multiple sequence alignment of all 40 sequences was performed. This is a multiple protein sequence alignment tool which finds a collection of pairwise constraint derived from conserved domain databases, protein motif database, and sequence similarity, using the RPS-BLAST, BLASTp and PHI-BLAST. Subsequently, pairwise constrains are incorporated into a progressive multiple alignment (12). Multiple aligned sequences of FBA from different species were carefully examined.

Based on COBALT multiple alignment, phylogenetic tree was generated which was incorporated into the same program itself. The phylogenetic guide tree was computed by the Fast Minimum Evolution (FME) method (13) which is a distance-based method for phylogeny reconstruction applied on large sets of taxa. The evolutionary distance between two sequences were calculated using the Grishin (protein) algorithm (14). Four methods i.e., Rectangle, Slanted, Radial and Force were used for rendering the phylogenetic tree and all of them show the same guide tree compute with a method selected in 'Tree method' option. Rectangle view is the rectangular shaped rooted tree, where root is placed in the longest edge. The Slanted view is similar to rectangle, but with triangular shape. The radial view is un-rooted tree. The Force view is similar to radial, where nodes are pushed away from one another for the improved appearance of the tree. The phylogenetic tree computed for all 40 FBA sequences were viewed in different forms and their evolutionary relationship was judiciously analyzed.

Proteomic analysis of Fructose biphosphate aldolase

Pfam prediction

The protein sequence of fructose biphosphate aldolase (FBA) in CA-MRSA was searched in the pfam database (http://pfam.sanger.ac.uk/) to identify the protein family of the protein. The Pfam database is a huge collection of protein families, each represented by *multiple sequence alignments* and *hidden Markov models* (HMMs) (15). This database has been classified into two classes Pfam-A and Pfam-B. *Pfam-A* entries are high quality, manually curated families. *Pfam-B* contains supplement of families which are commonly of lower quality. Using the pfam database searching, domain of the FBA has been identified which provides insight into their function.

Conserved domain prediction

In order to identify the conserved domain in fructose biphosphate aldolase (FBA) in CA-MRSA, the protein sequence was searched against the Conserved Domain Database (CDD) which has been provided by the NCBI. CDD is the collection of sequence alignments and profiles representing protein domains conserved during molecular evolution (http://www.ncbi.nlm.nih.gov/cdd) (16). It encompasses alignments of the domains to known 3-dimensional protein structures in the MMDB database as well. The amino acid sequence of FBA in FASTA file format was submitted to the CDD v3.10 database using the default parameters such as E-value (Expect value) of 0.01 and maximum number of hits 500, and the result was obtained in concise mode.

Motif prediction

The motif(s) in fructose biphosphate aldolase (FBA) of CA-MRSA was identified by the Motif Search tool provided by GenomeNet (http://www.genome.jp/tools/motif/). It searches with a protein query sequence against Motif Libraries (17). The amino acid sequence of FBA was submitted in FASTA file format, subsequently searched against the PROSITE pattern database, which is the secondary protein database derived from the Swiss-Prot database (primary database).

Prediction of physico-chemical properties

Various physico-chemical properties of the fructose biphosphate aldolase (FBA) in CA-MRSA were computed using the ProtParam tool, which is provided by ExPASy server (http://web.expasy.org/protparam/). This tool allows the computation of different physical and chemical parameters for a given protein that can be deduced from a protein sequence. Number of parameters were computed such as molecular weight, theoretical pI (Isoelectric point), amino acid composition, atomic composition, extinction coefficient, estimated half life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) (18).

Secondary structure prediction of FBA

The secondary structures for fructose biphosphate aldolase (FBA) were predicted using the GOR V server (http://gor.bb.iastate.edu/) (19). The alpha-helices, beta-sheets, coils, and turns comprise the fundamental elements of the secondary structure of proteins. The GOR (Garnier-Osguthorpe-Robson) method uses both information theory and Bayesian statistics for predicting the secondary structure of proteins (20). The knowledge of the secondary structure provides an input for prediction of three-dimensional structure of protein. By secondary structure prediction complex three-dimensional problem can be reduced to a much simpler one-dimensional problem, which helps to find the location of fundamental elements not in the three-dimensional space but along the protein amino acid sequence as well (19).

Protein globularity and disorder prediction

The tendency of protein disorder and globularity of the fructose biphophate aldolase (FBA) in CA-MRSA was predicted by using the GlobPlot v2.3 server. It is a CGI (Common Gateway Interface) based server accessible at http://globplot.embl.de, that allows the user to plot the tendency within the query protein for order/globularity and disorder (21). The amino acid sequence (in single letter IUPAC code) of FBA was submitted to the GlobePlot server.

RESULTS AND DISCUSSION

Phylogenetic analysis of fructose-bisphosphate aldolase (FBA)

The multiple sequence alignment (MSA) for forty (40) protein sequences of fructose 1, 6 biphosphate aldolase (FBA) from different bacterial species was performed using the COBALT program (12), and mutational regions within these sequences were examined carefully. Overall study of MSA indicates a high extent of similarity among these enzyme

sequences, and a number of one or two consecutive mutational regions throughout the entire length of sequences. Phylogenetic tree for these aligned sequences has been created to trace out their evolutionary relationship and direction of evolution. Different views of the tree have been generated for better understanding but all the views show the same guide tree computed with a Fast Minimum Evolution method (13**)**. This algorithm was used to produce a tree from given distances (or dissimilarities) between the sequences. The Rectangle view is rectangular shaped rooted tree, where root is placed in the longest edge, and Slanted view is similar to rectangle view, but with triangular tree shape. Radial view shows an un-rooted tree whereas force view is similar to radial view, where nodes are pushed away from one another for better presentation. The phylogenetic trees of FBA are shown in different views (Figure 1-3).

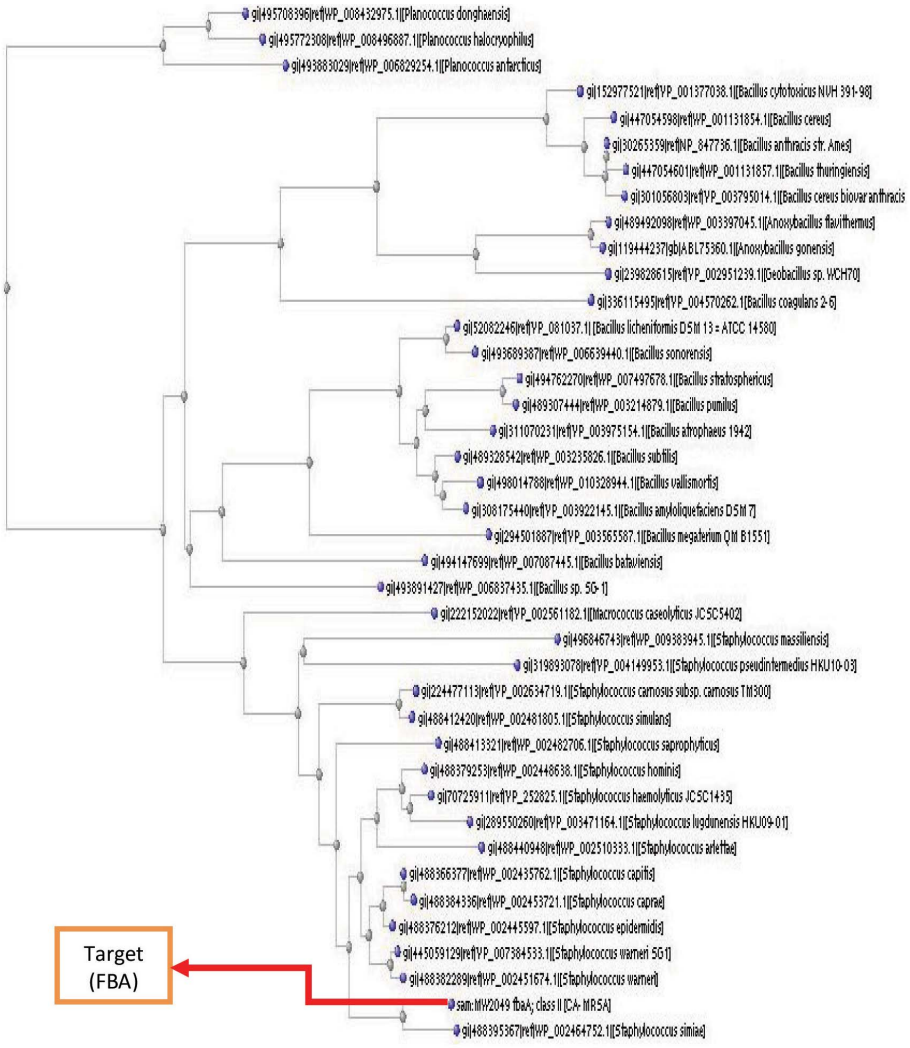

Fig. 1: Phylogenetic tree of FBA in rectangle view

The FBA proteins of all *Staphylococcus* genus are very close to each other and have very low evolutionary divergence among the same species. FBA sequence of community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) is phylogenetically closest to the *Staphylococcus simiae* (Figure 1, 2). Furthermore, FBA is closely related to other species of *Staphylococcus* such as *S. epidermidis, S. warneri, S. epidermidis, S. caprae, S. capitis, S. arlettae, S. lugdunensis, S. haemolyticus, S. saprophyticus, S. simulans, S. carnosus, S. pseudintermedius* and *S. massiliensis* (Figure 3).

FBA in all those organisms have been evolved from one lineage. Among those non-aureus species of Staphylococci, *S. lugdunensis, S.haemolyticus, S. saprophyticus* and *S. simulanss* cause rare but severe infections in human. *S. lugdunensis and S. saprophyticus* are notorious for urinary tract infections (UTIs) and skin and soft-tissue infections (SSTI) respectively (22 and 23). Since the FBA of CA-MRSA is evolutionarily closely related to above species, it might be considered as drug target in those pathogens as well.

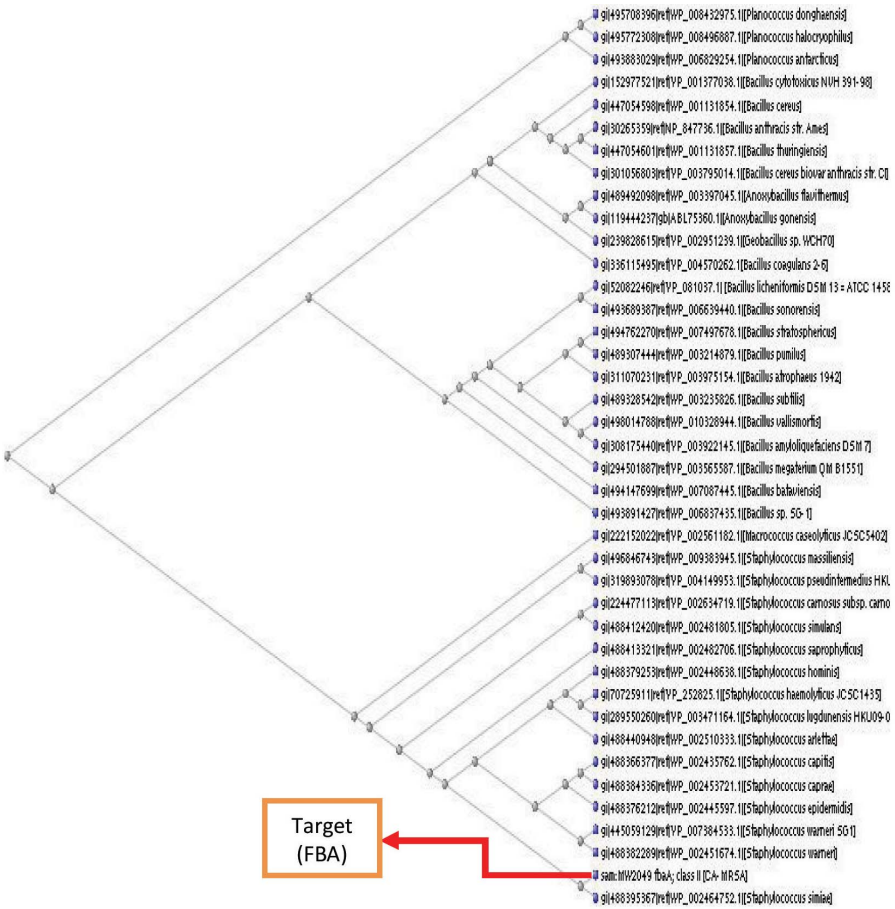

Fig. 2: Phylogenetic tree of FBA in slanted view

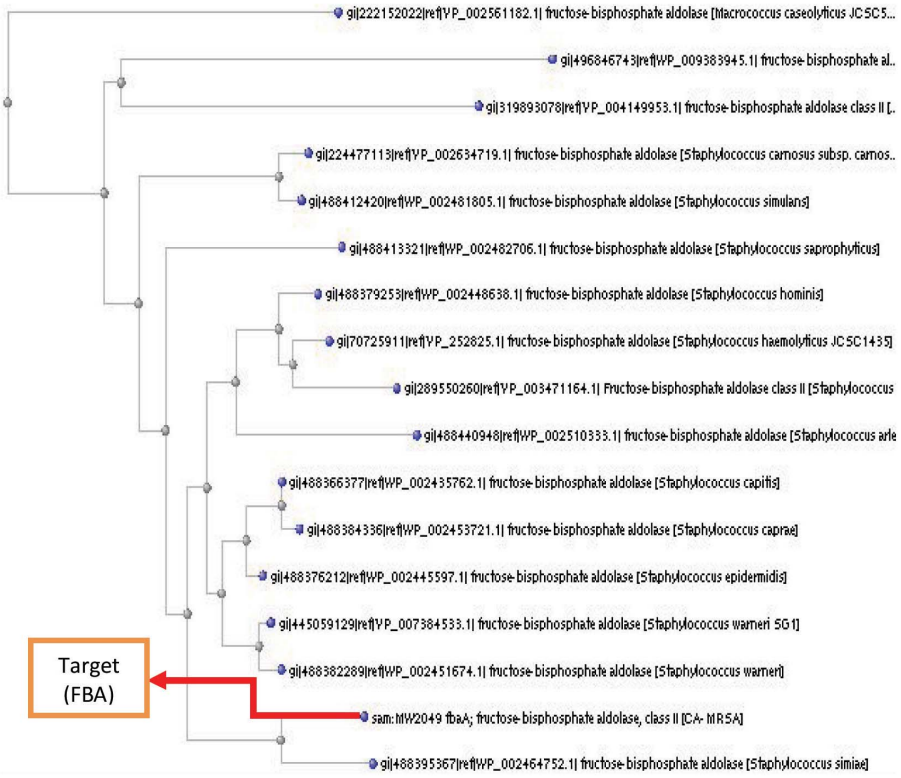

Fig. 3: Phylogenetic tree of FBA in rectangle view (sub-tree)

In the second lineage of evolution high degree of closeness has been observed among FBA of different *Bacillus* species (Figure 1and 2). *Anoxybacillus flavithermus* and *Anoxybacillus gonensis* species are very close to each other, while *Anoxybacillus* and *Geobacillus* species have a common ancestral origin. *Bacillus* sp. *SG-1* is looking distinct and distantly related with other remaining *Bacillus* species. *Bacillus coagulans* has been represented as a unique species of *Bacillus* with entirely different way of origin and evolution with a little closeness with two common clusters of *Bacillus* species. Evolutionary divergent nature of *Bacillus* sp. *SG-1* and *Bacillus coagulans* has been observed.

Macrococcus caseolyticus is very distantly related with *planococcus.* The FBA in *Bacillus* species have been evolved from common ancestor of *Staphylococcus*. In the third and distinct lineage, FBA from three different species of *Planococcus* i.e. *donghaensis, halocryophilus, antarcticus* are evolutionarily related to FBA from *Bacillus* and staphylococcal species. *Planococcus donghaensis a*nd *Planococcus halocryophilus* are very closely related (Figure 1 and 2).

The phylogenetic analysis results of FBA suggest that, apart from different *S. aureus* strains, it is closely related to other pathogenic non-aureus staphylococcal species as well. In addition, FBA is evolutionarily conserved in different pathogenic species of

Bacillus. Therefore, FBA might be used as important drug target in wide range of *Staphylococcal aureus* and non-aureus species, and *Bacillus* species as well.

Proteomic analysis of Fructose biphosphate aldolase (FBA)

i) Pfam prediction

The protein family of fructose biphosphate aldolase (FBA) was predicted by Pfam database searching. Two Pfam-A matches were found in the database, one significant and another insignificant. It was found that FBA belongs to F_bP_aldolase protein family and it is class-II aldolase which makes it absolutely distinct from mammals. Therefore, this protein might be used as drug target in CA-MRSA. The active sites in the protein were also predicted which will be helpful in locating the active site. The predicted active site was located as D85 (Aspartic acid at position 85) in FBA (Table 1).

According to clan entry, this protein belongs to common phosphate binding-site TIM barrel superfamily. A clan is higher-level groupings of related families. In this large superfamily of TIM barrel enzymes, all contain a common phosphate binding site.

ii) Conserved domain prediction

Proteins usually consist of one or more functional regions, commonly known as *domains*. Different combinations of domains give rise to the diverse range of proteins found in nature. The prediction of domains that occur within proteins can therefore provide insights into their function. The conserved domain of the FBA was predicted from the Conserved Domain Database (CDD). The enzyme FBA has a significant conserved domain which belongs to FTBP_ aldolase_II super family (Figure 4). It indicates correct prediction, as predicted domain is found only in bacteria and fungi that makes FBA as unique drug target for CA-MRSA.

iii) Motif prediction

The motifs in fructose-bisphosphate aldolase were predicted by using the Motif Search tool. Following motif was predicted in the amino acid sequence of FBA:

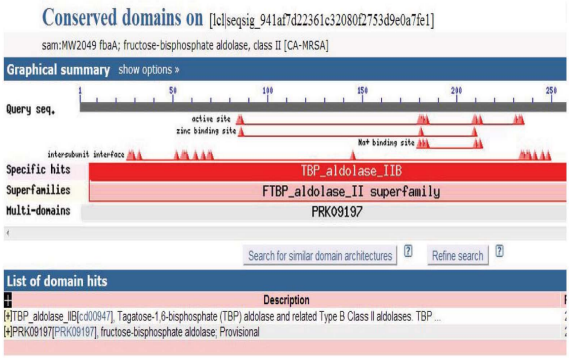

Fig. 4: Conserved domain in Fructose biphosphate aldolase

Table 1: Significant Pfam-A matches

Family	Description	Entry type	clan	Envelop		Alignment		HMM		Bit score	E-value	Pred.active sites
				start	end	from	to	from	to			
F_bP_ aldolase	Fructose biphosphate aldolase class-II	Domain	CL0036	2	285	3	285	2	287	354.3	3.8e-106	85(D85)

Prosite ID: ALDOLASE_CLASS_II_2 (PS00806)

Description: Fructose-bisphosphate aldolase class-II signature 2.

Pattern: [LIVM]-E-x-E-[LIVM]-G-x(2)-[GM]-[GSTA]-x-E.

Appearance:

Position	Predicted Motif
134..145	VEAELGTVGGQE

The motifs are the conserved regions in multiple protein sequences which play important structural or functional role in the proteins. The predicted motif 'VEAELGTVGGQE' might play significant role in functioning of the fructose-bisphosphate aldolase enzyme. It may also have important role in the process evolution of FBA across different organisms especially in bacteria and fungi.

iv) Prediction of physico-chemical properties

Different physicochemical properties of FBA such as molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) were computed by ProtParam tool at the ExPASy server [18].

Table 2: Physico-chemical properties of Fructose biphosphate aldolase

Sr. No.	Physico-chemical properties of FBA	Value
1.	Number of amino acids	286
2.	Molecular weight	30836.0
3.	Theoretical pI	5.01
4.	Instability index	33.22
5.	Aliphatic index	88.32
6.	Grand average of hydropathicity (GRAVY)	-0.228
7.	Estimated half life	> 10 hrs (E. coli)

Table 3: Amino acid composition of FBA

Sr. No.	Amino acid residue	No. of residues	% of residues
1.	Ala (A)	27	9.4%
2.	Arg (R)	5	1.7%
3.	Asn (N)	13	4.5%
4.	Asp (D)	14	4.9%
5.	Cys (C)	2	0.7%
6.	Gln (Q)	7	2.4%
7.	Glu (E)	29	10.1%
8.	Gly (G)	27	9.4%

Contd.

Sr. No.	Amino acid residue	No. of residues	% of residues
9.	His (H)	7	2.4%
10.	Ile (I)	21	7.3%
11.	Leu (L)	19	6.6%
12.	Lys (K)	24	8.4%
13.	Met (M)	8	2.8%
14.	Phe (F)	8	2.8%
15.	Pro (P)	13	4.5%
16.	Ser (S)	14	4.9%
17.	Thr (T)	15	5.2%
18.	Trp (W)	0	0.0%
19.	Tyr (Y)	9	3.1%
20.	Val (V)	24	8.4%

The physicochemical properties of the fructose biphosphate aldolase were analyzed from the Table 2. The molecular weight of FBA was found to be 30836.0, which reveals that protein is of moderate size. The theoretical isoelectric point (pI) was 5.01. The instability index was found to be 33.22, which is less than 40. The instability index provides an estimate of the stability of protein in a test tube (24). According to instability index, the fructose biphosphate aldolase enzyme is classified as stable protein. The aliphatic index was found to be 88.32, which reveals that protein is composed of majority of aliphatic amino acid residues. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It may be considered as a positive factor for the increase of thermostability of globular proteins (25). The aliphatic index value indicates that FBA protein is thermostable. The Grand average of hydropathicity (GRAVY) was found to be -0.228. The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. The GRAVY index indicates solubility of the proteins: positive (hydrophobic), negative (hydrophilic) (26). The GRAVY index indicates that FBA protein is hydrophilic in nature. Estimated half life of FBA was computed which was found to be >10. The half-life is a prediction of the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell (27). The amino acid composition of FBA was analyzed, and it was found that Glutamic acid (E) residue is present in highest percentage (10.1%) while Tryptophan (W) is not found in the enzyme. Rest of the amino acids are present in different ratios (Table 3).

v) Secondary structure prediction of FBA

The secondary structure of the fructose biphosphate aldolase in CA-MRSA was predicted using the GOR IV server. The predicted secondary structures from the amino acid residues of FBA are shown in Figure 5.

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MPLVSMKEMLIDAKENGYAVGQYNINNLEFTQAILEASQEENAPVILGVSEGAARYMSGF
ccccchhhhhhhhhccceeeeeccccchhhhhhhhhhhhhcccceeeeccccchhhhhccc
YTIVKMVEGLMHDLNITIPVAIHLDHGSSFEKCKEAIDAGFTSVMIDASHSPFEENVATT
eeeeeeeeccccccccccceeeeccccccchhhhhhhcccccceeeeecccccchhhhhch
KKVVEYAHEKGVSVEAELGTVGGQEDDVVADGIIYADPKECQELVEKTGIDALAPALGSV
hhhhhhhhccceeeeeecccccccccceeeceeeecccccchhhhhhhcchhhhhccccccc
HGPYKGEPKLGFKEMEEIGLSTGLPLVLHGGTGIPTKDIQKAIPFGTAKINVNTENQIAS
ccccccccccchhhhhhhcccccceeeecccccccccccccccccceeecccchhhhhh
AKAVRDVLNNDKEVYDPRKYLGPAREAIKETVKGKIKEFGTSNRAK
hhhhhhhhccccccccccccccchhhhhhhhhhcceeeecccceec
Sequence length :   286
GOR4 :
   Alpha helix      (Hh) :    91 is  31.82%
   3₁₀ helix        (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    52 is  18.18%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   143 is  50.00%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Fig. 5: Secendary structure of FBA predicted by GOR IV server

The protein fructose biphosphate aldolase consists of alpha helix (Hh), Extended stand (Ee) and random coil (Cc). The highest number of amino acid residues (143) contribute to the random coil (50.00%), 91 residues contribute to the alpha helix (31.82%) and 52 residues contributed to the extended stand (18.18%) in overall secondary structure of protein (Figure 5).

Protein globularity and disorder prediction

The GlobPlot program was used to explore the protein sequences of fructose biphosphate aldolase for predicting the globularity and disorder, which are shown in Figure 6. GlobPlot is a web service that allows users to plot the tendency within the query protein for order/globularity and disorder [21]. The plot depicts two (2) disordered regions in the amino acid sequence of fructose biphosphate aldolase (FBA). The non-globular sequence segments or disordered regions commonly contain short linear peptide motifs which are important for protein functions. The first disordered region is 'ALGSV HGPYKGEPK' between the sequence positions 176-189, while second disordered region is 'LPLVLHG GTGIPTKD' between the positions 204-218.

These disordered regions might play an important role in the catalytic activity of FBA in CA-MRSA. Another important region in the sequence of FBA corresponds to globular proteins which range between 2-175 amino acid positions. These globular regions contribute in the domains of the FBA protein, needed for the biochemical reactions.

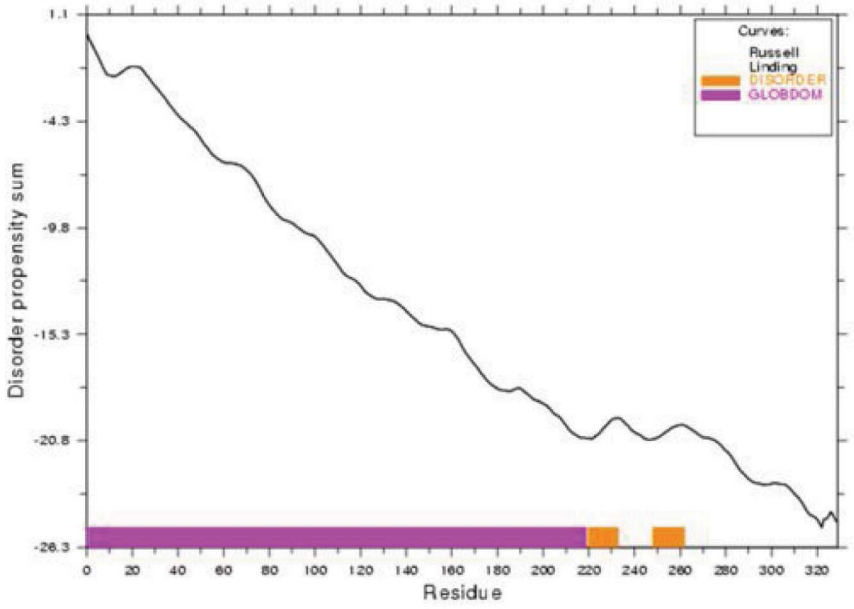

Fig. 6: GlobPlot output depicting globular regions and disordered segments in FBA

CONCLUSION

The phylogenetic analysis results of fructose biphosphate aldolase (FBA) in CA-MRSA reveal that apart from various *S. aureus* strains, it is closely related to other pathogenic non-aureus staphylococcal species as well. In addition, FBA is evolutionarily conserved in other pathogenic species of *Bacillus*. Therefore, FBA might be exploited as potential therapeutic drug target for various aureus and non-aureus species of Staphylococci. The proteomic analysis of FBA reveals that this protein belongs to Aldolase class-II family, which is absolutely distinct from the mammals. The physico-chemical properties data suggest that the FBA protein is stable in nature.

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