

Identification of potential targets in *Staphylococcus aureus* N315 using computer aided protein data analysis

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Abstract:

Staphylococcus aureus is a gram positive bacterium, responsible for both community-acquired and hospital-acquired infection, resulting in a mortality rate of 39%. 43.2% resistance to methicillin and emerging resistance to Fluroquinolone and Oxazolidinone, have evoked the necessity of the establishment of alternative and effective therapeutic approach to treat this bacteria. In this computational study, various database and online software are used to determine some specific targets of *Staphylococcus aureus* N315 other than those used by Penicillin, Quinolone and Oxazolidinone. For this purpose, among 302 essential proteins, 101 non-homologous proteins were accrued and 64 proteins which are unique in several metabolic pathways of *S. aureus* were isolated by using metabolic pathway analysis tools. Furthermore, 7 essentially unique enzymes involved in exclusive metabolic pathways were revealed by this research, which can be potential drug target. Along with these important enzymes, 15 non-homologous proteins located on membrane were identified, which can play a vital role as potential therapeutic targets for the future researchers.

Keywords: *Staphylococcus aureus*, Essential proteins, Surface, Therapeutic targets.

Background:

Staphylococcus aureus is a vanguard for both nosocomial and community-acquired infections. It is the primary cause of surgical site infections and lower respiratory tract infections [1] and the second leading cause of cardiovascular infections, pneumonia [1]. Because of evolved resistance to antimicrobial drugs such as penicillin and newer narrow-spectrum β -lactamase-resistant penicillin antimicrobial drugs (e.g., methicillin, oxacillin) infections with *S. aureus* are especially difficult to treat and this resistance appeared soon after they were introduced into clinical practice respectively in the 1940s and 1960s [2]. Initially resistance to penicillin was restrained to a small number of hospitalized patients, but as use of penicillin increased, resistance spread first to other hospitals and then into the community [3]. Greater than 80% of community- and hospital-acquired *S. aureus* isolated were resistant to penicillin by the late 1960s [2]. Current report suggests that the spread

and evolution of methicillin-resistant *S. aureus* (MRSA) seems to be following an identical wavelike emergence pattern [3]. In many US hospitals, MRSA is now endemic and even epidemic along with long-term care facilities and communities [4]. National Nosocomial Infections Surveillance system's data suggest that the proportion of *S. aureus* isolates that are resistant to methicillin has increased to 59.5%-64.4%, in intensive care units [5]. Accurate national estimates of incidence are needed for an understanding of the magnitude of the problem. Nevertheless, national studies examining the effect of *S. aureus* or MRSA on the healthcare system are greater than 5 years old [6]. Noskin *et al.* estimated that there were 290,000 *S. aureus* - related whereas Kuehnert *et al.* estimated a similar number of *S. aureus*-related hospitalizations for 1999-2000 and reported that 125,969 (43.2%) were likely resistant to methicillin [6]. The infections caused by this pathogen range from mild infections such as skin infections, food poisoning to life threatening

infections such as pneumonia, sepsis, osteomyelitis and infectious endocarditis. Methicillin-resistant *Staphylococcus aureus* is considered as superbug which was first reported in 1961 and now-a-days cause mortality rate of 39% while MSSA cause 24% death [7].

The currently known target of *Staphylococcus* sp. includes PBP (penicillin binding protein) of peptidoglycan biosynthesis pathway. Previously, beta-lactam antibiotics were known to be effective against them, but due to production of altered form of PBP protein as well as their beta-lactamase enzyme synthesis those drugs are not effective now. Hence, our current study involves identifying targets apart from PBP [8]. Another antibiotic prescribed is Fluroquinolone. Fluroquinolone target DNA Gyrase A enzyme-essential for the replication, supercoiling of DNA. But according to Stephen et al., a highly significant association between Levofloxacin and Ciprofloxacin treatment and subsequent isolation of MRSA is reported [9]. Linzolid, a new class of antibiotic called Oxazolidinones is used to treat MRSA, which involves the mechanism of binding to the bacterial 23s ribosomal RNA of the 50s subunit and thus inhibiting the formation of Functional 70s initiation complex. But between April 13 & June 26, 2008, 12 patients were identified with LRSA (Linzolid resistant *Staphylococcus aureus*). All the isolates were detected with a point mutation in 23s rRNA. It was concluded that clinical outbreak of LRSA mediated by the *cfr* gene was related with extensive usage of Linzolid [10]. In this study, we have tried to search for some potential therapeutic targets other than the targets discussed above. We have implemented an approach considering two important criteria. First of all, the identified target should be essential to that pathogen in the sense that it must be associated with replication as well as viability of the pathogen. Secondly, the target should not be homologous to human. The non-homolog property of these proteins helps to establish highly selective drug against the pathogen preventing the possibility of the cross-reaction with the human host. This may help to minimize the side effects of the proposed drug [11]. By this approach, we have found some targets which are not only essential & non-human homolog but also bypass the resistant mechanism of existing targets and some targets are involved in the metabolic pathway of pathogen which is not exploited as potential target area [12]. We have also identified the membrane-bound Essential, non-human homolog proteins emphasizing the fact that 60% of the drug target is membrane-bound [13].

Methodology:

The systematic identification & characterization of the putative drug target of *Staphylococcus aureus* N315 was done sequentially by the following methods:

Retrieval of Essential proteins of *S. aureus*

At first, according to the Database of essential genes (DEG) [14], 302 essential proteins of *S. aureus* N315 were retrieved from NCBI [15] in FASTA format.

Identification of non-human homologous essential proteins in *S. aureus*

These 302 essential proteins were subjected to BlastP at NCBI server against *Homo sapiens* with default parameters. To identify human non-homologous essential proteins of *S. aureus*,

proteins having threshold expectation value greater than 10(-4) were taken and for this purpose Refseq protein database was selected [16].

Metabolic pathway analysis

The human non-homologous essential proteins of *Staphylococcus aureus* obtained through BlastP were then subjected to metabolic pathway analysis, which was done by KAAS server at KEGG. Functional annotation of genes is provided by KAAS (KEGG Automatic Annotation Server) [17]. This functional annotation was done by this server through BLAST comparison of the genes against the manually curated KEGG GENES database.

Unique pathway identification

After this, unique metabolic pathways of *Staphylococcus aureus* N315 were identified through the comparison of metabolic pathways of both *Staphylococcus aureus* & *Homo sapiens* by using KEGG (Kyoto Encyclopedia of Genes & Genomes) [18] Genome Database. Among unique metabolic pathways of *S. aureus*, only those proteins were identified which were human non-homologue essential proteins.

Surface protein identification

Sub-cellular localization of metabolic proteins (essential non-human homologues) of *S. aureus* was done by PSORTb [19] to identify the surface membrane proteins which can be used as putative therapeutic targets.

Uncharacterized membrane-bound protein characterization

Uncharacterized membrane-bound Hypothetical Proteins were characterized by SVM-PROT [20].

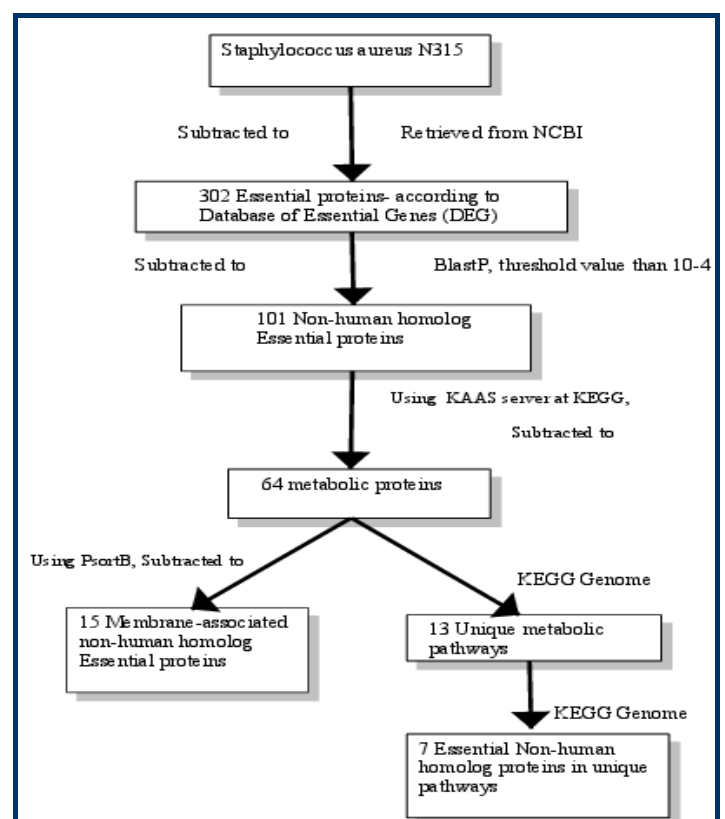


Figure 1: Flowchart- A schematic representation of process analysis and interpretations.

Result & Discussion:

The aim of this investigation was to determine the potential therapeutic targets for alternative treatment of MRSA and to categorize and analyze the targets. Subtractive genomic approach was used in this approach. The basic principle of subtractive genomic approach is, "a good therapeutic target is a gene/protein essential for the bacterial survival, which cannot be found in host" [21]. In the present study, at first essential proteins of *S. aureus* N315 were retrieved from 'Database of Essential Gene (DEG)'. Essential genes can be defined as a minimal gene set which is adequate for survival of a cellular life form under favorable condition [22]. The results obtained by this approach are shown in (Figure 1).

With a purpose to forsake any cross-reactivity of established drug with the human host, BlastP was done against the 302 essential proteins then 101 non-human homolog proteins were resulted having a threshold expectation value greater than 10^4 . For further specification of the non-human homolog Essential proteins of *S. aureus*, metabolic pathway analysis was done by KAAS server at KEGG, which resulted in a set of 64 proteins Table 1 (see supplementary material). With a view to further reduce the number of 64 metabolic proteins as probable drug targets, comparative analysis of metabolic pathways of human host & *S. aureus* at KEGG Genome database was done. This comparison reveals 13 unique pathways that mean these metabolic pathways are present in *S. aureus* (bacteria), but not in human. Then, each selected unique pathway was tested for the presence of pathogen specific essential proteins. At last, 7 proteins were found to be present on two different unique pathways. Six out of the seven proteins are present in peptidoglycan biosynthesis pathway and one protein-Phosphoenolpyruvate-protein phosphatase is present in Phosphotransferase system (PTS). For further characterization, Computational characterization of hypothetical proteins and prediction of subcellular localization of pathogenic proteins was done which provides a quicker way to identify cell surface drug targets Table 2 (see supplementary material) [22].

Potential Drug targets from unique metabolic pathways of *S. aureus*

In this section we analyze some potential therapeutic targets identified from unique metabolic pathways. The Penicillin Binding Proteins (PBPs), involved in the final step of Peptidoglycan Biosynthesis pathway was the one of the first conventional targets of Beta-lactam antibiotics- Penicillins & Cephalosporins. The beta-lactam ring of these drugs has the ability to bind to transpeptidase (TPase) of PBPs and thus prevent normal cross-linking of peptide chains in the peptidoglycan layer. Unfortunately, expression of beta-lactamase and an altered form of PBP2 has lead to Methicillin resistant *Staphylococcus aureus*. Thus one of the approaches of establishing new anti-MRSA agents should be to target on molecule other than PBPs to modulate the methicillin resistance in *S. aureus* [8].

Our study finds six essential non-human homolog proteins in the peptidoglycan pathway-all excluding the PBP, they are: UDP-N-acetylglucosamine 1-carboxyvinyltransferase(murA), UDP-N-acetylmuramate- L-alanine ligase (murC), UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase (murD), UDP-N-acetylmuramoylalanyl-D-D-glutamyl-2,6-

diaminopimelate-D-alanyl-D-alanyl ligase (murF), protein-D-alanyl-alanine synthetase A (ddl), Undecaprenyl pyrophosphate phosphatase (uppP). Among these six proteins four are from Mur ligase family catalyzing the synthesis of Peptidoglycan precursor (UDP-MurNAc-pentapeptide) - the first step in the Peptidoglycan synthesis, thus fulfilling the prerequisite of being targets other than PBP.

One potential drawback of murA target is that the presence of two different genes-murA1 and murA2- encoding proteins with similar enzymatic activity. Both the genes should be mutated in order to cause bacterial death, but as both murA1 and murA2 has only 60% sequence similarity, the encoded enzymes has different active sites for which a unique murA-specific antibiotic development is difficult. Another potential drug target can be D-alanyl-alanine synthetase A (ddl), which catalyzes the synthesis of D-alanyl:D-alanine dipeptide-a prime building block in peptidoglycan biosynthesis and any disruption in this step would lead to a tender cell wall resulting in cell-death. Another essential protein is Undecaprenyl pyrophosphate phosphatase encoded by uppP. Bacteriocin and drugs of similar mechanism will not be suitable for targeting the *S. aureus* N315, as uppP is responsible for coding protein conferring resistance to bacteriocin [23].

Potential Drug targets from common metabolic pathways

In this study, we have found essential metabolic non-human homolog proteins in energy metabolism pathways. *In vitro* and *In vivo* studies suggest that inhibitors designed for targeting these proteins can effectively hinder bacterial growth by bypassing the bacterial mutation site for drug resistance. The logic behind this is almost all existing antibiotics target only four pathways (cell wall synthesis, nucleic acid synthesis, protein synthesis, folate synthesis), thus repeated exposure to similar site increases the chance of mutation to the site, resulting in mutant bacteria [24, 25]. Targets of these pathways have shown their potential for anti-bacterial agents (e.g., Oligomycin, Antimycin A), but not used in case of human, due to harmful effect both to human & bacteria. But the proteins revealed by our study can be considered as non-human homolog, thus reducing the baneful effect of the drug to human host [26].

Membrane-bound protein

Our study revealed 15 membrane-bound proteins. Special attention was given to membrane proteins, as among all the drug targets- 60% is membrane proteins. Membrane proteins are also easier to study by computational method rather than experimental methods due to their uniform structure & interaction. The high tendency of proteins to construct secondary structure in turn lowers the complexity of the prediction of protein structure by computational method. If a high resolution structure is not found, computer-based structure prediction can be done with membrane protein easily & then structure-based drug prediction can done [13].

Conclusion:

Our study working in several points of *Staphylococcus aureus* N315 genome reveals essential non-homolog proteins, 64 essential non-homolog metabolic proteins- among which the proteins of energy metabolism and 7 metabolic proteins in unique pathways, 15 essential non-homolog metabolic membrane-bound proteins- can be potential drug target. The

drug would be specific for the pathogen and not lethal to the host as subtractive genomic approach is applied in this case. Molecular modeling of the targets will help to discover the best possible active sites that can be targeted for drug design. Virtual screening against these novel targets might be useful in the discovery of potential therapeutic agents against *Staphylococcus aureus*.

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Supplementary material:

Table 1: List of KO (KEGG Orthology) Numbers & genes of the Essential Non-human homolog metabolic proteins of *S. aureus* N315

Metabolic pathway	KO number (gene name)
Energy Metabolism	
(a) Carbonfixation pathways in prokaryotes	KO0925(ackA), KO0625(eutD)
(b) Methane metabolism	KO0925(ackA), KO1624(fbaA), KO0625(eutD)
(c) Oxidative phosphorylation	KO2109(TpF)
Carbohydrate metabolism	
(a) Pyruvate metabolism	KO0625(eutD), KO0925(ackA), K01962(accA)
(b) Propanoate metabolism	KO0925(ackA), KO065(eutD), K01962(accA)
(c) Pentose phosphate pathway	KO1839(drm), KO1624(fbaA)
(d) Fructose and Mannose metabolism	KO2768(fruA), KO2769(fruA), KO2770(fruA), KO1624(fbaA), KO2798(mtlA), KO2793, KO2794, KO2795, KO1809(pmi), KO0009(mtlD)
(e) Amino sugar and Nucleotide sugar metabolism	KO1809(pmi), KO0790(murA)
(f) Glycolysis (Gluconeogenesis)	KO1624(fbaA)
Lipid metabolism	
Glycerolipid metabolism	KO3621(plsX), KO3691(plsY)
Nucleotide metabolism	
(a) Purine metabolism	KO1428(ureC), KO1839(drm), KO3040(rpoA), K02335(polA)
Pyrimidine metabolism	KO3040(rpoA), K02335(polA)
Amino acid metabolism	
(a) Tryptophan metabolism	KO1696(trpB)
(b) Arginine and Proline metabolism	KO1428(ureC)
(c) Lysine biosynthesis	KO1929(murF)
Metabolism of other amino acids	
Taurine and Hypotaurine metabolism	KO0925(ackA), KO0625(eutD)
(b) D-glutamate and D-glutamine metabolism	KO1925(Mur D)
(c) D-Alanine metabolism	KO1921(ddl)
Glycan biosynthesis & metabolism	
Peptidoglycan Biosynthesis	KO1924(murC), KO1925(murD), KO6153(uppP), KO0790(murA), KO1921(ddl), KO1929(murF)
Metabolism of Terpenoids and Polyketides	
(a) Tetracycline biosynthesis	KO1962(aceA)
Membrane transport	
(a) Phosphotransferase system	KO8483(PTS I), KO2768(fruA), KO2769(fruA), KO2770(fruA), KO2763(ptsG), KO2764(ptsG), KO2765(ptsG), KO2798(mtlA), KO2793, KO2794, KO2795
(b) Bacterial secretion system	KO3070(secA), KO3076(secY), KO3070(azi, dix)
(c) ABC transporters	KO2073
Protein export	KO3070(secA), KO3076(secY)
Genetic information processing	
(a) Ribosome	KO2911(rpmF), KO2864(rplJ) KO2909(rpmE2), KO2939(rplI), KO2916(rpmI), KO2990(rpsF), KO2946(rpsJ,nusE), KO2892(rplW) KO2965(rpsS), KO2982(rpsC), KO2904(rpmC), KO2961(rpsQ), KO2994(rpsH), KO2933(rplF), KO2881(rplR), KO2907(rpmD), KO2876(rplO), KO2919(rpmJ), KO2968(rpsT), KO2970(rpsU)
(b) Nucleotide excision repair	KO3703(UvrC)
(c) DNA replication	KO2337(dnaE), KO2314(dnaC), KO2316(dnaG)
(d) Homologous recombination	KO2337(dnaE), KO3626(recF)
(e) Mismatch repair	KO2337(dnaE)
(f) RNA degradation	KO3628(Rho)
(g) RNA polymerase	KO3040(rpoA)

(h)Cell-cycle Caulobacter KO3590(ftsA), KO3531(ftsZ), KO2314(dnaC), KO2313(dnaA)
 Epithelial cell signaling in Helicobacter pylori infection KO1428(ureC)

Table 2: List of Essential non-human homolog membrane proteins in *S. aureus* N315 strain identified by PSORTb and Protein Families Of Membrane-bound Hypothetical Proteins identified by SVM-PROT

SN.	Accession No.	Name of Protein
1.	GI:15926360	UDP pyrophosphate phosphatase
2.	GI: 15926377	Fructose specific permease
3.	GI:15927459	Two-component sensor histidine kinase
4.	GI:15927681	F0F1 ATP synthase subunit B
5.	GI:15927652	potassium-transporting ATPase subunit A
6.	GI:15928117	PTS system, glucose-specific IIABC component
7.	GI:15927975	Respiratory nitrate reductase alpha chain
8.	GI:15927810	Preprotein translocase subunit SecY
SN.	Accession No	Protein family of Hypothetical proteins generated by svm-prot
9.	GI:15926499	Lipid-binding Protein
10.	GI:15926454	Transmembrane, Electrochemical potential-driven transporter
11.	GI:15926933	Transmembrane, Manganese-binding Protein
12.	GI:15928227	Transmembrane, Transferase
13.	GI:15927946	Electrochemical potential driven transporter
14.	GI:15927800	Transmembrane, Zinc-binding protein
15.	GI:15926141	Lipid Binding Protein