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MULTIPLE SEQUENCE ALIGNMENT AND PHYLOGENTIC ANALYSIS OF 16S RNA OF DIFFERENT STRAINS OF *STAPHYLOCOCCUS AUREUS*

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ABSTRACT: Phylogenetics is the study of evolutionary relationships among organisms or genes. Below, we will refer to the objects whose phylogeny we are studying as organisms or species, but the discussion of methods is valid for the phylogeny of genes as well. In this work, we have analyzed the phylogenetic relationships of 16s RNA sequences of different Methicillin resistant *Staphylococcus aureus* strains. This analysis has focused on 16s RNA families for which initial characterizations have been achieved for individual members. We used BLAST programme and Clustalw for alignment. Later phylogenetic tree was constructed based on the similar regions.

KEYWORDS: *Staphylococcus aureus*, Phylogenetic analysis, 16s RNA sequence, Methicillin resistance.

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1.INTRODUCTION

Over the past few decades, microbes have responded to the selective pressure of antimicrobial drugs by evolving resistance to the commonly used antibiotics. Some of the microbes have developed multi drug resistance. Methicillin resistant *Staphylococcus aureus* (MRSA) is now a pandemic pathogen for hospital and community acquired infections [1, 2]. Increased incidence of resistance among microbes and decreased rate of discovery of antimicrobial agents has resulted in a serious situation which may ultimately lead to a medical disaster. The situation demands new strategies and new avenues for the discovery of antimicrobial compounds against such resistant microbes. β-lactam antibiotics such as Methicillin inhibit bacterial cell wall synthesis by inhibiting the Penicillin binding proteins (PBPs) 1, 2, and 3 in the bacterial cell wall [3]. The Methicillin resistant

Staphylococcus aureus (MRSA) however acquired a new gene called MecA, which encodes for an additional PBP called PBP2a with a low affinicity for β- lactam as compared to PBP1, 2, and 3 and therefore does not interfere with the cell wall synthesis of microbes, thus conferring resistance to these organisms against Methicillin and other β- lactam antibiotics [4]. Since the difference between MRSA and Methicillin Sensitive *Staphylococcus aureus* (MSSA) is due to presence of protein PBP2a, research is directed towards finding inhibitors of proteins PBP2a from various sources. A phylogenetic tree is described as, a branching diagram that shows, for each species, with which other species it shares its most recent common ancestor. The evolutionary tree or cladograms were traditionally used to draw evolutionary relationship among the organism; a more modern version of the same is phylogenetic tree which uses gene / protein sequences to draw the evolutionary relationship. These trees dictate the relationship among the organisms based on the similarity and dissimilarity among the nucleotide or nucleic acid sequences [5-8]. The tree construction can be done through variety of tree-building methods which include methods based on distances, likelihood and characters. After a phylogenetic tree is constructed, it is important to test its accuracy which refers to the degree to which a tree is close to the true tree. Phylogenetics is the study of evolutionary relationships among organisms or genes. Below, we will refer to the objects whose phylogeny we are studying as organisms or species, but the discussion of methods is valid for the phylogeny of genes as well. We construct phylogenetic trees to illustrate the evolutionary relationships among a group of organisms [9-11]. The purpose of phylogenetic studies is to reconstruct evolutionary ties between organisms and to estimate the time of divergence between organisms since they last shared a common ancestor [12-16]. There are several types of data that can be used to build phylogenetic trees: Traditionally, phylogenetic trees were built from morphological features (e.g., beak shapes, presence of feathers, number of legs, etc). Today, we use mostly molecular data like DNA sequences and protein sequences.

2. MATERIALS AND METHODS

The initial model of 16s RNA was built by using homology-modeling methods and the MODELLER software. The sequence of 16s RNA was obtained from Uniprot. The query sequence from *Staphylococcus aureus* was submitted to domain fishing server for 16s RNA domain prediction. The predicted domain *was* searched to find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) program against PDB (Protein Data bank). Sequence that showed maximum identity with high score and less e-value was aligned and was used as a reference structure to build a 3D model for 16s RNA. The co-ordinates for the structurally conserved regions (SCRs) for 16s RNA were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm. Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved. The final structure obtained was analyzed by

Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) and environment profile using ERRAT graph (Structure Evaluation server). This model was used for the identification of active site and for docking of the substrate with the enzyme [17-21].

Ramchandran Plot Analysis

Ramachandran plot (also known as a Ramachandran map or a Ramachandran diagram), developed by Gopalasamudram Narayana Ramachandran, is a way to visualize dihedral angles φ against ψ of amino acid residues in protein structure. It shows the possible conformations of φ and ψ angles for a polypeptide. The domain of this function is the torus. Hence, the conventional Ramachandran plot is a projection of the torus on the plane, resulting in a distorted view and the presence of discontinuities. One would expect that larger side chains would result in more restrictions and consequently a smaller allowable region in the Ramachandran plot. In practice this does not appear the case; only the methylene group at the β position has an influence. Glycine has a hydrogen atom, with a smaller van der Waals radius, instead of a methyl group at the β position. Hence it is least restricted and this is apparent in the Ramachandran plot for Glycine for which the allowable area is considerably larger. In contrast, the Ramachandran plot for proline shows only a very limited number of possible combinations of ψ and φ.

Molecular Dynamics

Molecular dynamics (**MD**) is a form of computer simulation in which atoms and molecules are allowed to interact for a period of time by approximations of known physics, giving a view of the motion of the particles. This kind of simulation is frequently used in the study of proteins and biomolecules, as well as in materials science. It is tempting, though not entirely accurate, to describe the technique as a "virtual microscope" with high temporal and spatial resolution. Whereas it is possible to take "still snapshots" of crystal structures and probe features of the motion of molecules through NMR, no experiment allows access to all the time scales of motion with atomic resolution. Richard Feynman once said that "If we were to name the most powerful assumption of all, which leads one on and on in an attempt to understand life, it is that all things are made of atoms, and that everything that living things do can be understood in terms of the jigglings and wigglings of atoms." Molecular dynamics lets scientists peer into the motion of individual atoms in a way which is not possible in laboratory experiments. Molecular dynamics is a specialized discipline of molecular modeling and computer simulation based on statistical mechanics; the main justification of the MD method is that statistical ensemble averages are equal to time averages of the system, known as the ergodic hypothesis. MD has also been termed "statistical mechanics by numbers" and "Laplace's vision of Newtonian mechanics" of predicting the future by animating nature's forces and allowing insight into molecular motion on an atomic scale. However, long MD simulations are mathematically ill-conditioned, generating cumulative errors in numerical integration that can be

minimized with proper selection of algorithms and parameters, but not eliminated entirely. Furthermore, current potential functions are, in many cases, not sufficiently accurate to reproduce the dynamics of molecular systems, so the much more computationally demanding Ab Initio Molecular Dynamics method must be used. Nevertheless, molecular dynamics techniques allow detailed time and space resolution into representative behavior in phase space for carefully selected systems [22-28]. Before it became possible to simulate molecular dynamics with computers, some undertook the hard work of trying it with physical models such as macroscopic spheres. The idea was to arrange them to replicate the properties of a liquid. J.D. Bernal said, in 1962: "... I took a number of rubber balls and stuck them together with rods of a selection of different lengths ranging from 2.75 to 4 inches. I tried to do this in the first place as casually as possible, working in my own office, being interrupted every five minutes or so and not remembering what I had done before the interruption." Fortunately, now computers keep track of bonds during a simulation. Because molecular systems generally consist of a vast number of particles, it is in general impossible to find the properties of such complex systems analytically. When the number of particles interacting is higher than two, the result is chaotic motion (see n-body problem). MD simulation circumvents the analytical intractability by using numerical methods. It represents an interface between laboratory experiments and theory, and can be understood as a "virtual experiment". MD probes the relationship between molecular structure, movement and function. Molecular dynamics is a multidisciplinary method. Its laws and theories stem from mathematics, physics, and chemistry, and it employs algorithms from computer science and information theory. It was originally conceived within theoretical physics in the late 1950s and early 1960s, but is applied today mostly in materials science and the modeling of biomolecules [29-33].

Energy minimization

Deviations in the protein structure geometry, which have been introduced by the modeling algorithm when joining rigid fragments are regularized in the last modeling step by steepest descent energy minimization using the GROMOS96 force field. Empirical force fields are useful to detect parts of the model with conformational errors. In our own experience and the work of others, energy minimization or molecular dynamics methods are in general not able to improve the accuracy of the models, and are used in SWISS-MODEL only to regularize the structure. However, the successful application of restricted molecular dynamics for improving homology models has recently been reported for a few test cases. To derive more general rules of engagement of molecular dynamics, further systematic experiments have to be conducted.

The four modeling steps:

1. Template superposition,
2. Target-template alignment,
3. Model building

4. Energy minimization have to be implemented in the program ProModII in ANCI.

Phylogenetics studies

Reference proteins of well-established molecular function, representing each of the 16s RNA families investigated, were chosen as query sequences for searches in the *Staphylococcus aureus* genome databases. Searches were made using the TBLASTN tool against GenBank database non-redundant (NR), with search specifications. The other databases used were SWISSPROT and Universal Protein resource Uniprot (http://www.ebi.uniprot.org/uniprot-srv/protein/uniProtView). The BLAST server used was that of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov/BLAST/). As selection criteria of BLAST hits for genomic sequences, a cut off e-value of e- 10 was previously set. The genomic sequences found were used to predict putative genes contained within them. Whenever possible, genes were predicted on the basis of sequences generated by the *Staphylococcus aureus* Genome database, since these sequences present a higher degree of accuracy. To that end, a mixed procedure was adopted combining ab initio gene prediction algorithms of genomic sequence alignments with similar sequences from expressed genes (ESTs and cDNAs). The prediction algorithms were GenScan (Burge and Karlin, 1997; http:// genes.mit.edu/ GENSCAN.html), GenomeScan; http://genes.mit.edu/ genomescan.html), FGENESH [31]; http://www.softberry. - Com/berry.phtml? topic=gfind), GeneMark.hmm (Borodovsky and Lukashin, unpublished; http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi) and GrailEXP; http://compbio. ornl.gov/grailexp/). Such expressed sequences were found by BLAST searches against EST and NR databases of GenBank, using the genomic sequence as query. The algorithm of choice for the multiple alignments of protein sequences was ClustalX1.8, available through the BCM Search Launcher server (http://searchlauncher.bcm.tmc.edu/multi-align/multialign. html). The multiple alignments were edited with the help of GENEDOC (Free Software Foundation Inc.). All the genes with greater than 30% identity, with at least one of the reference proteins used in the searches, were regarded as functionally similar (homologous) to the reference proteins, receiving the same name. Those sequences that did not conform to this criterion were discarded. Prediction of homology and signature sequences for the putative transporter proteins were carried out with PROSITE (http://www.ebi.ac.uk/InterProScan/) and Pfam databases. Sequences were included into families based on homology and presence of signature sequences. Protein alignments obtained with ClustalX 1.8 were used as starting points for phylogenetic analysis. Unrooted trees were prepared by the neighbor-joining method using either Clustal, PHYLIP, or and 1000 bootstrap replicates were performed. Bold lines on trees indicate protein sequences that were confirmed by cDNA sequencing. In this work, we aimed to reveal phylogenetic distances across the species using experimental values, rather than sequence information in the graphs. Hence we used the data of 16sRNA experimental values. In the relation network, enzymes and genes are represented as nodes, while the substrate and product compounds are represented as edges. The

related structural information from the graphs was used for computing phylogenetic distances.

3. RESULTS AND DISCUSSION

Homology Modeling of Ribosomal RNA small subunit methyltransferase A

The amino acid sequence of Ribosomal RNA small subunit methyltransferase A was obtained from the protein sequence databank in the Uniprot_KB

QUERY SEQUENCE OF Ribosomal RNA Small Subunit Methyltransferase A

>sp|Q932G1|RSMA_STAAM Ribosomal RNA small subunit methyltransferase A OS=*Staphylococcus aureus* (strain Mu50 / ATCC 700699) GN=rsmA PE=3 SV=3

MLDNKDIATPSRTRALLDKYGFNFKKSLGQNFLIDVNIINNIIDASDIDAQTGVIEIGPG
MGSLTEQLARHAKRVLAFEIDQRLIPVLNDTLSPYDNVTVINEDILKANIKEAVENHLQD
CEKIMVVANLPYYITTPILLNLMQQDIPIDGYVVMMQKEVGERLNAEVGSKAYGSLSIVV
QYYTETSKVLTVPKSVFMPPPNVDSIVVKLMQRTEPLVTVDNEEAFFKLAKAAFAQRRKT
INNNYQNYFKDGKQHKEVILQWLEQAGIDPRRRGETLSIQDFAKLYEEKKKFPQLEN

Template selection is a process of identifying a suitable protein which shares nearly the same structure of the query protein which doesn't possess the 3D structure. Template selection is very important in comparative protein modeling. Templates can be chosen by various tools such as BLAST, FASTA, Swiss-model, etc. In the case of Blast and Fasta the sequence of protein in fasta format can be uploaded and the templates can be manually selected by considering the score value and the E value. In the case of Swiss-Model server, it automatically chooses the template and models the protein structure.

BLAST Search

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one-reference protein 1CKJ has a high level of sequence identity and the identity of the reference protein with the **Ribosomal RNA Small Subunit Methyltransferase A** domain are 70% (Fig 1).

Sequences producing significant alignments:		Score (Bits)	E Value
pdb|1CKJ|A	Chain A, Casein Kinase I Delta Truncation Mutant C...	168	2e-42
pdb|2C47|A	Chain A, Structure Of Casein Kinase 1 Gamma 2 >pdb...	154	4e-38
pdb|2CMW|A	Chain A, Structure Of Human Casein Kinase 1 Gamma-...	147	5e-36
pdb|2CHL|A	Chain A, Structure Of Casein Kinase 1 Gamma 3	144	3e-35
pdb|2IZR|A	Chain A, Structure Of Casein Kinase Gamma 3 In Com...	144	4e-35
pdb|2CSN|A	Chain A, Binary Complex Of Casein Kinase-1 With Cki7	134	3e-32
pdb|1EH4|A	Chain A, Binary Complex Of Casein Kinase-1 From S....	134	3e-32
pdb|2V62|A	Chain A, Structure Of Vaccinia-Related Kinase 2 >p...	99.4	1e-21

Fig 1: BLAST result

Sequence Alignment

In the following study, we have chosen 1CKJ A as a reference structure for modeling **Ribosomal RNA Small Subunit Methyltransferase A**. Coordinates from the reference protein (1CKJ) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. Sequence of the reference structures were extracted from the respective structure files and aligned with the target sequence using the default parameters in ClustalW (Fig 2).

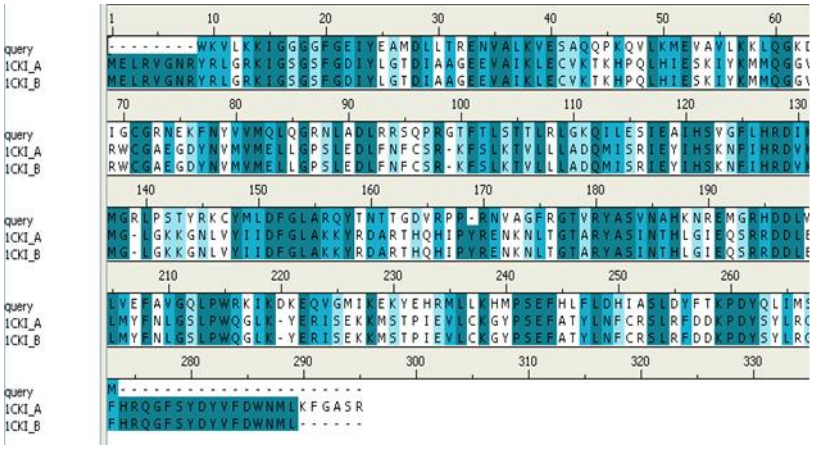


Figure 2: Aligning the query sequence with template

Three-Dimensional Structure Of TAU-Protein Kinase: MOLSOFT

The 1CKJ structure were used as the templates for building the 3D model of the **Ribosomal RNA Small Subunit Methyltransferase A** using Swissmodel (Fig 3, 4).

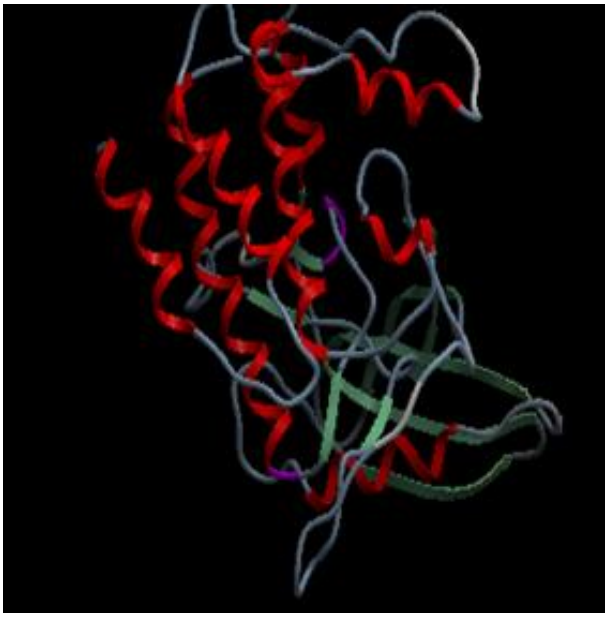


Figure 3: The final stable structure of the **Ribosomal RNA Small Subunit Methyltransferase A** protein

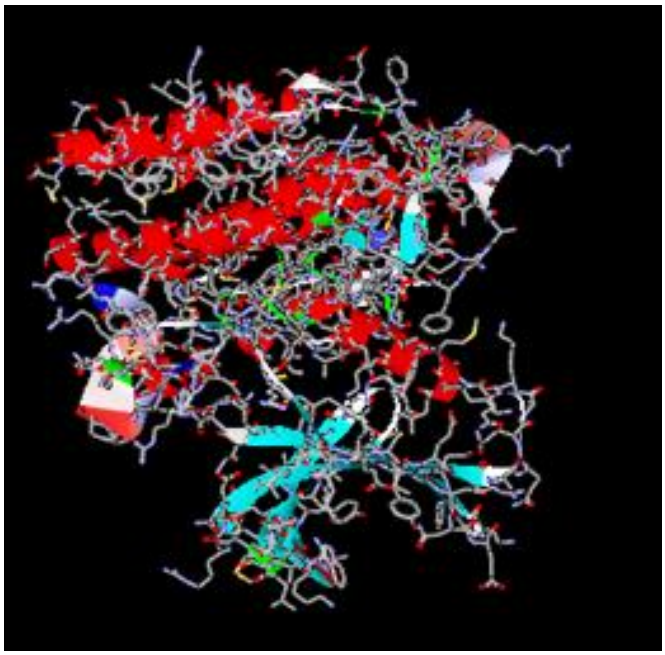


Figure 4: Secondary structure visualization of **Ribosomal RNA Small Subunit Methyltransferase A**

Superimposition Query structure with templates

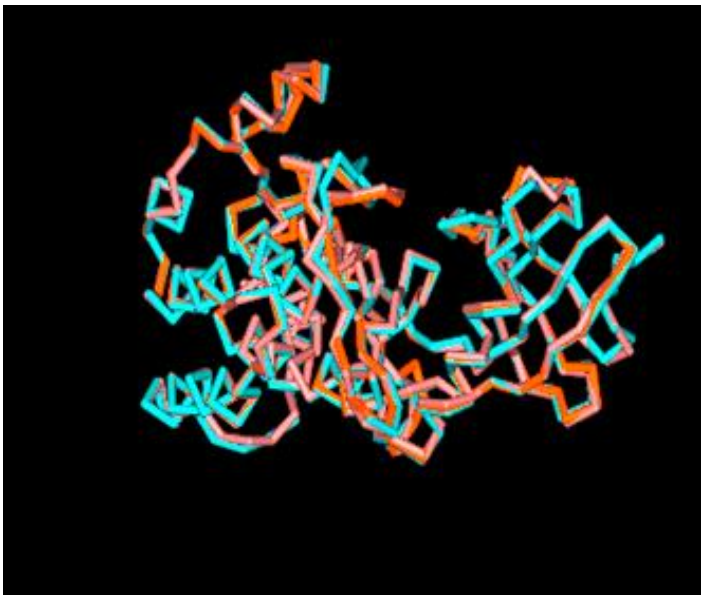
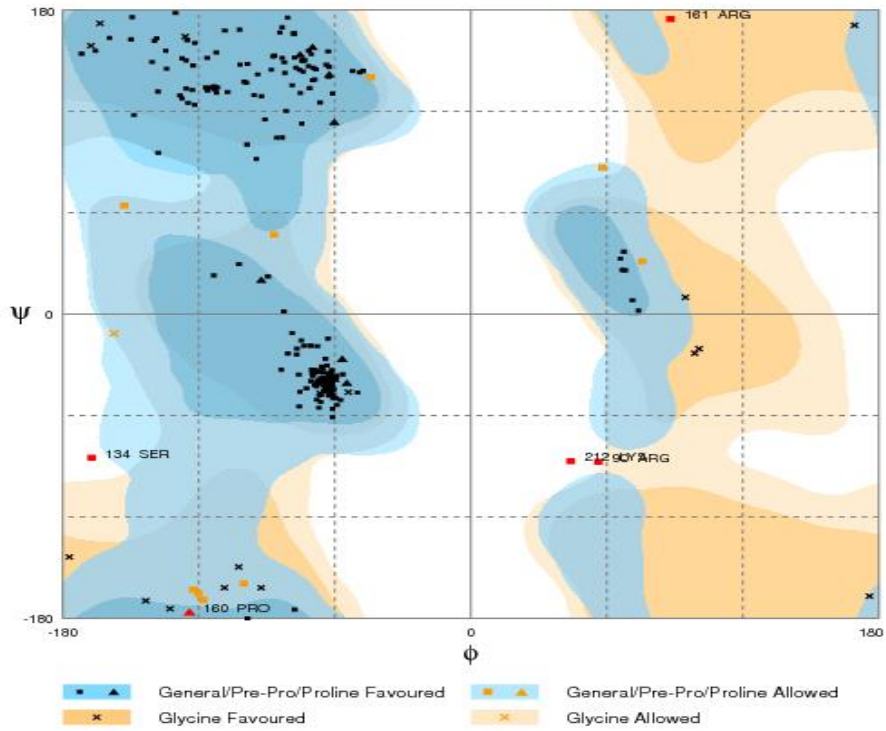


Figure 5: Superimposition of **Ribosomal RNA Small Subunit Methyltransferase A** structure with Template, the RMS deviation is 0.75.

Model Verification by Ramachandran Plot analysis



Number of residues in favoured region	(~98.0% expected)	:	247 (94.3%)
Number of residues in allowed region	(~2.0% expected)	:	10 (3.8%)
Number of residues in outlier region		:	5 (1.9%)

Figure 6: Ramachandran plot analysis

Molecular Dynamics Simulation Studies by VMD/NAMD

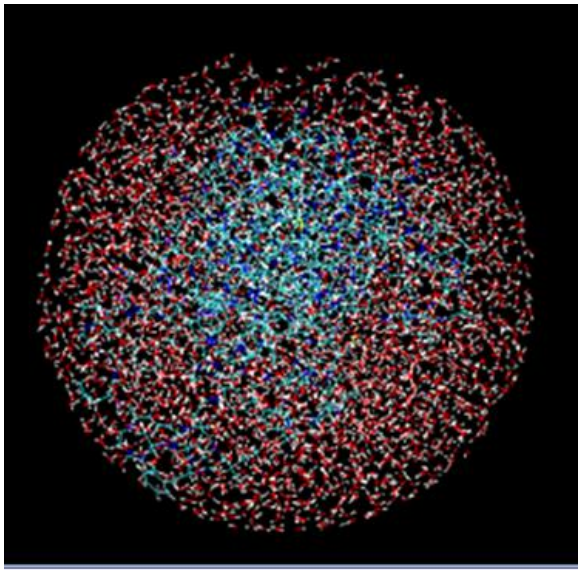


Figure 7: Structure having least energy with low Rmsd which was obtained by NAMD in water molecule (TIP)

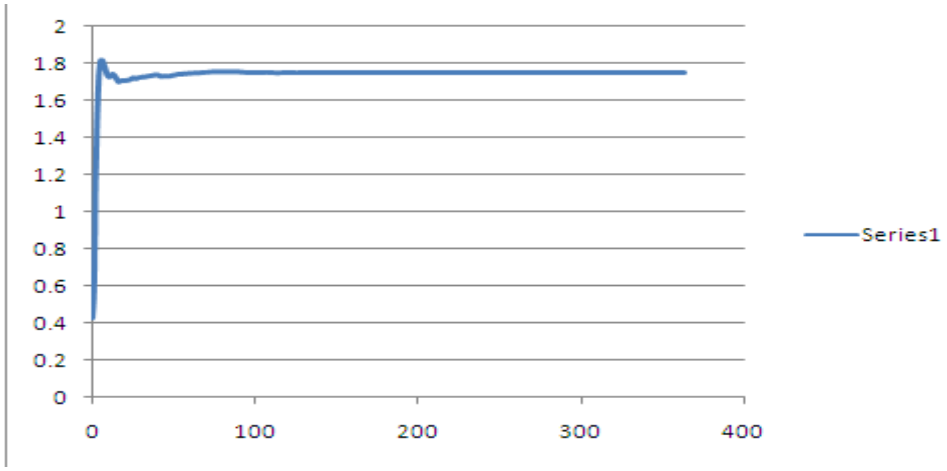


Figure 8: Graphical Representation of RMSD value of **Ribosomal RNA Small Subunit Methyltransferase A**

Collection of 16sRNA sequence from data base

Using NCBI and UNIPROT, we have collected 16sRNA sequences from *Staphylococcus aureus* different strains for phylogenetic analysis using the algorithm and treeview softwares**.** The developed algorithm was used to align the different 16sRNA sequences, and the aligned sequences were converted into phylogenetic tree. With the availability of the data in GenBank, it was possible to construct an overview of 16s RNA in *Staphylococcus aureus*. As a starting point, the protein families in 16s RNA which have positive molecular implications on pain transport, intracellular targeting and storage in *Staphylococcus aureus* strains, were chosen for analysis. Taking specific members of these families as query sequences, searches were carried out for orthologous sequences in GenBank, and Uniprot current databases using TBLASTN. After searching the databanks with TBLASTN sequences, clones having genomic sequences to the related family were taken and converted to amino acid sequences. In each family, similar sequences were removed and the sequences were subjected to PROSITE and Pfam databases to see the presence of signature sequences for the corresponding families. After subjecting the sequences to PROSITE 13 putative genes were predicted in 16s RNA of different strains in Staphyloccus aureus. The percent identity for all the sequences was calculated in each family with the corresponding query sequence using GENEDOC. Phylogenetic analysis of the sequences of transporters revealed that the COX proteins were divergent, showing branches in tree view. The phylogentic analysis shows four branches indicating different transporting function to each family. Some of the orthologous sequences are available as full-length cDNA clones. The expressed sequence tags were mentioned as accession numbers for the sequences (Fig 9).

Sequence Alignment

These sequences were aligned using CLUSTALW program. The multiple alignment was used to develop phylogenetic tree.

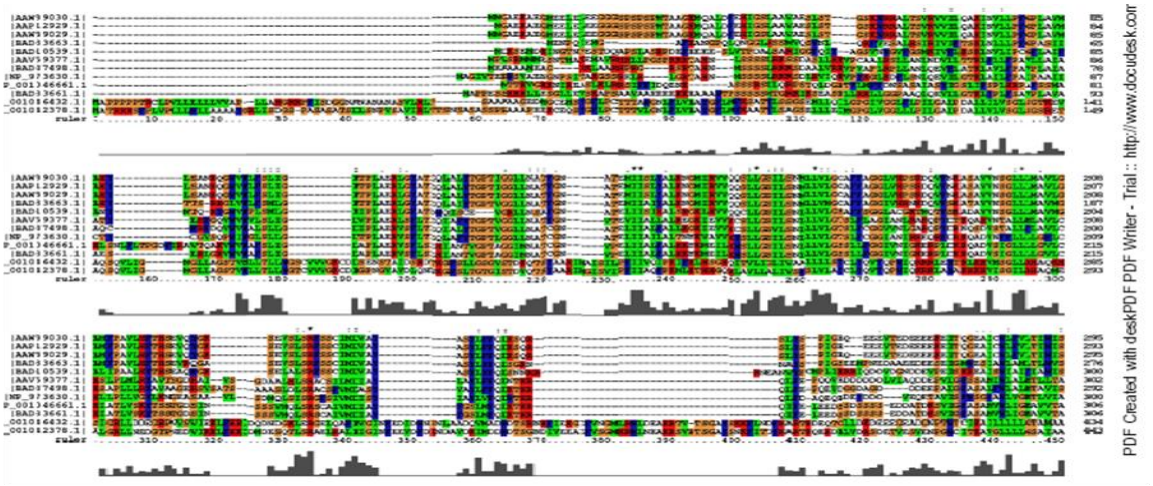


Figure 9: Multiple sequence alignment of 16s RNA of different strains of *Staphylococcus aureus*

16s RNA family is an important family and these are numbered based on their alignment. The genes, which are showing more than 30% identity with query, are said to be homologous sequences (Fig 10, 11).

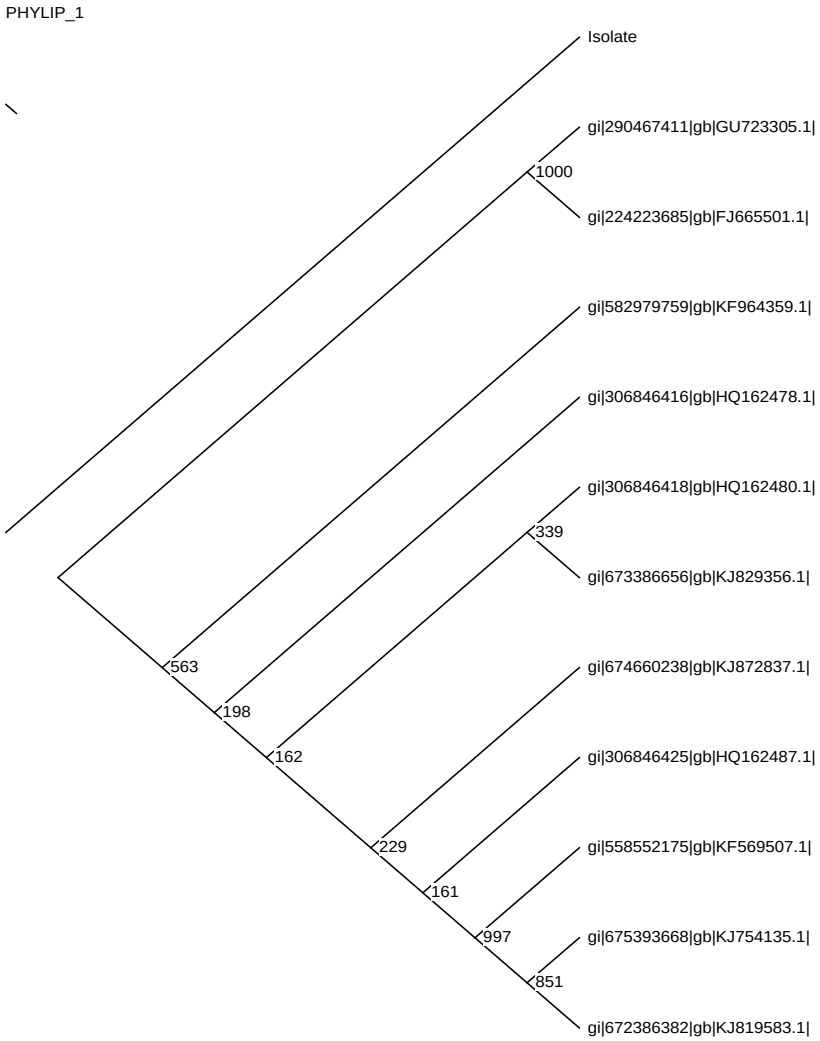


Figure 10: Unrooted Phylogenetic tree of different *staphylococcus aureus* strains

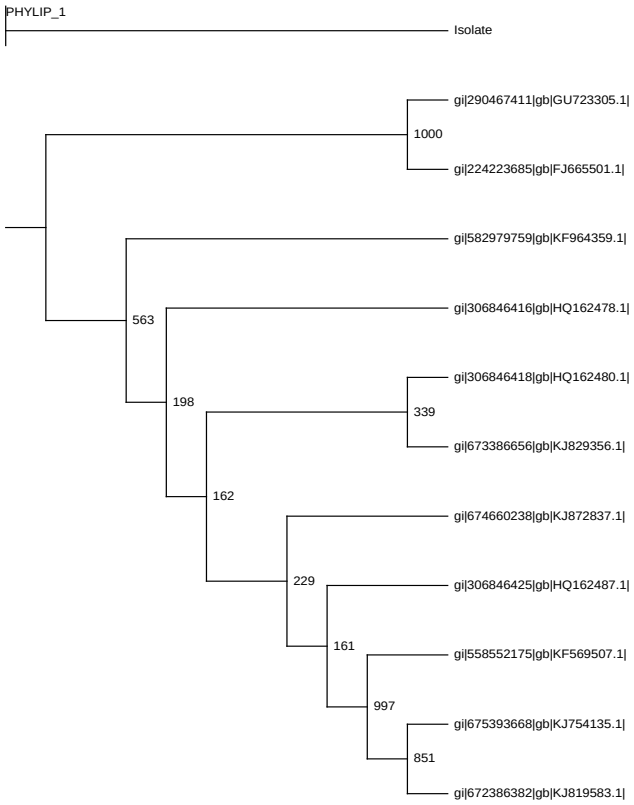


Figure 11: Rooted Phylogenetic Tree with branch length (UPGMA)

4. CONCLUSION

In this work, we have analyzed the phylogenetic relationships of 16s RNA sequences of different *Staphylococcus aureus* strains. This analysis has focused on 16s RNA families for which initial characterizations have been achieved for individual members. Phylogenetic trees of each family define the evolutionary relationships of the members to each other. These families contain numerous members, indicating diverse functions in vivo. Closely related isoforms and separate subfamilies exist within many of these gene families, indicating possible redundancies and specialized functions. To facilitate their further study, which includes alignment of the analyzed genes.

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CONFLICT OF INTEREST

Authors declare that they don't have any conflict of interest

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