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Phenotypic characterization of *sarR* mutant in *Staphylococcus aureus*

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ABSTRACT

Multiple factors of *Staphylococcus aureus* are involved in infection. Expression of these factors is controlled by multiple regulatory systems such as, the Sar family of transcriptional regulators. The staphylococcal specific Sar family of proteins are involved in expression of numerous target genes involving virulence, autolysis, biofilm formation, antibiotic resistance, oxidative stresses, and metabolic processes. Genetic and biochemical characterization of several *sar* family genes have been studied. However, less is known about the phenotypic properties of the *sar* family mutants, except *sarA* mutant in *S. aureus*. In this report, various studies such as phenotype microarray, autolytic, hemolytic, protease and DNase assays were performed to study the phenotypic properties of *sarR* mutant, a member of the *sar* family mutants. Phenotypic microarray for growth kinetic analysis identified eight substances (e.g., chlorhexidine, ceslodin, 3,5-dinitrobenzene, plumbagin, minocycline, dipeptide Arg–Ser, phenylarsine oxide and piperacillin), whose mode of actions were more specific towards cell wall or membrane. These findings were confirmed by various independent growth study experiments. Overall, the results from various phenotypic assays such as growth kinetics, autolysis, protease and DNase suggest that a *sarR* mutant strain is more sensitive to autolytic activities compared to the wild type, while less sensitive with respect to a *sarA* mutant strain.

1. Introduction

Staphylococcus aureus is an opportunistic pathogen that causes a broad range of human and animal infections. The spectrum of infections include: acute food poisoning, pneumonia, meningitis, skin conditions (e.g., acne, boils or cellulits), arthritis, osteomyelitis, endocarditis, and toxic shock syndrome. *S. aureus* is a major cause of hospital acquired (nosocomial) infections due to surgical wounds and infections associated with in-dwelling medical devices [2,13,22,23]. In the US, staphylococcal related infections account for 19% surgical sites and catheter related infections, 12% of all nosocomial infections, 16% of bacteremia cases, and 20% of lower respiratory tract infections [15,22,23]. Most of these infections begin as an infection of intravenous cannulas that subsequently spread to the bloodstream. Once bacteria enter the bloodstream they invade various host tissues, increasing the risk of developing endocarditis and other metastatic complications especially in immunocompromised patients. Despite antimicrobial therapies, the morbidity and mortality associated with *S. aureus* infections remains high, due to the organism's ability to develop resistance to virtually all antibiotics, including vancomycin. Also alarming, once inside the host, *S. aureus* can thwart host defense systems, primarily provided by neutrophils and macrophages; consequently allowing for persistent infections [13,15,20]. Recent development and spread of community-associated methicillin resistant *S. aureus* (CA-MRSA) strains in the various parts of world as well as the annual direct costs associated with the staphylococcal infections are a major health concern that has created an urgent need and a window of opportunity to introduce new treatment options.

Multiple factors expressed by *S. aureus* including those associated with life threatening infections, antibiotic resistance, and survival in distinct adverse environments, are tightly regulated. Expression of these factors is controlled either by two-component systems (e.g., *agr, saeRS, srrAB, arlRS, lytSR*) and/or by transcriptional regulators (e.g., *sarA*, *sigB*, *sar* family genes, *tcaRA*) [4,6–12,16–19,27,30–40,42,43,46–48]. Frequently in *S. aureus*, one target gene can be under the influence of several regulators or regulatory systems, which influence each other to ensure the expression of the target gene under favorable conditions. For example, the *agr* locus is regulated by several regulatory systems:

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auto-regulated by *AgrA* [21,37,38], regulated by *SrrAB* (at low oxygen) [40], as well as regulated by several members of *Sar* family transcriptional regulators [4,10]. More specifically, among the *Sar* family regulators, *SarA* and *SarR* partly activate *agr* transcription in the early phase of growth [35], whereas *MgrA*, *SarX* and *SarZ* are involved in the exponential to the stationary phases of growth [4,34]. *SarX* is a repressor [34] and others are putative activators for the *agr* transcription [4,10]. Similarly, expression of specific cytotoxins (*hla* and *hlb),* proteases *(sspA, aur, sspB, splA-F),* surface proteins (*spa, fnbA, fnbB*, *clfA, clfB*), and capsular polysaccharides (*cap5* and *8*) are regulated by several regulatory systems, including the *Sar* family proteins [8,10,38]. All these reports suggest the existence of a fine network for target gene regulation.

Among the ten major *sar* family genes, phenotypic, genetic and biochemical properties of the *sarA* gene has been well studied [4,10], although the exact mechanism of target gene regulation remains largely unknown. The *sarA* locus is known to up-regulate the synthesis of fibronectin and fibrinogen binding proteins, hemolysins (α-, β-, and δ-), enterotoxins, TSST-1 toxin and capsule biosynthesis genes and to down-regulate proteases, protein A and a collagen binding protein. *SarA* has been shown to bind to several regulatory and target gene promoter regions (e.g., *agr*, *sarS, rot, sarV*, *sarT*, *hla*, *fnb*, *spa, cna, bap, icaRA*) to modulate gene transcription, thus implicating both *agr*-dependent and *agr*-independent pathways as well as direct and indirect mode for *SarA*-mediated regulation [4,9–12,35,42]. In addition, other nine *Sar*-family proteins (e.g., *SarR*, *SarS*, *SarT*, *SarU, SarV*, *SarX*, *SarZ*, *Rot*, and *MgrA*) are partially characterized in *S. aureus* [4,9,19,27,31,34,36,45–48,50,51]. The *Sar* family proteins are homologous to each other (22–35% identity and 48–65% similarity to *SarA*) as well as homologous to the *MarR* family of transcriptional regulators (about 45% homology and 29% identity) in Gram-negative bacteria [1,24–26]. The *Sar* protein family members are able to regulate numerous target genes either by binding directly to promoter regions or by indirectly via other regulatory systems [4,10,38]. In fact, transcriptional profiling has shown that individual deletion or inactivation of *sarA*, *rot*, *mgrA,* and *sarZ* alters expression of roughly 120 genes, 168 genes, 355 genes, and 87 genes, respectively [14,28,45,51].

Structural analysis for four *Sar* family proteins suggests that smaller *Sar* family proteins exist as dimers (e.g., *SarA*, *SarR*, *MgrA*), while the larger proteins with two homologous domains are monomers (e.g., *SarS*) [24–26]. Crystal structures of the known *Sar* family proteins are homologous to the winged-helix family of transcription factors [1,24–26]. Structural analysis also suggests that these proteins have similar DNA-binding domains as evident from the site-directed mutagenesis of *SarA* and *SarR* proteins [26,35]. In spite of the conserved DNA binding domains among the *Sar* family proteins, the DNA-binding sites mapped by DNase I footprinting for four *Sar* family proteins, *SarA* [12,35,42], *SarR* [31,35], *MgrA* [32] and *SarT* [47] are different. Thus, overall published literature suggest that *sar* family genes are important in regulating large numbers of target genes both directly and indirectly, although much of the phenotypic, biochemical, and exact mechanisms of target gene regulation by most of the *sar* family genes remains largely unknown.

SarR was identified as a 13.6 kDa protein using DNA-affinity column and the gene has been characterized by both genetically and biochemically [30,31,35]. The *SarR* binds to the upstream promoter region of the P1 *sarA* to partially down-regulate the expression of *SarA* [31]. In fact, expression of the *sarA* gene is also autoregulated [11]. Analysis for expression of *SarR* in six different *S. aureus* strains demonstrated that the maximum expression is in the exponential phase of growth [5]. Inactivation of *sarR* has positive effects on the transcription of the *agr* locus by directly binding to the intergenic promoter region of the *agr* locus [35]. Inactivation of the *sarR* gene has a positive effect on the maximal transcription of *aur* (aureolysin) and *sspA* (serine protease) in *S. aureus* [18]. Regulation of transcription of the *sarR* gene affected by the salicylate [44], indicating that small molecule can modulate the expression of the *sarR* gene and also it has been shown that adjacent gene, *rsr*, repress the transcription of the *sarR* gene [48]. Taken together, published results on genetic, biochemical, and structural studies of *SarR* clearly indicate the importance of the *sarR* gene in regulation of different target genes in *S. aureus*. But little is known regarding the phenotypic characterization, and the exact mechanism of target gene regulation in *S. aureus*. Therefore, this study will focus on the phenotype characterization of the *sarR* gene using various approaches such as phenotypic microarray (PM), protease, hemolytic, and autolytic activities.

2. Results and discussion

2.1. Phenotypic microarray (PM) analysis of the sarR mutant

Sar family transcriptional regulators in *S. aureus,* are known to be involved in regulation of numerous genes both directly and indirectly. Considering the importance of the *Sar* family regulators in *S. aureus* virulence gene regulation, the goal of this study is to determine cellular phenotypes of the *sarR* mutant. To assess the cellular phenotype of the *sarR* gene and/or to determine agents or chemicals that interfere with the function of the *sarR* gene in *S. aureus*, phenotypic microarray analysis was performed using various metabolic, pH, osmotic, toxic ion, and chemical and antibiotic panels. Phenotypic microarray analysis was used to provide a comprehensive cellular profile which is consequently useful to identify gene function. Notably, this study is one of the first kinds to investigate the cellular phenotype of a regulatory system in *S. aureus.*

PM analysis relies on bacterial growth in media containing various growth sources, inhibitors or toxic compounds. Thus, analysis to measure and compare the growth kinetics of the *sarR* mutant with the wild type strain under nutrient-rich standard growth conditions was conducted. Fig. 1A demonstrates the growth kinetics for the wild type and isogenic *sarR* mutant strains for the SH1000 background in half strength MH media. Additional growth analysis in a different background (CA-MRSA strain MW2) as well as in different media (TSB and minimum M9) showed similar growth patterns, hence, demonstrating no defect in growth due to inactivation of the *sarR* gene – irrespective of media or background used. A complete PM analysis for the wild type SH1000 and isogenic *sarR* mutant strains is shown Fig. 1B, which consists of the consensus plot, derived from two duplicate experimental runs. Results from the PM metabolic panels (PM1-8) clearly indicate that there is no significant difference in the carbon utilization panels (PM1-2). The signals for the rest of the metabolic panels (PM3-8) were very low, which made the comparison somewhat difficult, with the exception of growth kinetics in plate PM06, C3 containing Arg–Ser dipeptide. Therefore, these results demonstrate that most of the metabolic compounds tested in PM1-8 panels under PM growth conditions, do not have any effect on the growth of the *sarR* mutant. Overall the PM analysis of the *sarR* mutant against the wild type *S. aureus* SH1000 identified 14 potentially significant agents (Table 1) which showed either a gain or a loss of growth phenotype in the *sarR* mutant strain. Results from the PM consensus report indicate the *sarR* mutant showed a gain of growth phenotype to chlorhexidine (PM19C4 – a membrane and electron transport inhibitor), plumbagin (PM18H10 – oxidizing inhibitor), 3, 5-dinitrobenzene (PM20 G7-8 – respiration and ionophore inhibitor), and cefsulodin (PM17H3-4 – cell wall inhibitor). The PM also showed a gain of phenotype in the *sarR* mutant to seven antibiotics