Potential Antibacterial Targets in Bacterial Central Metabolism

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***Abstract*—The emerging antibiotic resistant bacteria and their abilities for rapid evolution have pushed the need to explore alternative antibiotics less prone to drug resistance. In this study, we employed methicillin/multidrug-resistant *Staphylococcus aureus* (MRSA) as a model bacterial system to initiate novel antibiotic development. An *in silico* identification of drug targets in *MRSA* 252 strain and *MRSA* Mu50 strain respectively was described. The identified potential targets were classified according to their known or putative functions. We discovered that a class of essential non-human homologous, central metabolic enzymes falls into the scope of potential drug targets for two reasons: 1) the identified targets either do not have human counterparts or use alternative catalytic mechanisms. Based on major differences in active site structure and catalytic mechanism, an inhibitor of such a bacterial enzyme can be designed which will not inhibit its human cousin. 2) attacking bacterial energy-making machinery bypasses the usual drug resistance sites, paving the road to multi-faceted approaches to combat antibiotic resistance.**

Keywords-antibiotic resistance; Methicillin/multidrug-resistant Staphylococcus aureus; essential genes; drug targets; central metabolism.

I. INTRODUCTION

This paper is an extended version of the previously published conference paper [1]. The earlier paper detailed *in silico* identification of drug targets in MRSA 252 strain and MRSA Mu50 strain respectively and proposed that the development of a new class of antibiotics may be a potential solution to avoid bacterial antibiotic resistance.

One of the biggest medical breakthroughs of the twentieth century is the discovery of antibiotics [2], which was immediately followed by the unfortunate emergence of bacterial antibiotic resistance [3].The rapid rate of bacterial evolution to overcome the antibiotic action, the ability of a single pathogen strain to resist multiple drugs, as well as the stunning frequency of resistance occurring constitute a major challenge to the medical profession [3] and thus raised retrospective discussions of currently existing antibiotics [4-6]. Although there are hundreds of antibiotics on the market, it remains a fact that almost all existing antibiotics target only four cellular functions: protein synthesis, nucleic acid synthesis, cell walls synthesis or folate synthesis [6, 7, 8]. Bacterial resistance usually arises as the result of evolutionary adaptation of the target proteins that are subject to direct antibiotic attack [3]. Repetitively striking the same cellular sites leads to defensive bacterial gene mutation, which remains the primary cause of the prevalence of antibiotic-resistant bacteria [9], such as Methicillin/multidrug-resistant *Staphylococcus aureus* (MRSA) [10], Multidrug or Extensively Drug-Resistant Tuberculosis (MDR TB or XDR TB) [11,12], NDM-1 induced antibiotic -resistant *Escherichia coli* [13], *etc.* Hence, exploration of novel antibiotics with alternative modes of action is of great urgency [8]. The task falls on the shoulders of academia due to the fact that the pharmaceutical industry has ceased investing in antibiotic discovery owing to high cost, lengthening developing cycles, complexities and low profits along with failure of several recent investments in target-based approaches [14].

In this study, we employed MRSA as a model bacterial system because it is the most common bacterial pathogen isolated from humans [15, 16] with a significant morbidity and mortality [17]. The first MRSA case presented in the United Kingdom in 1961[18]. Shortly after, more variations were identified to be immune to β-lactam antibiotics (including penicillin, methicillin, oxacillin, and cephalosporins [19, 20]). Newly discovered MRSA strains have evolved to survive sulfa drugs, such as tetracyclines, and clindamycin [21]. Glycopeptide antibiotics, such as vancomycin and teicoplanin, considered drugs of "last resort", were used for the treatment of MRSA infections [22, 23]. However, recently discovered MRSA strains showed resistance even to vancomycin and teicoplanin [24, 25]. As of 2007, one variant found was resistant to six major kinds of antibiotics [26]. The beginning signs of MRSA infections are skin infections that resemble pimples, boils or spider bites. In immune-deficient patients, localized skin infections quickly spread through the bloodstream causing vital organ infections and possible death [27]. In a 2007 Centers for Disease Control and Prevention press release, there were about 94,000 cases of MRSA infections, contributing to around 19,000 deaths in the United States, which implies a mortality rate higher than that caused by HIV [28, 29]. The current treatment for MRSA infections is

still traditional broad-spectrum antibiotics such as lincosamides, sulfa drugs, glycopeptides [30-32], among which linezolids [33] daptomycin [34], Trimethoprim-sulfamethoxazole and MoxifloxacinHCl were considered relatively more effective [35, 36] though MRSA infections have become increasingly difficult to treat [31-33]. Thus, alternative treatments precisely targeting the root cause of MRSA infections needs to be established.

Novel antibiotic development starts with target screening [37]. In this paper, we reported the preliminary results of anti-MRSA drug development, *i.e.*, a systematic *in silico* approach for the identification of drug targets in two MRSA strains, MRSA 252 and MRSA Mu50 based on the following two criteria: essentiality to pathogen survival and absence from the human genome [38, 39]. A special list of enzymes targeting bacterial metabolism was identified, shedding light on a potentially new approach for antibiotic development.

II. METHODS

The objective of this study was to determine potential drug targets for alternative treatment of MRSA infections, to predict their enzymatic functions and to further shorten the list. We employed a reported *in silico* approach through a systematic and justified method [39, 40] for the identification of drug targets of MRSA infections following two criteria: essential to the survival MRSA and absent in the humans [38, 39].

MRSA genome National Center for Biotechnology Information (NCBI) gene bank contains at present complete genomic sequences of 13 MRSA strains. In this study, genomic sequences of MRSA 252 strain and MRSA Mu50 stain were studied respectively.

Sequence retrieval The genomic sequences of MRSA 252 and MRSA Mu50 were retrieved from the NCBI database respectively [41]. A total of 2656 genes from MRSA 252 strain and 2697 genes from MRSA Mu50 stain were purged at 90 % and 60% using CD-HIT [42] to remove paralogous or duplicate proteins.

Blasp against the database of essential genes (DEG) The resulting sequences were run through DEG [43**]** at an expectation (E-value) cutoff of 10^{-4}. The database of essential genes includes genes required for basic survival of *Staphylococcus aureus*, as well as more than 10 other bacteria, such as *E. coli*, *B. subtilis*, *H. pylori*, *S. pneumoniae*, *M. genitalium* and *H. influenzae,etc.*

Blasp against human genome The essential genes identified were subjected to BLASTP against the human genome (both refseq and nonrefseq) [44] to exclude any genes that have a significant match (E-value cutoff of 10^{-3} and lower) with human homologs. Genes having BLAST E-scores less than 10^{-3} were considered as having no close relatives in human.

Protein function assignment Information on the function of the identified proteins was derived from the annotated genome sequence through Integr8-Inquisitor [45] and/or EMBL/EBI/InterProScan [46].

Metabolic pathway study MRSA Metabolic pathways were obtained from KEGG database [47].

Amino Acid Alignment Analysis The interested protein sequences were submitted to SDSC Biology Workbench [48] for alignment in order to identify orthologs.

III. RESULTS AND DISCUSSION

The goal of this investigation was to determine potential drug targets for alternative treatment of MRSA infections and to classify and to analyze the identified targets. Out of the complete genomes of 13 MRSA strains that were sequenced and deposited in the NCBI gene bank, MRSA 252 and MRSA Mu50 were selected due to the fact that the former is a common strain in the USA [49] and the UK [50] and the latter, a methicillin and vancomycin resistant strain isolated in Japan [51] is commercially available for future molecular biological study (ATCC). The common method of drug target identification encompasses two steps: the identification of essential genes for bacterial viability [37] and the identification of genes absent in the human genome [38]. The former was performed by adopting the DEG database in our approach because this database compiles a list of all currently available essential genes in more than 10 prokaryotes including *Staphylococcus aureus* [41] and proved to be more accessible than conventional tools [39, 40]. On the other hand, the availability of the human genome sequence [52, 53] renders the latter step feasible. Following two newly published genomic analysis methods [39, 40], 2656 MRSA 250 and 2697 Mu50 genes were purged at 90 % and 60% using CD-HIT to remove

TABLE 1: GENOMICS ANALYSES OF MRSA 252 AND MRSA MU50 STRAINS REPECTIVELY.

Genes	**MRSA 252**	**MRSA Mu50**
Total number	2656	2697
Duplicates (>60% identical)	88	105
Non-paralogs	2568	2592
Essential genes [cut-off *E*-value < 10^{-4}]	499	496
Essential genes w/o human homologs[cut-off *E*-value < 10^{-3}]	133	134

TABLE 2. 133 ESSENTIAL, NON-HUMAN HOMOLOGOUS GENES IN BOTH MRSA 252 AND MRSA MU50 STRANS ENCODING DIFFERENT CLASSES OF PROTEINS AND THEIR KNOWN OR PUTATIVE FUNCTIONS

Categories	Classes	Groups	MRSA 252 *NCBI Gene Accession#*	MRSAMu50 *NCBI Gene Accession #*	Known or putative functions
Metabolism	Cellular respiration	Carbohydrate Catabolism	49482458	15923216	Formate acetyltransferase
			49482459	15923217	Formate acetyltransferase activating enzyme
			49482486	15923242	Xylitol dehydrogenase
			49483017	15923750	HPr kinase/phosphorylase
			49483247	15924074	Phosphoenolpyruvate-protein phosphatase ptsI
			49483033	15923765	Phosphoglyceromutase
			49483952	15924701	Acetate kinase
			49484267	15925031	Sucrose-6-phosphate hydrolase
			49484349	15925115	Fructose-bisphosphate aldolase
			49484367	15925133	Mannose-6-phosphate isomerase
			49484381	15925149	Mannitol-1-phosphate 5-dehydrogenase
			49484415	15925185	Galactose-6-phosphate isomerase subunit LacA
		Lipid Catabolism	49483384	15924216	Phosphatase/ dihydroxyacetone kinase
			49483425	15924288	Glycerol uptake operon antiterminator regulatory protein
		Amino acid catabolism	49482426	15923174	N-acetyl-γ-glutamyl-phosphate reductase
			49482779	15923539	N-acyl-L-amino acid amidohydrolase
			49483163	15923990	Thimet oligopeptidase homolog
			49483313	15924141	Glutamate racemase
			49483846	15924589	5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase
			49484504	15925279	Urease subunit β
			49484120	15924869	Aminopeptidase ampS
			49484649	15925422	Glycerate kinase
			49484868	15925663	HisF cyclase-like protein
				15923177	Cystein Hydrolase
			49483520	15924318	Homoserine dehydrogenase
			49483584	15924384	Aspartate semialdehyde dehydrogenase
				15925319	Amino acid amidohydrolase
		Common metabolic pathway	49482818	15923578	Phosphotransacetylase
			49484161	15924909	Putative manganese-dependent inorganic pyrophosphatase
			49484002	57634637	Probable NAD(FAD)-utilizing dehydrogenase
	Bio-synthesis	Amino acid biosynthesis	49484873	15925668	Histidinol dehydrogenase
			49482425	15923173	Ornithine acetyltransferase
			49482586	15923346	5-methyltetrahydropter-oyltriglutamatehomo- cysteine methyltransferase
			49482696	15923462	Glutamate synthase, large subunit
			49483565	15924362	Tryptophan synthase β subunit
			49483583	15924383	Aspartokinase II
			49483655	15924456	Chorismate synthase
			49484279	15925043	dihydroxy acid dehydratase
			49484281	15925046	Ketol-acid reductoisomerase
			4948429	15925060	Alanine racease
			49484794	15925588	Pantoate--β-alanine ligase
		Fatty acid biosynthesis	49483392	15924219	Fatty acid/phospholipid synthesis protein
		Nucleotide biosynthesis	49482382	15923129	Phosphopentomutase
			49483421	15924248	Uridylate kinase
			49483664	15924468	Cytidylate kinase
		Cell wall biosynthesis	49484627	15925401	FemAB family protein
			49483567	15924364	FemA protein
			49482490	15923244	Teichoic acid biosynthesis protein (truncated TagF)
			49482939	15923673	Undecaprenyl Pyrophosphate Phosphatase
			49482995	15923728	UDP-N acetylenolpyruvoyl-glucosamine reductase
			49483182	15924008	UDP-N-acetylmuramoylalanyl-D-glutamate-2, 6-diaminopimelate ligase
			49484307	15925072	UDP-N-acetylmuramoylalanyl-D-glutamyl-2, 6-diaminopimelate-D-alanyl-D-alanyl ligase
			49484133	15924882	UDP-N-acetylmuramyl tripeptide synthetase

			49483346	15924173	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
			49484348	15925114	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
			49484309	15925074	Rod shape determining protein RodA
			49483587	15924387	Tetrahydrodipicolinate acetyltransferase
			49483980	15924730	UDP-N-acetyl-muramoyl-L-alanine synthetase
				57634647	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
		Other biosynthesis	49482716	15923479	tetrapyrrole(corrin/porphy-rin) methylase
			49482722	15923485	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
			49484013	15924759	Riboflavin biosynthesis
			49484795	15925589	3-methyl-2-oxobutanoate hydroxymethyltransferase
Transmission of genetic information	DNA replication, recombination and repair		49482254	15922991	Chromosomal replication initiation protein
			49482255	15922992	DNA polymerase III β subunit
			49482269	15923006	Replicative DNA helicase (DnaB-like)
			49483309	15924136	Excinuclease ABC subunit C
			49483633	15924434	Methyltransferase
			49483747	15924487	Integrase/recombinase
			49483811	15924552	DNA primase
			49483834	15924577	DNA polymerase III subunit delta
			49483926	15924674	Primosomal protein DnaI
			49483944	15924693	DNA polymerase III, β chain
			49484385	15925153	DisA bacterial checkpoint controller nucleotide binding
	Transcription and RNA processing		49483418	15924245	Transcriptional repressor CodY
			49483550	15924347	Transcription antiterminator
			49484097	15924845	SpoU rRNA methylase family protein
			49484908	15925703	Ribonuclease P
			49483433	15924260	Ribosome-binding factor A
			49483855	15924600	Transcription elongation factor
			49482590	15923350	Transcription terminator
			49483976	15924726	Catabolite control protein A
	Translation and posttranslational modifications		49483000	15923733	peptidase T
			49483039	15923772	SsrA-binding protein
			49483384	15924211	Hypothetical translation and posttranslational modifications
			49483609	15924409	Gcn5-related acetyltransferases
			49483778	15924518	Elongation factor P
Trans-membrane Proteins	Antibiotic Resistance		49482275	15923012	Metallo- lactamase
			49483344	15924171	Penicillin-binding protein
	Regulation		49483168	15923996	GTP pyrophosphokinase
			49483425	15924252	Zinc metalloprotease yluc
	Transport		49482431	15923179	Glucose-specific PTS, IIABC component
			49482476	15923232	PTS, IIBC component
			49482956	15923690	Gructose-specific PTS, IIABC component
			49483966	15924716	N-acetylglucosamine specific PTS, IIABC component
			49484378	15925146	Mannitol-specific PTS, IIBC component
			49484380	15925148	Mannitol specific PTS, IIA component
			49484538	15925313	PTS, arbutin-like, IIBC component
			49484739	15925528	Glucose-specific PTS, II ABC component
			49484838	15925631	PTS, IIABC component
			49483148	15923977	Oligopeptide transport system permease protein
			49484706	15925495	Gluconate permease
			49482866	15923628	Teichoic acid ABC transporter permease
			49484434	15925210	Cobalt transport protein
			49484516	15925291	Na^+/H^+ antiporter
			49484891	15925688	Nickel transport protein
			49484846	15925639	Bifunctional Preprotein translocase subunit SecA
			49483881	15924627	Bifunctional preprotein translocase subunit SecD/SecF
			49483265	15924092	Spermidine/putrescine-binding protein precursor homolog
			49482314	15923062	Potassium-transporting ATPase subunit A
			49482353	15923100	L-lactate permease homolog
			49484303	15925067	potassium-transporting ATPase subunit A
			49484446	15925220	Preprotein translocase subunit SecY
			49483071	15923829	ABC transporter substrate-binding protein
			49483075	15923833	ABC transporter-associated protein
			49483078	15923836	ABC transporter-associated protein

Other Proteins	Carrier proteins	49483175	15924003	Sodium/proton-dependent alanine carrier protein
		49482688	15923454	Lipoprotein
	Regulation	49482271	15923008	Response regulator protein
	Cell division	49482736	15923499	C ell division
		49483349	15924176	C ell division protein FtsZ
		49484905	15925700	Glucose-inhibited division protein B
	Other	49484374	15925142	Haloacid dehalogenase-like hydrolase
		49484612	15925386	Nitrate reductase β chain
		49484613	15925387	Respiratory nitrate reductase alpha chain
	Unknown function	49482472	15923228	Unknown
		49483005	15923738	Unknown
		49483022	15923755	Unknown
		49483024	15923757	Unknown
		49483035	15923767	Unknown
		49483546	15924343	Unknown
		49483928	15924676	Unknown
		49484792	15925584	Unknown

paralogues, respectively. The resulting 2568 MRSA 250 and 2592 Mu50 sequences were run through the database of essential genes (DEG) at an expectation cut-off of 10-4, yielding 499 and 496 essential genes respectively. Those 499 and 496 essential genes identified were subjected to BLASTP against the human genome [52, 53] to exclude any genes that have a significant match (E-value threshold of 10-3 and lower) with human homologs. Consensually, 133 MRSA 252 and 134 Mu50 genes respectively were considered as having no close relatives in humans. The results are summarized in table 1. Their known or putative functions annotated by Integr8-Inquisitor [46] and/or EMBL/EBI/InterProScan [47] are listed in table 2.

Among the 133 and 134 essential non-human homologous genes in MRSA 252 and Mu50 strains, respectively, 133 encode proteins that are well conserved between the two strains. Out of this conserved set, 63 are involved in metabolism, 24 participate in the transmission of genetic information, 29 represent transmembrane proteins, 9 have other functions such as regulation cell division and carrier proteins, *etc*., and 8 have unknown functions.

Our approach identified 14 genes in cell wall biosynthesis, most of which were validated by other research groups [54-56]. Among them, 6 are involved in the elongation of peptidoglycan, in agreement with previous studies [54, 55]. FemA family proteins are currently considered novel anti-staphylococcal targets due to the fact that they are involved in cell wall biosynthesis and expression of a methicillin resistance gene [56]. They are found to be essential in both MRSA 252 (NCBI Gene Accession#: 49484627 and 49483567) and Mu50 (NCBI Gene Accession#: 15925401 and 15924364) strains by our approach. Gene GI#49484133 in MRSA 252 and GI#15924882 in Mu50 respectively represent Staphylococcus aureus murE gene encoding UDP-N-acetylmuramyl tripeptide synthetase, which was demonstrated to be essential in *Staphylococcus aureus* through a method incorporating an IPTG controllable promoter [57].

Although the cell wall has long been considered an attractive target for antibiotic development because of its absence in humans, what should not be overlooked is that one of the most common antibiotic resistance mechanisms is the metamorphosis of cell-wall proteins, leading to antibiotic resistance. For example, β- lactam resistance was attributed to the expression of a group of cell wall penicillin-binding proteins (PBP-2') encoded by the mecA gene [58, 59]. Glycopeptide resistance is also considered to be caused by cell wall thickening resulting in binding vancomysin extracellularly [60, 61] and/or alteration of the drug-acting site in the cell wall from D-alanine-D-alanine to D-alanine-D-lactate owing to the expression of *vanA* resistance gene [62]. Hence, for novel antibiotic development, substances that anchor in sites other than the bacterial cell wall may have more potential because resistance usually arises as the result of gene mutation on the target proteins that are subject to direct antibiotic attack [63]. A 2006 review on mechanisms of bacterial antibiotic resistance suggested the exploration of novel antibiotics with alternative mechanisms of action [5].

Genes involved in transmission of genetic information including DNA replication, recombination and repair, transcription and RNA processing, translation, post-translational modification remain viable targets for antibacterial agent development [39, 40]. Our approach identified 24 of these candidate genes.

Our approach identified 29 membrane bound proteins. A recent review of anti-MRSA drug development indicated that agents anchoring in the bacterial membrane (*e.g.*, ceragenins and lipopeptides) showed great bactericidal effect and less prone to drug resistance due to the inability of bacteria to modify their targeted cellular sites in a way that is compatible with their survival [64]. Among this pool of proteins, 19 are involved in membrane transport, which represent valid drug targets

because pathogens usually lose their biosynthetic capabilities and rely on their hosts for the supply of essential nutrients [65, 66]. Thus, certain membrane transport proteins are of great importance in maintaining pathogen viability.

Our approach identified 30 energy metabolic (*i.e.* cellular respiration) genes in both MRSA 252 and MRSA Mu50, which are essential to staphylococcal survival with *E*-score < 10^{-4} but absent in human genome with *E*-score <10^{-3}. Currently there are limited numbers of commercially available antibiotics targeting energy metabolism. Those existing are mainly biological reagents such as oligomycin [67] and pesticides or piscicides such as antimycin A [68], not commonly used for humans because they affect both bacterial and human cells. Surprisingly, nature has provided us with a group of energy metabolic enzymes which are essential to pathogen survival while absent in humans. The differentiation lies in that those enzymes function through alternative mechanisms other than their counterpart enzymes in humans. Accumulating *in vitro* [69] and *in vivo* [70] evidence suggests that enzymes catalyzing bacterial cellular respiration with differentiated mechanisms of action are promising targets for novel antibiotic development. The inhibitors against those enzymes are able to hinder bacterial growth by inhibition of those enzymes without interfering with their human cousins. Most importantly, attacking bacterial energy-making machinery bypasses the usual bacterial mutation

TABLE 3 - POTENTIAL CENTRAL METABOLIC DRUG TARGETS FROM MRSA MU50 BASED ON DATA BASE OF ESSENTIAL GENES (DEG) HOSTED RECORDS OF CURRENTLY AVAILABLE ESSENTIAL GENES.

Class	MRSA 252	MRSA Mu50	Known or putative function	EC #	Identity with DEG genes				human homolog or ortholog
	Accession #	Accession #			Organism	E-Value	% Identity	% Similarity	
Carbohydrate Catabolism	SAR0217	SAV0226	Formate acetyltransferase	2.3.1.54	*H.influenzae* Rd KW20	0	62%	77%	No
	49482486 SAR0247	15923242 SAV0252	Xylitol dehydrogenase	1.1.1.137	*S. aureus* NCTC 8325	1e-163	80%	91%	No
	49483017 SAR0814	15923750 SAV0760	HPr kinase /phosphorylase	2.7.11.- 2.7.4.-	*S. aureus* NCTC 8325	1e-155	95%	95%	No
	49483247 SAR1057	15924074 SAV1084	Phosphoenolpyruvate-protein phosphatase	2.7.3.9	*S. aureus* N315	0	97%	97%	No
	49483033 SAR0831	15923765 SAV0775	Phospho-glyceromutase	5.4.2.1	*S. aureus* NCTC 8325	0	97%	97%	No
	49483952 SAR1789	15924701 SAV1711	Acetate kinase	2.7.2.1	*S. aureus* N315	0	92%	92%	No
	49484349 SAR2213	15925115 SAV2125	Fructose-bisphosphate aldolase	4.1.2.40	*S. aureus* N315	1e-163	100%	100%	No
	49484381 SAR2247	15925149 SAV2159	Mannitol-1-phosphate 5-dehydrogenase	1.1.1.17	*S. aureus* N315	0	100%	100%	No
	49484415 SAR2286	15925185 SAV2195	Galactose-6-phosphate isomerase subunit LacA	5.3.1.26	*S. aureus* N315	1e-76	100%	100%	No
	49482818 SAR0594	15923578 SAV0588	Phospho-transacetylase	2.3.1.8	*S. aureus* NCTC 8325	1e-169	93%	93%	No
Lipid Catabolism	49483425 SAR1238	15924288 SAV1298	Glycerol uptake operon antiterminator regulator	Un-classified	*S. aureus* N315	7e-98	100%	100%	No
Protein Catabolism	49483313 SAR1123	15924141 SAV1151	Glutamate racemase	5.1.1.3	*S. aureus* N315	1e-154	100%	100%	No
	49484504 SAR2373	15925279 SAV2289	Urease subunit β	3.2.2.16	*S. aureus* N315	2e-77	100%	100%	No
	49484120 SAR1969	15924869 SAV1879	Aminopeptidase ampS	3.4.11.-	*S. aureus* N315	0	100%	100%	No
Common metabolic pathway	49484161 SAR2012	15924909 SAV1919	manganese-dependent inorganic pyrophosphatase	3.6.1.1	*S. aureus* NCTC 8325	1e-172	100%	100%	No

sites for drug resistance [71-72]. Hence, exploration of antibiotics targeting alternative cellular functions such as central metabolic pathways may be a promising direction, and selective inhibition of targets specific to bacterial energy metabolism may be a potentially efficacious alternative in the treatment of MRSA infections. The enzymes on the higher priority list include *MRSA* fructose-bisphosphate aldolase, *MRSA* acetate kinase, *MRSA* phosphotransacetylase, *MRSA* formate acetyltransferase *and MRSA* xylitol dehydrogenase, *etc.* (table 3), which either do not have human homologues or adopt dramatically different catalytic mechanisms compared to their human cousins.

MRSA fructose-1, 6-diphosphate aldolase (NCBI Gene Accession#: 49483952 and 15924701 respectively) showed a 100 % match to both *Staphylococcus aureus* NCTC 8325 and *Staphylococcus aureus* N315 in Database of Essential Gene (DEG) with an identical expectation value of e^{-163} [73,74], suggesting the essential nature of this protein. It is well known that FBPA is one of the key enzymes in the glycolytic pathway that involves the breakdown of glucose [75]. FBPA is divided into two classes based on structural properties and catalytic mechanisms [75, 76]. Class I FBPA is mainly found in higher order organisms (*e.g.*, humans and animals). Catalysis in class I FBPA proceeds via a Schiff base intermediate formed by an active site lysine residue [75]. Class II FBPA is usually found in yeasts, bacteria, fungi, and parasites [76]. Catalysis in class II FBPA centers on the participation of a Zn (II) cofactor that coordinates to an enolate anion intermediate [76]. Based on major differences in active site structure and catalytic mechanism, an inhibitor of class II FBPA can be designed which will not inhibit class I FBPA. Thus, class II FBPA has long been considered as potential drug target in the development of antibiotics [77]. Multiple alignment of the sequence of MRSA FBPA with class II giardia FBPA and class I human FBPA was shown in Figure 1. *MRSA* FBPA (NCBI Gene Accession#: 49484349 and 15925115 respectively) exhibits 40.8% sequence identity to class II *giardia* FBPA while it exhibits only 18.8 % sequence

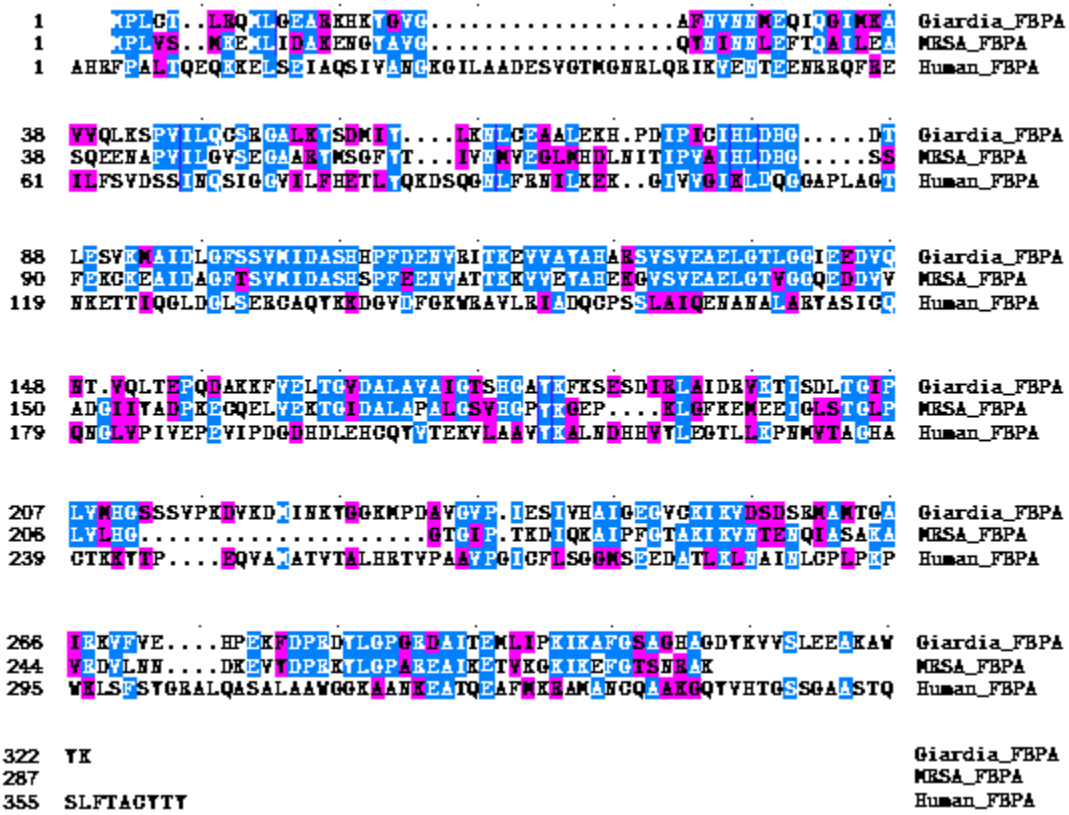

Figure.1 Alignment of the amino acid sequences of *MRSA* FBPA (NCBI GENE ACCESSION#:49484349 and 15925115 respectively) with class II *giardia* FBPA (2ISV) and class I *human* FBPA (1QO5). Numbering of the amino acids is indicated on the left. Identical amino acid residues in the alignment are indicated in light-blue shading and similar amino acid residues are indicated in purple shading. Gaps introduced during the alignment process are indicated as dots.

identity to class I *human* FBPA. Thus, *MRSA* FBPA should be putatively classified into class II FBPA, not class I FBPA. We have cloned and purified and characterized *MRSA* FBPA (unpublished result). Validation of the essential nature of class II *MRSA* FBPA through allelic replacement and inducible expression is underway in our research group.

MRSA acetate kinase (NCBI Gene Accession#: 49483952 and 15924701 respectively) demonstrated a 92 % match to *Staphylococcus aureus* N315 in Database of Essential Gene (DEG) with an expectation value of 0 [73, 74], suggesting the essential nature of this enzyme. Acetate kinase catalyzes the reversible phosphorylation of acetate to synthesize acetyl phosphate by transfer of a phosphoryl group from ATP. Acetate kinases are wildly distributed among prokaryotes [78] and some eukaryotes [79]. In aerobic conditions, this enzyme converts acetate to acetyl-CoA, a key intermediate in TCA cycle [80]. In anaerobic conditions, it plays a central role in synthesizing ATP from acetyl phosphate [81]. Prokaryotic acetate kinases are highly conservative. *MRSA* acetate kinase exhibits 44.6 % sequence identity to *E. coli*, 48.5 % sequence identity to *Salmonella typhimurium* acetate kinase, 51.3 % sequence identity to *Methanosarcina themophia* acetate kinase and 52.0 % sequence identity to *Lactobacillus sanfranciscensis* acetate kinase (Figure 2). Smith group has confirmed that it is a key enzyme in bacterial metabolism in a number of important fungal and protozoan pathogens. (*e.g.,* fungus *Cryptococcus neoformans* and protist *Entamoeba histolytica*). Its absence in humans suggests that it may also be a possible drug target [82]. We have cloned, purified and characterized *MRSA* acetate kinase (unpublished result). The development of crystal structures of *MRSA* acetate kinase is in progress at the laboratory of our collaborator Dr. Scott Lovell at the University of Kansas, which will allow us to preform structure-activity analysis as the basis of rational inhibitor design.

MRSA phosphotransacetylase (NCBI Gene Accession#: 49482818 and 15923578 respectively) demonstrated a 93 % match to *both Staphylococcus aureus* NCTC 8325 and *Staphylococcus aureus* N315 in Database of Essential Gene (DEG) with an identical expectation value of e^{-169} [73, 74], suggesting the essential nature of this enzyme. The gene encoding *MRSA* phosphotransacetylase has been cloned and the enzyme has been expressed in *E. coli* and purified. Kinetic assay of this enzyme is in progress.

Overall, we proposed that a class of essential, central metabolic enzymes, such as *MRSA* fructose-bisphosphate aldolase, *MRSA* acetate kinase, *MRSA* phosphotransacetyl-ase, *MRSA* formate acetyltransferase and *MRSA* xylitol dehydrogenase, *etc.* (table 3), which either do not have human homologues or functionally differentiate themselves from their human counterparts, are promising antibiotic drug targets. Because of the alterations in active site structure and mode of action of such a bacterial enzyme *v. s.* its human cousin (if there is one), through rational inhibitor design, an inhibitor of this enzyme can be designed which will not inhibit its human cousin. Nevertheless, this central metabolic inhibitor approach potentially decreases the risk of bacterial resistance against the antibacterial agents in that it bypasses the cellular sites where currently existing antibiotics regularly attack. In other words, since those cellular sites have not been repeatedly exposed to antibacterial agents, central metabolic inhibitors should be less prone to drug resistance induced by evolutionary adaptation.

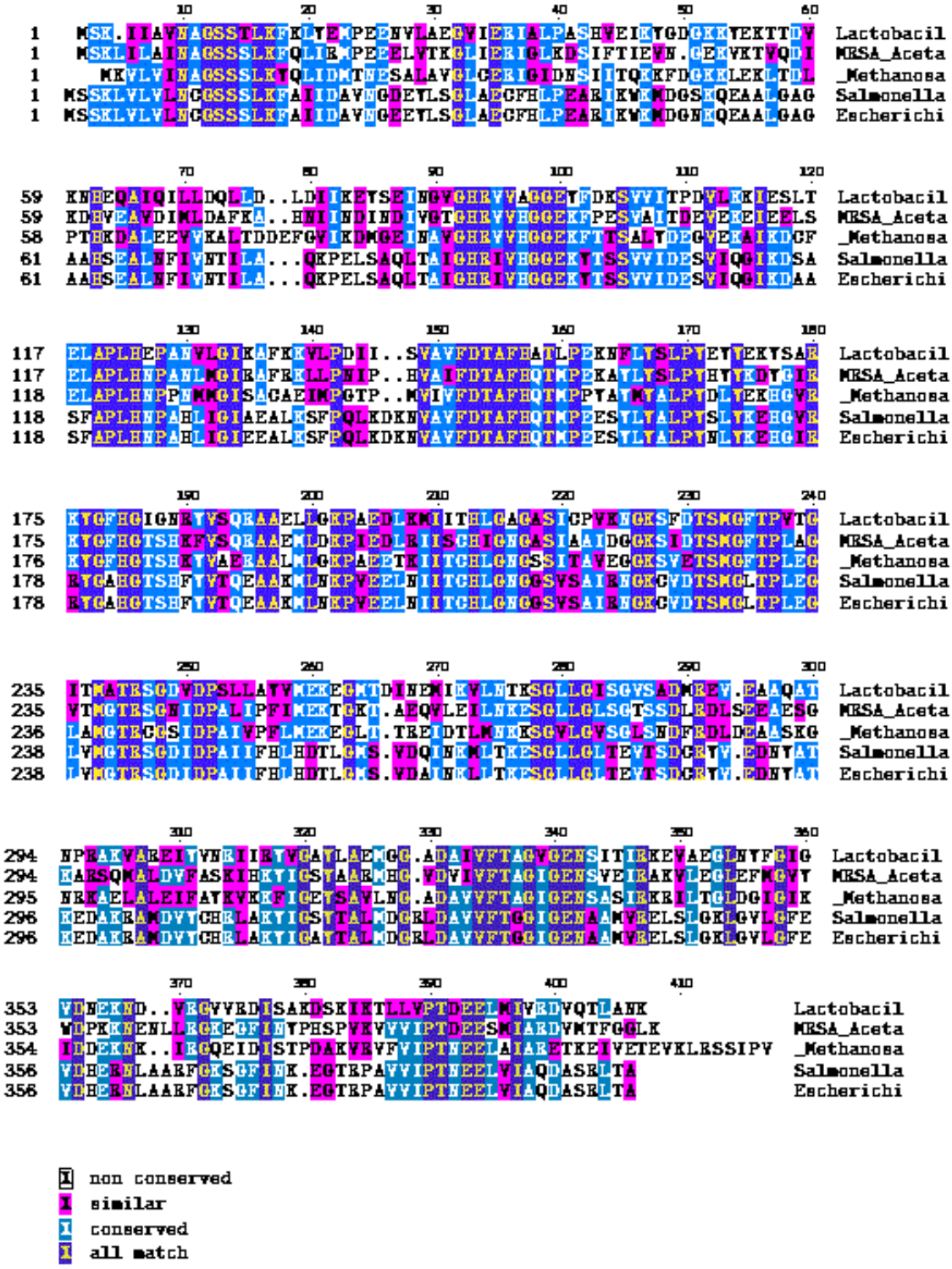

Figure.2 Alignment of the amino acid sequences of *MRSA* acetate kinase with *E. coli*, *Salmonella typhimurium* ,*Methanosarcina themophia* ,*Lactobacillus sanfranciscensis* acetate kinases. Numbering of the amino acids is indicated on the left. Identical amino acid residues in the alignment are indicated in light-blue shading and similar amino acid residues are indicated in purple shading. Gaps introduced during the alignment process are indicated as dote

IV. CONCLUSION AND FUTURE WORK

One of the crucial steps in narrow-spectrum antibiotics development is target identification. In this study, a putative set of candidate drug targets were elucidated by an *in silico* approach. The candidate genes are hypothetically required for survival of the candidate microorganisms and have no close human analogues. Many identified targets have been experimentally validated [56-59, 83-88]. By shortening the list of potential drug targets to a small pool of genes, the data presented in this paper facilitated our group and, may also aid other researchers in pursuing target validation and target characterization for alternative treatment of MRSA infections. Future directions include using a combination of kinetic assay and crystal structure development for enzyme characterization such as substrate recognition, catalytic site identification and reaction mechanism elucidation. Using ration drug design, tight-binding inhibitors will be designed followed by organic synthesis and *in vitro* evaluation. Once a nanomolar level inhibitor with high specificity is identified, development of X-ray crystal structures of enzyme-inhibitor complexes will be performed for further optimization. In principle, the premise is that the inhibitors of these targets should only be toxic to pathogens, but safe for use by humans. Proposed long-term work also includes extension of this approach to other bacterial systems to combat antibiotic resistance. It is even more crucial that this type of investigation is undertaken in academia than it would be if industry were still heavily investing in it.

This study sheds light on a potentially new class of MRSA antibiotics, which may pave the road to multi-faceted approaches to combat antibiotic resistance. From the broader perspective, blocking central metabolic pathways was usually considered as a forbidden area in drug development due to the possibility of affecting human central metabolism (*e.g.*, side effects of chemotherapies). If the assertion that certain central metabolic inhibitors are specific to pathogens not to humans is tested, it will reassure that we have moved in the right direction to tackle a major challenge.

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