

Bioinformatics and Molecular Biological Characterization of a Hypothetical Protein SAV1226 as a Potential Drug Target for Methicillin/Vancomycin-*Staphylococcus aureus* Infections

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Abstract—Methicillin/multiple-resistant *Staphylococcus aureus* (MRSA) are infectious bacteria that are resistant to common antibiotics. A previous *in silico* study in our group has identified a hypothetical protein SAV1226 as one of the potential drug targets. In this study, we reported the bioinformatics characterization, as well as cloning, expression, purification and kinetic assays of hypothetical protein SAV1226 from methicillin/vancomycin-resistant *Staphylococcus aureus* Mu50 strain. MALDI-TOF/MS analysis revealed a low degree of structural similarity with known proteins. Kinetic assays demonstrated that hypothetical protein SAV1226 is neither a domain of an ATP dependent dihydroxyacetone kinase nor of a phosphotransferase system (PTS) dihydroxyacetone kinase, suggesting that the function of hypothetical protein SAV1226 might be misannotated on public databases such as UniProt and InterProScan 5.

Keywords—Dihydroxyacetone kinase, essential genes, Methicillin-resistant *Staphylococcus aureus*, drug target.

I. INTRODUCTION

METHICILLIN/MULTIPLE-resistant *Staphylococcus aureus* (MRSA) is any strain of a bacterium, *Staphylococcus aureus*, that has developed resistance to antibiotic [1]. In 2005, MRSA caused a mortality rate higher than that caused by HIV [2], [3]. MRSA infections start out as small cellulitis, boils or impetigo on the skin and often progress to an open, inflamed wound, and then spread to other organs of the body in immunocompromised patients, which turn to be life-threatening [4]. MRSA is difficult to treat because of two reasons: 1) the fast speed of spreading when it gains access to the bloodstream [5] and 2) multiple antibiotics resistance [6].

Antibiotic resistance can be ascribed to two major origins: misuse or overuse of prescription antibiotics [7] and limitation

of current existing antibiotics [8], [9]. Whereas the former attracted national or even international attention from health providers, the latter was analyzed extensively as follows: although hundreds of antibiotics stay on the market, a fact that cannot be neglected is that almost all existing antibiotics only target enzymes in four classes of cellular functions: cell wall synthesis, protein synthesis, nucleic acid synthesis and foliate synthesis [8]. Bacterial resistance usually arises as the result of evolutionary adaptation of the target proteins that are subject to direct antibiotic attack. Repetitively striking the same cellular sites remains to be one of the major courses of defensive bacterial gene mutation [8], [10], [11]. Hence, for novel antibiotic development, substances that anchor in new sites may be promising, among which enzymes of core metabolism were suggested as one of the three sets of novel targets [8].

Reference [12] showed that a class of known, putative or hypothetical central metabolic enzymes such as MRSA acetate kinase (ACK) (SAV 1711), MRSA fructose biphosphate aldolase (FBPA) (SAV 2125), MRSA phosphotransacetylase (PTA) SAV (0588), MRSA putative Mannitol-1-phosphate 5-dehydrogenase (M1P5D) (SAV2159) and MRSA hypothetical dihydroxyacetone kinase (Dha kinase) (SAV1226), which are *in silico* essential to bacterial growth and absent in humans, are promising drug targets. MRSA ACK, MRSA FBPA, MRSA PTA were cloned and characterized in our lab [13].

Dha kinases are enzymes that transfer the phosphate group from high-energy donor molecules to dihydroxyacetone (Dha) [14], [15]. Dha kinases are classified into two classes according to the phosphoryl group donors: ATP dependent Dha kinases (Fig. 1) in animals, plants, and some bacteria such as

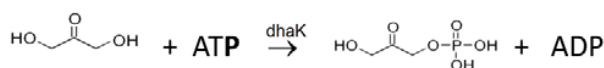


Fig. 1 Reaction catalyzed by ATP dependent Dha kinases

Citrobacter freundii [14] and a phosphoprotein of the bacterial phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) dependent Dha kinases (Fig. 2) in most bacteria such as *E. coli*. [15].

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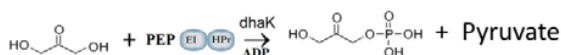


Fig. 2 Reaction catalyzed by PTS dependent Dha kinases

ATP-dependent Dha Kinases are two-domain proteins [14], while PTS-dependent Dha Kinases are mainly three-domain proteins [15]. However, both types of Dha kinases share the two upstream domains in common with high sequence/structure similarity, i.e. DhaK binds Dha while DhaL binds nucleotide of ATP in ATP-dependent Dha kinases and ADP, as a cofactor, in PTS-dependent Dha kinases [14]-[16]. PTS-dependent Dha kinases contain the third domain, DhaM, which serve as the shuttle for phosphoryl group is transferred from PEP to HPr, then to the Histidine residue of DhaM, eventually to Dha. Due to sequence conservation between two types of Dha kinases, the presence of a dhaM domain determines if an organism contains a PTS-dependent Dha kinase. In addition, DhaK/DhaL fusion is another indicator of a PTS-dependent Dha Kinase [16].

In this study, we reported bioinformatics and molecular biological characterization of hypothetical MRSA SAV1226 as an initial step of drug target identification for methicillin/vancomycin-*Staphylococcus aureus* Infections.

II. MATERIALS AND METHODS

A. Protein Function and Essentiality Prediction

Information on the function of hypothetical protein SAV1226 was derived through NCBI [17], KEGG [18], UniProt [19] and InterProScan5 [20] respectively. Hypothetical protein SAV1226 sequence was aligned with sequences acquired from Database of Essential Genes (DEG) [21] using BLASTP at an E-value cutoff of 10^{-5} .

B. Strains, plasmids, and culture conditions

Template DNA was from *Staphylococcus aureus* subspecies *aureus* Mu50 (ATCC, 700699D-5). DH5 α *E. coli* and BL21 (DE3)/pLysS *E. coli* were from Invitrogen. Sequencing of the MRSA DhaK gene was conducted by the Western South Dakota DNA Core Facility at Black Hills State University.

C. SAV1226 Gene Cloning and Expression

SAV1226 gene was PCR amplified from MRSA Mu50 chromosomal DNA using Roche High Fidelity PCR Master, sense 5'-GCG ATA GCG GGA TCC ATG ATT AGC AAA ATT AAT GGT A-3' and anti-sense 5'-GCG ATA GCG GGT ACC TTA TTC TAC TGA AAA GAA ATA TTG-3' primers (IDT). Both pRSET A vector (Invitrogen) and insert DNA were digested with BamHI and KpnI in a sequential digest. The resulting DNA fragments were agarose gel-purified and further cleaned using the Qiagen gel prep kit (Qiagen). SAV1226 gene fragment was ligated into pRSET A vector (Invitrogen) at the BamHI and KpnI sites (sites underlined in primer sequences). The recombinant plasmid was transformed into chemically competent DH5 α cells and positive transformants were tested by restriction analysis and DNA-sequencing. Fidelity clones were transformed into

chemically competent BL21(DE3)/pLysS cells for expression.

D. DhaM Gene (SAV0653) Cloning and Expression

DhaM gene (SAV0653) from *Staphylococcus aureus* genomic DNA Mu50 was amplified using Taq DNA polymerase (Qiagen), genomic DNA Mu50 template, and the following primers: forward 5'-AAGGTACCCCAACCCAACC-3', reverse 5'-AAGGATCCCAATCGGCGG-3' (IDT). Amplified DhaM gene and pRSET A vector (Invitrogen) were digested with BamHI and KpnI sequentially. Digest products were evaluated on a 0.9% agarose gel and further cleansed with the Qiagen gel prep kit (Qiagen). Purified DAK-M was ligated into pRSET A vector (Invitrogen) at the BamHI and KpnI sites (underlined). Recombinant plasmid was transformed into chemically competent DH5 α *E. coli* cells grown in ampicillin selective LB agar broth. Cells were isolated and purified with QIAspin miniprep spin kit (Qiagen), then analyzed with 0.9% agarose gel and DNA-sequencing. Diagnostic PCR of DAK-M recombinant plasmid was done to confirm digestion, ligation, recombination, and transformation were done correctly. Clones were transformed into chemically competent BL21(DE3)/pLysS cells for protein expression.

E. SAV1226 Protein Purification

250 mL of LB media with ampicillin and chloramphenicol (100 μ g/ml) were inoculated with 10 ml of overnight-grown cells harboring the plasmid. Cells were grown at 37°C to A600 = 0.6, when IPTG (1 mM) was added for induction. After 5 h, cells were collected by centrifugation and His-tagged recombinant proteins were purified using the QIAexpress Ni-NTA protein purification system (Qiagen) according to the manufacturer's suggestions. The purity and sizes of the recombinant proteins were assessed by SDS-PAGE.

F. SAV1226 Western Blot Analysis

Protein samples fractionated on a 12% SDS-polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane and probed with primary antibody (Mouse anti-His, Qiagen) and secondary antibody (Goat anti-mouse IgG-HRP, Millipore). Immunocomplexes were detected using chemiluminescence reagents (Immobilon Western HRP Substrate (Millipore)).

G. SAV1226 Kinetic Assay

Assuming hypothetical Protein SAV1226 is a ATP-dependent Dha kinase Initial velocities were measured at 25°C using 1-ml reaction solutions containing 0.224 mM NADH, 2 mM ATP, 4mM Dha, 20mM MgCl₂, 0.1 mM Imidazole, 2.5 units of glycerol-3-phosphate dehydrogenase, and varying concentrations of hypothetical protein SAV1226 in 50 mM K⁺HEPES (pH 7.5). The absorbance of the reaction solution was monitored at 340 nm (6.2 mM⁻¹cm⁻¹). Enzyme activity is expressed at a decrease of absorbance at 340 nm.

Assuming hypothetical Protein SAV1226 is a PTS-dependent Dha kinase Initial velocities were measured at 25°C using 1-ml reaction solutions containing 0.224 mM NADH, 4mM Dha, 20mM MgCl₂, 0.1 mM Imidazole, 2.5 units of

glycerol-3-phosphate dehydrogenase, varying concentrations of HPr, varying concentrations of EI, varying concentrations of DhaM, 1 mM PEP, 0.1 mM ADP, and varying concentrations of hypothetical protein SAV1226 in 50 mM K+HEPES (pH 7.5). The absorbance of the reaction solution was monitored at 340 nm ($6.2 \text{ mM}^{-1}\text{cm}^{-1}$). Enzyme activity is expressed at a decrease of absorbance at 340 nm.

H.SAV1226 Mass Spectrometry

Purified hypothetical protein SAV1226 was sent to the University of New Mexico Mass Spectrometry Facility to be tested through Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). It was also tested through Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI-TOF) for function.

III. RESULTS AND DISCUSSION

A. Bioinformatics Prediction of Hypothetic Protein sav1226

Hypothetic protein sav1226 demonstrates high similarity to two essential proteins in DEG: 34% identity and 56% similarity to GI:15828632 a predicted kinase related to dihydroxyacetone kinase, in *Mycoplasma pulmonis* with match score of $5e-97$ and 34% identity and 56% similarity, respectively and to GI:12045230, a DAK2 phosphatase domain protein *Mycoplasma genitalium* with match score of 1e-63 and 28% identity and 48% similarity, respectively [21]. Thus, whatever the function of hypothetic protein sav1226 is, it is *in silico* essential to MRSA survival.

The sequence of sav1226 gene and its deduced amino acid sequence are deposited in NCBI, KEGG and UniProt databases under accession number 15924216, SAV1226 and Q99UP2 respectively. The sav1226 gene encodes a 548-amino-acid-protein of a predicted molecular mass of 64,498 Da. *Bacillus subtilis* yloV (553 aa), an uncharacterized protein, demonstrates the most sequential similarity to sav1226 (55.5% identity and 68.1% similarity) with a match score of 8.6 E-211 (Fig. 3). MRSA SAV1226 is annotated as a hypothetical protein on NCBI [17] and KEGG [18] On UniProt, SAV1226 is annotated as an uncharacterized protein but also listed as DhaL domain of Dihydroxyacetone kinase [19]. EMBL/EBI/InterProScan5 suggested that SAV1226 is either DhaL domain or DAK2 domain-containing protein [20]. Polypeptide length comparison of SAV1226 (548 aa) with other corresponding DhaL domains (around 150 aa) of characterized Dha kinases [14], [15] implied that SAV 1226 is more like a fused DhaK-DhaL. Interestingly, MRSA Mu50 also contains a highly conservative putative PTS-dependent Dha kinase elsewhere in the genome, i.e. DhaK (SAV0650), DhaL (SAV0651) and DhaM (SAV0653). However, no significant sequence similarity was detected between MRSA SAV1226 and any of the three domains of MRSA putative PTS-dependent Dha kinase. On the other hands, putative PTS-dependent Dha kinase (DhaK, DhaL, and DhaM) have no hit in DEG, thus are of less medicinal value. Previous study reported that in some case an extra phosphocarrier protein of PTS-dependent Dha kinase was detected elsewhere in the

genome [16]. Thus, determining whether SAV1226 is such an extra phosphocarrier protein of a Dha kinase or not is an initial step to characterize SAV1226, as well as further explore SAV1226 as a potential drug target MRSA Infections.

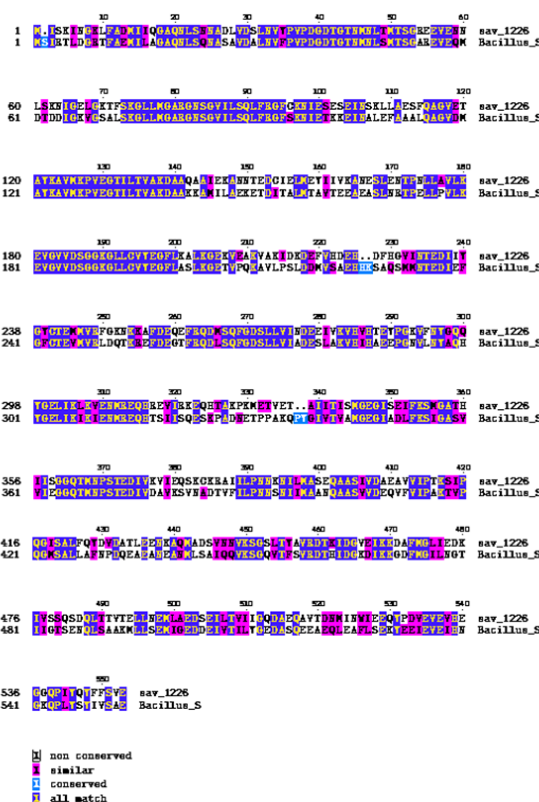


Fig. 3 Alignment of the amino acid sequences of MRSA SAV1226 with *Bacillus subtilis* yloV. Numbering of the amino acids is indicated on the left. Identical amino acid residues in the alignment are indicated in dark-blue shading and similar amino acid residues are indicated in purple shading. Gaps introduced during the alignment process are indicated as dots

B. Protein Cloning and Expression

The MRSA sav1226 gene was cloned from *Staphylococcus aureus* subspecies *aureus* Mu50 strain (ATCC, 700699D-5) into the BamH1 and KPN1 sites of pRSET-A vector and the ligation products were transformed into *Escherichia coli* DH5a. The restriction analysis of plasmid DNA digested by BamH1 and KpnI restriction endonucleases has revealed that the inserted fragment was about 1.6 kb as expected (Fig. 4).

DNA sequencing revealed that the sequence of inserted fragment was completely coincident with the sav1226 gene. *E. coli* BL21(DE3)pLysS Competent cells harboring the recombinant plasmid pRSET A/sav1226 with IPTG treatment at 37°C forms soluble proteins. His-tagged recombinant protein SAV1226 was purified using the QIAexpress Ni-NTA protein purification system

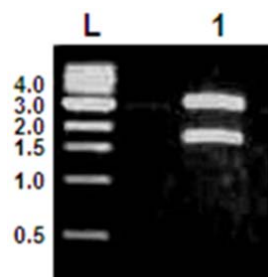


Fig. 4 Restriction analysis of recombinant pRSET A/SAV 1226 digested by BamHI and KpnI. Lane L: 1 kb DNA ladder; Lane 1: positive clone having a 2.9 kb backbone and a 1.6 kb SAV 1226 insert

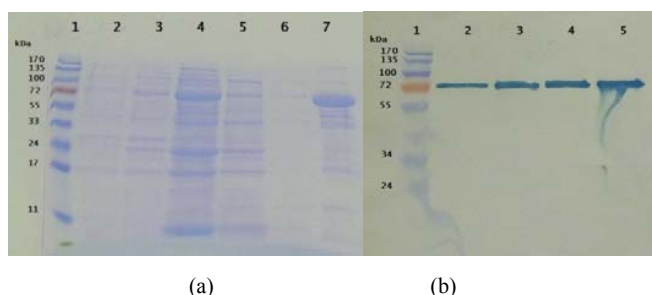


Fig. 5 Expression and Identification of MRSA SAV1226: (a) SDS-PAGE analysis. The samples were uninduced whole cells (lane 2), whole cells induced with IPTG (lane 3), cell lysate (lane 4), wash 1 from the Ni-NTA column (lane 5), wash 2 from the Ni-NTA column (lane 6), pooled fractions from the Ni-NTA column (lane 7), respectively. Lane 1, prestained protein ladder (Fisher BioReagents). (b) Western blot assay. Expression of MRSA SAV1226 was confirmed by Western blotting with mouse anti-His antibody. The samples were uninduced whole cells (lane 2), whole cells induced with IPTG (lane 3), cell lysate (lane 4), pooled fractions from the Ni-NTA column (lane 5), respectively. Lane 1, prestained protein ladder (Fisher BioReagents); protein purification system (Qiagen)

The purity and sizes of the recombinant proteins were assessed by SDS-PAGE (Fig. 5). Fig. 5 (a) shows SDS-PAGE analysis of the expression of N-terminal His tagged SAV 1226 protein (6.5 kDa of MRSA SAV 1226 plus 0.5 kDa of the length of the his tag), which was further confirmed by Western blotting with mouse anti-His antibody, as shown in Fig. 5 (b).

C. MS analysis ESI-TOF

Mass spectrometry was used to verify the protein size. The molecular weight of MRSA SAV1226 was measured to be 64,660.1 Da (Theoretical 64,498 Da) (Fig. 6). The extinction coefficient of MRSA SAV1226 at 280 nm was measured to be $24,045.8 \text{ cm}^{-1} \text{ Mol}^{-1}$ (Theoretical $33,663 \text{ cm}^{-1} \text{ Mol}^{-1}$).

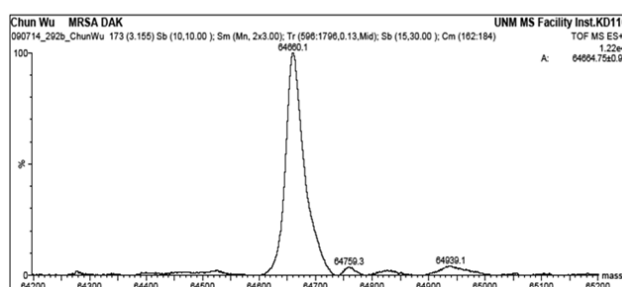
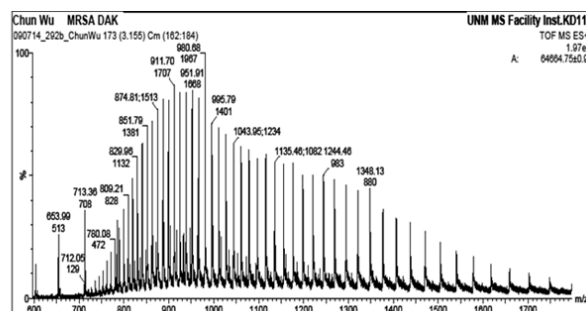


Fig. 6 ESI-TOF Results: (a) ESI mass spectrum of MRSA SAV1226; (b) deconvolution of MRSA SAV1226 mass spectrum for molecular weight determination

Although MALDI-TOF MS analysis (Table I) confirmed the bioinformatics prediction that the closest paralogue of MRSA SAV1226 is in *Bacillus subtilis*, the identity of MRSA SAV1226 remains unknown since none of the similar proteins to the hypothetical MRSA SAV1226 have been characterized. Work was continued under the assumption that the hypothetical MRSA SAV1226 is the fused DhaK-DhaL domain of Dha kinase. Kinetic assay was used to determine the phosphoryl group donor in the production of dihydroxyacetone phosphate.

D. Substrate Recognition

Two parallel kinetic assays were performed assuming hypothetical MRSA SAV1226 protein is either the ATP-dependent Dha kinase or the DhaK-DhaL fused subunits of PTS-dependent Dha kinase. For the latter DhaM, HPr and EI were added to the assay mixture [15]. No activity was detected in either condition.

TABLE I
MALDI-TOF MS ANALYSIS OF HYPOTHETICAL MRSA SAV1226

Protein Name	Species	Protein MW	Protein Score C.I. %	Protein Function
<i>hypothetical protein SARI202</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> MRSA252]		60463.35938	100	uncharacterized
<i>hypothetical protein MW1109</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> MW2]		60417.32813	100	uncharacterized
<i>DAK2 domain-containing protein</i> [<i>Staphylococcus epidermidis</i> RP62A]		60732.14844	100	uncharacterized
<i>Dihydroxyacetone kinase</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> USA300_TCH1516]		19520.89063	100	uncharacterized
<i>Dak phosphatase</i> [<i>Exiguobacterium sibiricum</i> 255-15]		60844.71875	100	uncharacterized
<i>Dihydroxyacetone kinase</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> USA300_TCH1516]		38611.23828	100	uncharacterized
<i>hypothetical protein MW1109</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> MW2]		60417.32813	100	uncharacterized
<i>hypothetical protein SE0901</i> [<i>Staphylococcus epidermidis</i> ATCC 12228]		60771.19141	100	uncharacterized
<i>Dihydroxyacetone kinase</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> USA300_TCH1516]		38611.23828	100	uncharacterized
<i>hypothetical protein SAV1226</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> Mu50]		60477.37891	100	uncharacterized
<i>dihydroxyacetone kinase family protein</i> [<i>Bacillus amyloliquefaciens</i> FZB42]		59617.23047	100	uncharacterized
<i>Dak phosphatase</i> [<i>Bacillus coahuilensis</i> m4-4]		60426.60156	100	uncharacterized
<i>hypothetical protein ABC2309</i> [<i>Bacillus clausii</i> KSM-K16]		60034.46094	100	uncharacterized
<i>hypothetical protein BSU15840</i> [<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168]		59459.75	99.998	uncharacterized
<i>Dihydroxyacetone kinase</i> [<i>Staphylococcus aureus</i> subsp. <i>aureus</i> USA300_TCH1516]		19520.89063	99.989	uncharacterized
<i>hypothetical protein ABC2309</i> [<i>Bacillus clausii</i> KSM-K16]		60034.46094	96.068	uncharacterized
<i>Dak phosphatase</i> [<i>Exiguobacterium sibiricum</i> 255-15]		60844.71875	93.768	uncharacterized
<i>Dak phosphatase</i> [<i>Bacillus coahuilensis</i> m4-4]		60426.60156	67.294	uncharacterized

IV. CONCLUSION

In this study, we cloned, expressed and purified a hypothetical protein SAV1226 from MRSA Mu50 strain for enzymological study in an attempt to explore a potentially novel drug target for MRSA infections. Although the results suggested that hypothetical protein SAV1226 is neither a domain of an ATP dependent Dha kinase nor of a PTS Dha kinase and the function of hypothetical protein SAV1226 remains unknown, our efforts in substrate recognition narrowed down the scope of the study. Overall, the essential nature of SAV1226 makes it an attractive potential drug target for MRSA infections.

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