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Staphylococcus aureus Virulence Factors Synthesis is Controlled by Central Metabolism

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STAPHYLOCOCCUS AUREUS VIRULENCE FACTORS SYNTHESIS IS CONTROLLED BY CENTRAL METABOLISM

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Staphylococcus aureus is a versatile pathogen that can survive in diverse host environments. This versatility depends on its ability to sense nutrients and respond by modulating gene expression, including the synthesis of virulence determinants. In addition to its ability to synthesize virulence factors, the capacity of *S. aureus* to form biofilms is an important mediator of virulence in certain infections. Biofilms are a complex aggregation of bacteria commonly encapsulated by an adhesive exopolysaccharide matrix (polysaccharide intercellular adhesin; PIA). To study *S. aureus* biofilm formation, we assessed the metabolic requirements of *S. aureus* growing in a biofilm and found the bacteria extracted glucose and accumulated lactate, acetate, formate, and acetoin. Additionally, *S. aureus* selectively extracted six amino acids from the culture medium (serine, proline, arginine, glutamine, glycine, and threonine). The major staphylococcal exopolysaccharide, PIA, is synthesized when the tricarboxylic acid (TCA) cycle is repressed. To better understand TCA cycle-dependent regulation of PIA and virulence factor synthesis in *S. aureus*, we artificially induced the TCA cycle by limiting its ability to exogenously acquire a TCA cycle-derived amino acid (*i.e.*, glutamine) by inactivating the glutamine permease gene (*glnP*) and assessed the effects on biofilm formation and virulence factor synthesis. We found that inactivation of this major glutamine transporter increased TCA cycle activity, transiently decreased PIA synthesis, and significantly reduced in *vivo* virulence in

a rabbit endocarditis model, establishing a causal relationship between TCA cycle activity and virulence factor synthesis. This causal relationship between the TCA cycle and virulence factor synthesis suggests there are regulatory proteins connecting metabolism and the regulation of virulence factor synthesis. This regulation is likely to occur when a metabolite-responsive regulator responds to changes in TCA cycle associated biosynthetic intermediates, the redox status, and/or ATP. In related work, NMR metabolomic analysis of *S. epidermidis* indicated that TCA cycle stress altered the intracellular concentration of ribose. Using this information, three putative ribose-responsive RpiR-family regulators (orfs SAV0317, SAV0193 and SAV2315) were identified in *S. aureus* strain UAMS-1. The proteins encoded by *sav0317* and *sav0193* regulate hexose monophosphate shunt transcription and alter virulence factor synthesis by increasing the transcription or stability of RNAIII. These data confirm a close linkage of central metabolism and virulence factor synthesis in *S. aureus* and establish that this metabolic linkage can be manipulated to alter infectious outcomes.

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I. Introduction

Staphylococcus aureus is a Gram-positive bacterium and an important human and animal pathogen. Humans and animals are the natural reservoir of *S. aureus*. In fact, approximately 20% of the population carry *S. aureus* chronically and 60% intermittently on the skin and mucosa, usually in the anterior nares (Sivaraman, Venkataraman et al. 2009). *S. aureus* causes a wide variety of infections ranging from mild skin infections, to life-threatening diseases such as necrotizing pneumonia and bacteremia. *S. aureus* is the most common cause of bloodstream, skin and soft tissue, and lower respiratory tract infections in the United States and Canada (Diekema, Pfaller et al. 2001). It is also the leading cause of hospital-associated infections in the United States (Styers, Sheehan et al. 2006). The economic impact on the U.S. healthcare system from *S. aureus* infections is $14.5 billion annually (Noskin, Rubin et al. 2007). With the increasing incidence of both community and hospital acquired staphylococcal infections and the prevalence of multi-drug resistance strains, *S. aureus* infections remain a huge challenge to scientists and physicians (Bamberger and Boyd 2005; Grundmann, Aires-de-Sousa et al. 2006).

S. aureus is a versatile bacterial pathogen that can survive in diverse host environments. This versatility depends on its ability to acquire and utilize nutrients from different sources; hence, *S. aureus* use nutrient availability as an indicator of their environment. Bacteria can sense nutrients and respond by modulating gene expression including the synthesis of virulence factors. Therefore, the metabolic signals encountered by bacteria not only help them survive in temporary adverse conditions, but also play a crucial role in pathogenesis. The evolutionary advantage of this linkage allows the

bacteria to adapt to ever-changing microenvironments during the establishment and progression of infections by regulating its virulence pattern. Recent advances have provided further support for the close linkage between metabolism and virulence synthesis, as evidenced by the fact that some metabolic regulatory protein, such as CcpA and MgrA, are regulators of virulence (Ingavale, van Wamel et al. 2005; Luong, Dunman et al. 2006; Seidl, Stucki et al. 2006; Seidl, Goerke et al. 2008; Seidl, Muller et al. 2009; Majerczyk, Dunman et al. 2010).

This review will first give an overview of staphylococcal central metabolism, virulence factors and their regulation. Subsequently, we will focus on how the metabolic regulators affect virulence in *S. aureus*.

II. Overview of Central Metabolism in *S. aureus*

Staphylococci metabolize glucose through the Embden-Meyerhof-Parnas (glycolytic), pentose phosphate, and tricarboxylic acid (TCA) cycle pathways. Glucose enters the glycolytic pathway in the form of glucose-6-phosphate that can be generated during the PTS-mediated transport or undergo direct phosphorylation by glucose kinase. The processing of glucose-6-phosphate by the glycolytic pathway produces 2 molecules of pyruvate. During anaerobic growth, pyruvate is primarily reduced to lactic acid with the concomitant oxidation of reducing equivalents. Under aerobic conditions, the end product of the glycolytic pathway, pyruvate, is decarboxylated to acetyl-coenzyme A. Acetyl-coenzyme A is converted into acetylphosphate that is used to produce ATP and

acetate by substrate level phosphorylation. Acetate accumulates in the culture medium during the exponential phase of growth. When the growth becomes nutrient-limited, bacteria exit the exponential growth phase and utilize the accumulated acetate as a carbon source; this process requires a fully functional TCA cycle (Somerville, Chaussee et al. 2002). The glycolytic pathway and TCA cycle produce ATP, as well as large quantities of reducing equivalents in the form of NADH and $FADH_2$. To maintain redox balance, these reducing equivalents must be oxidized. Under aerobic conditions, staphylococci use the electron transport chain to fulfill this purpose. Electrons flow from NADH and $FADH_2$ to menaquinone, then transfer to cytochrome c, finally to oxygen to produce water and proton gradient. The proton gradient drives the transmembrane F_0F_1ATPase to produce ATP.

Bacteria need to shift their metabolic pattern to grow most efficiently under different carbon sources and growth phases. For example, when glucose is abundantly available during the exponential phase, the TCA cycle is repressed. In contrast, during the post-exponential phase of growth, *S. aureus* begin to reintroduce the products of carbon overflow, such as acetate, into central metabolism through activation of the TCA cycle. In this process, bacteria drive not only gluconeogenesis but the synthesis of biosynthetic precursors of amino acids and nucleic acids, and the generation of reducing potential. Biosynthetic reactions often require oxidized dinucleotides as cofactors; hence, TCA cycle generated reducing potential must be oxidized. In staphylococci, oxidation of reduced dinucleotides is achieved through the electron transfer chain and oxidative phosphorylation. In other words, the growth phase change leads to dramatic changes in

the metabolic status of the bacterium. At the same time, virulence factor synthesis parallels the events of the metabolic changes during the phase change, namely, the bacterial transition from cell wall-associated to secreted virulence factor synthesis (Novick and Geisinger 2008). In the next chapter I will discuss those changes in detail.

III. Overview of Virulence Factors and Their Regulation in *S. aureus*

Bacteria cause disease by two major mechanisms: (1) Invasion and inflammation. This includes mechanisms for colonization, synthesis of extracellular molecules that facilitate adherence and the ability to evade host defenses. (2) Toxin production. Exotoxins produced by *S. aureus* are responsible for damaging host tissue and promoting dissemination. A third virulence mechanism that is important in certain infections is the ability to form biofilms. Biofilms are a complex aggregation of bacteria encapsulated by an extracellular polymeric matrix and are usually attached to a surface (Costerton, Stewart et al. 1999; Götz 2002; Otto 2008). Biofilms are of great clinical significance because bacteria in a biofilm have increased resistance to environmental stress, antimicrobial agents and host immunological defenses (Otto 2008). In the first section, I will give a brief introduction to cell wall-associated virulence factors that are involved in invasion and inflammation, and, in the latter sections, toxins and biofilms will be discussed.

Overview of virulence factors

Cell wall-associated virulence factors include adhesins, exopolysaccharides, and cell wall components such as peptidoglycan and teichoic acid. *S. aureus* synthesizes two broad categories of adhesins, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and secreted expanded-repertoire adhesive molecules (SERAMs) (Clarke and Foster 2006). MSCRAMMs are covalently bound to the peptidoglycan and function in helping bacteria adhere to different host extracellular matrices such as collagen, fibrinogen and fibronectin. This group of adhesins include protein A (Spa), collagen adhesin (Cna), clumping factor (ClfA and ClfB), and fibronectin-binding proteins (FnBPs). In addition to adhesion functions, they also participate in other aspects of pathogenesis such as evasion of the host immune response. As an example, protein A interferes with the host immune response by sensitizing B cells or depleting innate-like B cells (Goodyear and Silverman 2004; Bekeredjian-Ding, Inamura et al. 2007). Protein A can enhance platelet aggregation and undermine phagocytosis by binding to antibodies including IgG, IgA and IgE (O'Brien, Kerrigan et al. 2002). Protein A also contributes to the adherence to damaged endothelial surfaces by binding to von-Willebrand factor (Hartleib, Kohler et al. 2000), and induces local inflammation by binding to tumor-necrosis factor receptor-1 (TNFR-1) (Gomez, Lee et al. 2004).

The second group of adhesins, SERAMs, is secreted but partially bound to the cell wall. SERAMs include the extracellular adhesive protein (Eap), extracellular fibrinogen-binding protein (Efb), and extracellular matrix protein (Emp), and their main function is to modulate the host immune system. For instance, Eap inhibits leukocyte

migration by interacting with inter-cellular adhesion molecule-1 (ICAM-1) on the endothelial cell surface and altering T cell function (Chavakis, Wiechmann et al. 2005). Efb can prevent complement activation by changing the conformation of component 3 (C3) (Hammel, Nemecek et al. 2007).

Exopolysaccharides are important virulence factors in *S. aureus*. There are two types of exopolysaccharides: capsule polysaccharides and polysaccharide intercellular adhesin (PIA). There are a total of 11 capsule serotypes identified in *S. aureus,* with serotype 5 and 8 being the most common (O'Riordan and Lee 2004). Capsular polysaccharides are involved in the protection of bacteria from the host immune response by interfering with phagocytosis (Peterson, Wilkinson et al. 1978; Guidry, Oliver et al. 1991). Capsule also contributes to the survival of bacteria in neutrophils (Kampen, Tollersrud et al. 2005). PIA, a biofilm exopolysaccharide, is chemically composed of poly-*N*-acetyl-glucosamine (Götz 2002) and it is important for bacterial aggregation of staphylococci during biofilm formation.

The staphylococcal secreted toxins include cytotoxins, superantigens, proteases, lipase and coagulase. Cytotoxins are a group of toxins that can lyse host cells and include α-toxin (Hla), β-toxin (Hlb), γ-toxin (Hld), and leukocidins (LukF-PV). These toxins not only lyse host cells, but also alter the host immune response such as inducing caspase-dependent and caspase-independent apoptosis (Haslinger, Strangfeld et al. 2003). Superantigens are a class of protein toxins that can cause nonspecific T-cell activation and massive cytokines release (Fraser and Proft 2008). *S. aureus* superantigens include several enterotoxins (SEs), toxic shock toxin-1 (TSST-1), and exfoliative toxins (ETs).

Enterotoxins cause food poisoning, whereas toxic shock toxin is responsible for toxic shock syndrome (Fraser and Proft 2008). Exfoliative toxin can change the conformation of desmoglein-1, a cell-cell adhesin in the epidermis, eventually causing degradation and exfoliation (Nishifuji, Sugai et al. 2008). Proteases (SspA, SspB) are involved in tissue invasion and immunomodulation (Rudack, Sachse et al. 2009). For example, proteases can degrade or activate extracellular proteins such as host immunoglobulin, antimicrobial peptides, and host tissues (Bergmann and Hammerschmidt 2007).

Recently biofilm formation by *S. aureus* has been recognized as an important mechanism for causing disease (Stewart and Costerton 2001; Otto 2008). Biofilm formation is a well regulated multi-step process in which bacteria undergo a dramatic lifestyle switch from a planktonic unicellular state to an adherent multicellular state. Biofilms can form by attaching to a biotic or an abiotic surface where they will grow and secrete several small molecules (adhesin) that bind the bacterial cells together. In an *S. aureus* biofilm, the predominant adhesin that facilitates binding bacteria together is the exopolysaccharide PIA (Götz 2002). In some cases, cell wall-related proteins may function as alternative aggregating substances, such as accumulation-associated protein (Aap) (Hennig, Nyunt Wai et al. 2007), fibronectin binding protein (FnBP) and biofilm-associated protein (Bap) (O'Neill, Humphreys et al. 2009). Additionally, extracellular genomic DNA (eDNA) and teichoic acids from lysed cells may also facilitate aggregation presumably due to their polyanionic nature (Gross, Cramton et al. 2001; Rice, Mann et al. 2007). Upon maturation, portions of biofilms detach from the surface and disseminate. Two mechanisms of biofilm detachment have been proposed in *S. aureus*: enzymatic

degradation of proteinaceous aggregation factor and disruption of non-covalent forces by surfactant-like small molecules (Kong, Vuong et al. 2006; Boles and Horswill 2008). During infection, attachment is a crucial part of the colonization on host tissues or on indwelling medical devices, whereas detachment is a prerequisite for the dissemination of an infection.

Regulatory mechanisms of virulence factors

Expression of virulence factors is controlled by bacterial cell density and many environmental factors such as pH, oxygen and carbon dioxide. These factors function through different regulatory systems which can be divided into two broad categories: two-component signal transduction systems and global transcriptional regulators (Cheung and Zhang 2002; Cheung, Bayer et al. 2004). The best studied two-component regulatory system is the accessory gene regulator (*agr)* quorum sensing system. Bacteria communicate their presence by releasing and sensing signaling molecules, a phenomenon called quorum sensing. Many bacterial behaviors including growth, virulence, and antibiotic resistance are regulated by quorum sensing (Keller and Surette 2006). Quorum sensing is also involved in biofilm formation and dispersal (Boles and Horswill 2008).

The *agr* locus regulates more than 70 genes including 23 virulence genes (Ziebandt, Becher et al. 2004). It contains two divergent promoters, P2 and P3. The P2 promoter drives transcription of an auto-inducing signal transduction module which is composed of four genes, *agrBDCA*. *agrD* encodes the auto-inducing peptide precursor

which is processed by the trans-membrane endopeptidase AgrB. The mature AIP is a short peptide of 7-9 amino acids with a pentopeptide thiolactone ring. AgrC and AgrA comprise a two-component histidine kinase system, where AgrC is histidine kinase, and AgrA is the response regulator. AgrC has an N-terminal transmembrane sensor domain, and a C-terminal histidine protein kinase domain (Novick and Geisinger 2008). When the level of AIP reaches a threshold concentration, AgrC will be auto-phosphorylated leading to the phosphorylation of AgrA. Phosphorylated AgrA activates transcription from the P2 and P3 promoters. Activation of the P2 promoter completes the auto-inducing circuit. P3 promoter activation produces a regulatory RNA called RNAIII that is the effector molecule of the system. RNAIII is a large regulatory RNA with fourteen hairpin stem loops (Benito, Kolb et al. 2000) and it also encodes for δ-toxin (*hld*) (Janzon and Arvidson 1990). RNAIII activates the expression of exoprotein genes such as *hla* (α-toxin) (Morfeldt, Taylor et al. 1995) and *sspA* (serine proteinase) (Oscarsson, Tegmark-Wisell et al. 2006), and represses several cell wall-associated protein genes, such as *fnbA, fnbB* (fibronectin binding protein A, B) and *spa* (protein A) (Novick, Ross et al. 1993). RNAIII acts at both the transcriptional and post-transcriptional levels. For example, the interaction of RNAIII and *spa* mRNA involves base-pairing of these two RNAs, which prevents hair-pin formation in the ribosome-binding region of the *spa* mRNA (Novick, Ross et al. 1993; Gao and Stewart 2004). RNAIII also can function as an antisense RNA at the post-transcriptional level, which leads to a rapid degradation of target mRNA (Huntzinger, Boisset et al. 2005; Chevalier, Boisset et al. 2010).

The staphylococcal accessory element (*sae*) locus codes for another "two-component" system that is comprised of *SaePQRS* (Giraudo, Calzolari et al. 1999). SaeR and SaeS display strong homology to a response regulator (RR) and histidine kinase (HK) of typical bacterial two-component systems. The function of SaeP and SaeQ in regulation is unknown (Steinhuber, Goerke et al. 2003). The *sae* system regulates the expression of many virulence factors involved in bacterial adhesion, toxicity and immune evasion (Rogasch, Ruhmling et al. 2006). For example, α, β, and γ-hemolysins are positively regulated by SaeR (Goerke, Fluckiger et al. 2005; Liang, Yu et al. 2006), and protein A is negatively regulated by SaeR (Giraudo, Cheung et al. 1997). Additionally, the *sae* locus is important for in vivo virulence in *S. aureus* animal infection models, and inactivation of *sae* locus decreases the virulence (Novick and Jiang 2003; Goerke, Fluckiger et al. 2005; Liang, Yu et al. 2006).

In addition to two-component systems, several global transcriptional regulators function in the regulation of virulence in *S. aureus* (Cheung, Nishina et al. 2008). Staphylococcal accessory regulator A (SarA) is a well-studied example of a global transcriptional regulator. SarA was originally thought to antagonize the effect of *agr*, but later was found to have an additive effect (Cheung, Koomey et al. 1992; Gillaspy, Lee et al. 1998; Bronner, Monteil et al. 2004). Mutations in either *agr* or *sarA* attenuate virulence, however, *agr-sarA* double mutant leads to the loss of virulence (Cheung, Nishina et al. 2008). SarA regulates the expression of a numerous virulence factors including increasing the expression of FnBPs, α-toxin and decreasing the expression of protein A and proteases. SarA acts by either directly binding to the promoter region,

stabilizing mRNA, or indirectly through other regulators such as the *agr* system (Bronner, Monteil et al. 2004). In addition, SarA is a positive regulator of *ica* operon transcription, and enhances biofilm formation (Tsang, Cassat et al. 2008). Mutation of *sarA* leads to decreased biofilm formation and increased antimicrobial susceptibility in *S. aureus* (Weiss, Spencer et al. 2009). Staphylococci have several *sar* homologues which fall into two categories: small proteins and large proteins. Both categories are members of the MarR winged-helix family of transcriptional regulators (Bronner, Monteil et al. 2004). The small proteins are 13-16kDa in size and include SarA, SarR, SarT and SarX. The large proteins are 29-30kDa in size and include Rot, SarS, SarT, SarU, SarV, MgrA and TcaR.

In bacteria, the RNA polymerase holoenzyme is composed of core enzyme and σ factor. The core enzyme contains four subunits α_2, β and β'. The holoenzyme can recognize specific promoter regions and initiate transcription. The alternative sigma factor σ^B was first identified as a master regulator of non-growing *Bacillus subtilis* (Haldenwang and Losick 1979). σ^B regulates more than 150 genes involved in the general stress response during stress and energy depletion conditions (Hecker, Pane-Farre et al. 2007). Similarly, σ^B in *S. aureus* participates in the general stress response; however, it also has a significant function during an infection (Wu, de Lencastre et al. 1996; Kullik, Giachino et al. 1998). σ^B affects at least 30 virulence genes including *sarA* transcription, by binding to the P3 promoter region of *sarA* (Bischoff, Entenza et al. 2001). It is a positive regulator of capsule, fibronectin A, coagulase and a negative regulator of hemolysins and serine protease A (Bischoff, Dunman et al. 2004; Ziebandt, Becher et al.

2004; Entenza, Moreillon et al. 2005). In *S. epidermidis*, σ^B promotes biofilm formation by repressing *icaR* transcription (Knobloch, Jager et al. 2004); however, it has only a minor function in *S. aureus* biofilm formation (Valle, Toledo-Arana et al. 2003). The regulatory activity of σ^B is modulated by *rsb* genes (regulator of sigma B) that are located within the four-gene *sigB* operon (Kullik and Giachino 1997). In *S. aureus* there are three Rsb proteins: RsbU, RsbV and RsbW (Gertz, Engelmann et al. 2000). RsbU is a strong activator of σ^B, functioning as a putative sensor that can respond to micro-environmental changes by autophosphorylation (Jonsson, Arvidson et al. 2004; Palma, Bayer et al. 2006). Mutation of RsbU leads to defects in biofilm formation in *S. epidermidis* (Knobloch, Jager et al. 2004). RsbW and RsbV act as an anti-σ^B and an anti-anti-σ^B. The anti-σ^B RsbW can bind to the σ^B protein, which prevents interaction of σ^B with RNA polymerase. Phosphorylated RsbU dephosphorylates RsbV, which then binds to the anti-σ^B RsbW. This binding disassociates RsbW from σ^B (Miyazaki, Chen et al. 1999); consequently, σ^B is free to associate with RNA polymerase core enzyme to activate the target genes (Bischoff, Dunman et al. 2004).

IV. Metabolic regulation of virulence

Catabolite-responsive regulators

Bacteria have evolved intricate mechanisms to use preferred carbohydrates that allow fastest growth (Görke and Stulke 2008). These mechanisms include induction of specific carbohydrate transport and utilization systems in the presence of the

corresponding carbon source and their repression when a more efficiently utilizable carbohydrate is present. This phenomenon is called carbon catabolite repression and it is a common mechanism by which both Gram-positive and Gram-negative bacteria regulate gene expression in response to carbon availability. In many low-GC Gram-positive bacteria such as *B. subtilis* (Sonenshein 2007; Fujita 2009), *Lactococcus lactis* (Zomer, Buist et al. 2007), and *Streptococcus pneumonia* (Iyer, Baliga et al. 2005) carbon catabolite regulation is mediated by the Catabolite control protein A (CcpA). CcpA is a member of the LacI/GalR family of transcription regulators that binds to catabolite responsive elements sites (*cre*-sites), a *cis*-acting sequence usually in the operator sequence.

The activity of CcpA depends on binding the co-repressor HPr (histidine-containing protein) or Crh (catabolite repression Hpr) in *B. subtilis* (Jankovic and Bruckner 2002; Jankovic and Bruckner 2007). However, in *S. aureus* only Hpr homologues has been identified (Deutscher, Francke et al. 2006). HPr is a dual function protein that functions as a component of the sugar phosphotransferase system (PTS system) and as a co-repressor of CcpA. Hpr has two phosphorylation sites, one is Ser46, and the other one is His15. When His15 is phosphorylated, HPr functions as a component of the PTS system. In the presence of a PTS sugar such as glucose, Ser46 is phosphorylated and HPr functions as a corepressor. The phosphorylation of Ser46 is catalyzed by Hpr kinase/phosphatase. A high concentration of ATP or fructose-1, 6-bisphosphate (FBP) enhances the kinase function of Hpr kinase/phosphatase, whereas a high concentration of inorganic phosphate favors its phosphatase activity. The complex of

CcpA and phosphorylated Hpr (CcpA-Hpr-Ser46-P) has an increased affinity to *cre*-sites, and enhances or represses gene expression (Seidel, Diel et al. 2005; Singh, Schmalisch et al. 2008).

CcpA regulates more than 100 genes in *S. aureus* (Seidl, Muller et al. 2009). Considering the importance of carbon metabolism in bacteria for energy and biosynthesis, it is reasonable to expect that virulence factor synthesis also responds to the availability of carbon sources; this is in fact the case. In addition to genes involving carbon acquisition and metabolism, several genes associated with virulence and antibiotic resistance are also regulated by CcpA (Iyer, Baliga et al. 2005; Seidl, Stucki et al. 2006; Abranches, Nascimento et al. 2008). The effect of CcpA on virulence regulation is complex, involving both direct and indirect effects. An example of direct regulation is *tst* gene encoding toxic shock toxin-1 (TSST-1) in *S. aureus* (Seidl, Bischoff et al. 2008). There are *cre*-sites present in all known *tst* genes. CcpA binding represses *tst* transcription and deletion of *ccpA* derepress the expression of TSST-1 (Seidl, Bischoff et al. 2008). Since a majority of CcpA regulated genes lack apparent *cre*-sites, CcpA may perform regulation without binding to *cre*-sites (Seidl, Muller et al. 2009). Staphylococci possess many metabolite responsive regulators, so the regulation may be mediated by those regulators that can sense the dramatic metabolic changes created by CcpA regulation. Some global regulators affected by CcpA, such as RNAIII may be involved in this indirect regulation, as inactivation of *ccpA* decreases RNAIII transcripts (Seidl, Stucki et al. 2006). Considering RNAIII is the negative regulator of *spa* (protein A) and a positive regulator of *hla* (α-toxin), CcpA may be the true regulator of *spa* and *hla*.

Support for this conjecture was observed when it was shown that a *ccpA* mutant has an increased protein A expression (Huntzinger, Boisset et al. 2005). Despite the CcpA-dependent regulation of *spa*, mutation of *ccpA* does not affect *hla* transcription (Seidl, Goerke et al. 2008), even with a putative *cre*-site present in the *hla* promoter region. Taken together, the mechanism of CcpA-dependent regulation remains to be clarified.

In addition to affecting virulence factor synthesis, CcpA positively affects biofilm formation. Deletion of *ccpA* in *S. aureus* strain SA113 significantly decreased biofilm formation under both stationary and flow-cell conditions (Seidl, Goerke et al. 2008). The decreased biofilm formation is likely due to its inability to synthesize PIA and release eDNA, which are two key components of the extracellular matrix of a staphylococcal biofilm (Mann, Rice et al. 2009). The enzymes necessary for PIA biosynthesis are encoded within the *ica* operon. The holin-like protein CidA enhances cell lysis and the release of eDNA (Rice, Mann et al. 2007). Transcription of both of *ica* and *cidA* are up-regulated by CcpA.

In addition to CcpA, CodY is another catabolite-responsive regulator involving virulence regulation in staphylococci. CodY was first identified in the non-pathogenic bacterium *B. subtilis* as a nutrient and stress condition responder (Sonenshein 2005; Sonenshein 2007). In *B. subtilis*, CodY represses the transcription of more than 100 genes during the exponential phase and derepresses them as nutrients become limited, making this a key regulator for entering the stationary phase and during sporulation (Slack, Serror et al. 1995; Sonenshein 2007). CodY regulated genes are primarily involved in carbon and nitrogen metabolism such as proteases, peptide transport, and amino acid synthesis.

CodY binds as a dimer to a conserved A/T rich region of DNA (AATTTTCWGAAATT) (Belitsky and Sonenshein 2008) in response to changes in the concentration of GTP, which is an indicator of the energy status of the cell (Sonenshein 2007). The intracellular concentration of GTP varies dramatically between rapidly growing bacteria (2mM) and stationary phase bacteria (300μM). When the concentration of GTP is high, the probability that it will bind CodY is increased and late gene expression is repressed. During the post-exponential phase, the lower GTP level results in a decreased affinity of CodY for its operator site, de-repressing transcription of the CodY regulon (Inaoka, Takahashi et al. 2003). In addition to GTP, branched-chain amino acids (BCAAs) also enhance CodY repressor activity by increasing its affinity for target DNA. Hence, the biosynthesis of BCAAs such as isoleucine, leucine, and valine are under CodY control (Shivers and Sonenshein 2004). In total, CodY activity is controlled by GTP and BCAAs, particularly isoleucine or valine. Decreased concentrations of any of these molecules will derepress CodY regulated genes.

Homologues of CodY have been identified in most low G+C Gram-positive bacteria including *Listeria monocytogenes* (Bennett, Pearce et al. 2007), *Streptococcus pneumoniae* (Hendriksen, Bootsma et al. 2008), *Clostridium difficile* (Dineen, Villapakkam et al. 2007) and *S. aureus* (Majerczyk, Sadykov et al. 2008; Pohl, Francois et al. 2009). In *S. aureus* more than 200 genes are direct targets of CodY (Majerczyk, Dunman et al. 2010). In addition to its role in amino acid metabolism and transport, CodY regulates synthesis of many virulence factors. Some of them are regulated directly by CodY, whereas others are regulated indirectly through the *agr* system. Several

virulence genes including the gene for α-toxin (*hla*) and the genes coding for capsule synthesis are regulated by CodY. The effect of CodY on α-toxin appears to be mediated by RNAIII as CodY inactivation leads to increased RNAIII transcription and a corresponding increase in *hla* transcription (Majerczyk, Sadykov et al. 2008). Similarly, CodY affects biofilm formation, as inactivation of CodY leads to increased *ica* transcription, and biofilm formation in *S. aureus* (Majerczyk, Sadykov et al. 2008). Since increased expression of hemolysins and proteases can provide amino acids and peptides to the bacteria, the regulatory linkage between CodY and virulence genes may provide evolutionary advantages especially during infections.

Nitrogen-responsive regulators

Nitrogen metabolism is critical for bacterial survival and is therefore tightly regulated. Glutamine and glutamate are the primary nitrogen donors in the bacteria; glutamine is also important for maintaining the osmotic balance (Anderson and Witter 1982). In *B. subtilis*, there are two repressors involved in glutamine metabolism, GlnR and TnrA (Schreier, Brown et al. 1989; Wray, Ferson et al. 1996). Both of regulators belong to the MerR transcriptional regulator family (Brown, Stoyanov et al. 2003). The *glnRA* operon is composed of two genes *glnA* which encodes glutamine synthetase and *glnR* (Schreier, Caruso et al. 2000). Together GlnR and TnrA regulate gene expression in response to the availability of nitrogen (Sonenshein 2007). GlnR only represses genes with excess nitrogen, whereas TnrA can repress or activate the target genes when nitrogen is limited. Both GlnR and TnrA functions require the transcription of *glnA*, which is repressed when nitrogen is excessive (Hendriksen, Kloosterman et al. 2008).

Despite its importance in regulating nitrogen metabolism in *B. subtilis*, a TnrA homologue has not been identified in staphylococci. Importantly, inactivation of *glnR* increased methicillin sensitivity by altering peptidoglycan synthesis and structure in methicillin resistance *S. aureus* (MRSA) (Gustafson, Strassle et al. 1994).

S. aureus is a facultative anaerobic bacterium and it can grow in a low-oxygen environment by fermentation or nitrate respiration (Burke and Lascelles 1975). So adaptation to different levels of oxygen, nitrogen, or other electron acceptor is very important. The two-component Staphylococcal respiratory response AB (SrrAB) system is the major regulatory system responsible for anaerobic gene regulation (Pragman, Yarwood et al. 2004). The SrrAB system will be discussed in the oxygen response regulator portion. The NreBC system is another two-component system that regulates nitrogen metabolism and it was first identified in *S. carnosus* (Schlag, Fuchs et al. 2008). NreB is a sensor kinase and NreC is a response regulator. Under low-oxygen conditions, the histidine kinase activity of NreB is increased, which activates the response regulator NreC. This system activates genes involving nitrogen metabolism including *narT* (nitrite and nitrate transport), *narGHJI* (respiratory nitrate reductase), and *nasDE* (nitrite reductase). The *narGHJI* operon encodes the membrane-bound respiratory nitrate reductase that reduces nitrate to nitrite, and nitrite can be further reduced to ammonia by nitrite reductase (*nasDE*). The first reductase is coupled with a proton pump, while the latter reaction does not produce proton force. Both *narGHJI* and *nasDE* operons are found in *S. aureus* genome. In the absence of oxygen and nitrate, NreC activates transcription of fermentative metabolism including the lactate dehydrogenase (*lctE*) and

the 2,3-butanediol pathway (*alsS*) (Schlag, Fuchs et al. 2008). It has been shown that nitrite affects biofilm formation. Nitrite decreases PIA biosynthesis and inhibits biofilm formation in *S. aureus* strain SA113 and *S. epidermidis* strain RP62A (Schlag, Nerz et al. 2007).

Metal-dependent regulator

Metal ions, especially iron, magnesium, and manganese are important enzymatic cofactors for bacteria, and they have multiple roles in bacterial metabolism. These metals can be used in many biochemical reactions as cofactors as well as in maintaining a redox potential. In addition to being necessary for enzymatic function, transition metals produce harmful reactive oxygen species (ROS) by Fenton chemistry; therefore, staphylococci have evolved mechanisms to maintain metal ion homeostasis. Regulation of metal ion homeostasis is achieved by regulators belonging to two protein families, the ferric uptake regulator (Fur) family, and diphtheria toxin regulator (DtxR) family. There are three Fur family members in *S. aureus* including the ferric uptake regulator (Fur), iron storage and antioxidant protein regulator (PerR), and zinc homeostasis regulator (Zur). Only one DtxR family member has been identified named MntR, which is a manganese dependent regulator.

Free iron is present in a eukaryotic host at a concentration of 10^{-18} M, a concentration well below what is required to support bacterial growth. Considering the great difficulty for bacteria to obtain iron from the host, *S. aureus* has evolved multiple means to acquire iron. One of the primary mechanisms is siderophore-based iron

acquisition, which plays an essential role in getting the iron from transferrin (Park, Sun et al. 2005). Consistent with idea, defects in siderophore biosynthesis significantly decrease the virulence of bacteria in a kidney abscess model (Dale, Sebulsky et al. 2004). In many bacteria, regulation of siderophore biosynthesis and iron transport is mediated by Fur. Fur acts by binding to an inverted repeat motif called the Fur box located in the promoter region. The *S. aureus* strain N315 genome contains 11 confirmed Fur boxes and 11 putative Fur boxes (Horsburgh, Ingham et al. 2001). When iron is abundant in a bacterium, Fur binds to DNA and represses transcription of target genes. On the other hand, when iron is limiting supply, the dissociation of Fur derepresses the target genes. Fur mutants demonstrate a significant defect of growth, which is caused by unregulated iron transport, which dramatically increases the intracellular iron concentration (Horsburgh, Ingham et al. 2001). At the same time the iron concentration is increasing, transcription of catalase decreases because Fur is a positive regulator of *katA* (encodes catalase). In addition to regulation iron homeostasis, inactivation *fur* in *S. aureus* decreased in vivo virulence by altering expression of virulence factors in a murine pneumonia model (Torres, Attia et al. 2010). Taken together, these observations indicate that Fur has an important function in the overall fitness of staphylococci.

PerR, the second Fur homologue in *S. aureus*, is a transcriptional repressor of peroxide resistance operons (Horsburgh, Clements et al. 2001). PerR is a redox sensor that can sense hydrogen peroxide by metal-catalyzed histidine oxidation, using Mn (II) as a co-repressor (Lee and Helmann 2006; Traore, El Ghazouani et al. 2009). Based on the level of Mn (II) and iron, PerR has different functions. The PerR regulon is repressed in

the presence of Mn (II), whereas the regulon is derepressed in the presence of iron. Generally the concentration of iron has a dominant role (Horsburgh, Clements et al. 2001). PerR regulated genes include *katA*, *trxB* (thioredoxin reductase), two putative iron storage genes *ftn* (ferritin), and *mrgA* (ferritin-like DNA-binding proteins from starved cells homologue). Consistent with its proposed regulatory function, an *S. aureus perR* mutant has an increased resistance to hydrogen peroxide due to the over expression of catalase. In contrast, *perR* mutants of *S. aureus* strain 8325-4 have reduced virulence relative to the wild-type strain (Horsburgh, Ingham et al. 2001). However, the mechanism of decreased virulence is still unknown.

Manganese is an important metal ion that is used as a cofactor in many enzymatic reactions, signal transduction, and to protect the cell from oxidative stress. *S. aureus* has two manganese transporters, an ABC type transporter encoded by *mntABC* operon that is proposed to play a major role in manganese transport, and a proton-coupled metal ion transporter encoded by *mntH* (Horsburgh, Wharton et al. 2002). Both of them are regulated by MntR, a DtxR like protein (Que and Helmann 2000). Transcription of the *mntABC* operon is repressed by Mn, which is MntR dependent. By contrast, the transcription of *mntH* is not repressed by high Mn concentration (Que and Helmann 2000). Although single mutants of *mntA* and *mntH* genes do not decrease virulence, the double *mntAH* mutant has decreased survival in a host, which indicates manganese transport is important during an infection (Horsburgh, Wharton et al. 2002).

The Zinc uptake regulator (Zur) is a third Fur-like protein (Lee and Helmann 2007). Zur is the third gene following two putative zinc transporters. Zur binds as a dimer

to a conserved DNA region called Zur box (Gaballa, Wang et al. 2002) and represses transcription. Zur is the regulator of Zn uptake system and ribosomal protein paralogs (Panina, Mironov et al. 2003). Zur also involved in the regulation of some potential virulence factors such as metalloprotease in *Streptococcus suis* (Feng, Li et al. 2008) and *zur* mutant has attenuated virulence in a murine intraperitoneal infection model of *Salmonella enterica* (Campoy, Jara et al. 2002). However, *zur* inactivation seems no attenuation in virulence in a *S. aureus* mouse skin infection model (Lindsay and Foster 2001).

Oxygen-responsive regulator

S. aureus is a facultative anaerobe, meaning that it is able to grow under both aerobic and anaerobic conditions; thus, oxygen sensing and regulation of genes necessary for survival in an oxygen containing environment is critical. There is a dramatic metabolic change during the switch from aerobic to anaerobic growth. In *S. aureus* this change is mediated by the staphylococcal respiratory response regulator SrrAB (staphylococcal respiratory response) (Throup, Zappacosta et al. 2001). SrrAB is a homologue of the ResDE two-component regulatory system in *B. subtilis*, which is regulated by oxygen. Mutation of s*rrAB* leads to slower growth during anaerobiosis, a diminished ability to ferment carbohydrates, and decreased transcription of lactate dehydrogenase and alcohol dehydrogenase (Yarwood, McCormick et al. 2001). SrrB is a membrane-associated histidine kinase that is capable of autophosphorylation (Pragman, Yarwood et al. 2004). SrrA is a cytoplasmic DNA binding protein. Once phosphorylated by SrrB, SrrA activates or represses target genes including downregulating toxic shock

syndrome toxin (TSST-1) and protein A expression. SrrA also affects RNAIII expression and strongly downregulates RsbW (Pragman, Yarwood et al. 2004). In anaerobic conditions, phosphorylated SrrA directly binds to the *ica* operon promoter region in *S. aureus* and up-regulates its transcription (Ulrich, Bastian et al. 2007).

MgrA is a transcriptional regulator belonging to the MarR protein family that has a dimerization domain and a conserved helix-turn-helix domain (Chen, Bae et al. 2006). MgrA was first identified as a negative regulator of bacterial autolysis (Ingavale, Van Wamel et al. 2003); however, it was later found to be a positive regulator of RNAIII (Ingavale, van Wamel et al. 2005). The MgrA regulation pathway is involved in thiol-based oxidation. The only cysteine residue Cys 12 in the MgrA sequence is located in the dimerization region (Chen, Bae et al. 2006). Oxidation of this Cys residue leads to the dissociation of MgrA from the DNA, derepressing transcription of antibiotic resistance genes such as *norA, norB,* and *tet38* and more than 300 other genes including *cap*, *spa*, *coa*, *hla,* and global regulatory genes such as *agr*, *sarS, sarZ,* and *sarV* (Ingavale, van Wamel et al. 2005; Luong, Dunman et al. 2006; Ballal, Ray et al. 2009; Chen, Nishida et al. 2009; Trotonda, Xiong et al. 2009). Despite the derepression of antibiotic resistance genes, mutation of MgrA decreases resistance to cell wall active antibiotics (Trotonda, Xiong et al. 2009). MgrA also represses *S. aureus* biofilm formation by negative regulation of CidA and LrgA expression, which are involved in eDNA release (Luong, Dunman et al. 2006).

SarZ is another Cys-based oxidation sensor protein that is similar to MgrA and was first identified as a regulator of hemolysin expression (Kaito, Morishita et al. 2006).

Recent studies have demonstrated that SarZ indirectly regulates virulence factors by synthesis by repressing *sarA* transcription and activating the *agr* system (Tamber and Cheung 2009). SarZ can also directly regulate transcription by binding to the promoter region of a target gene, such as *hla*. The overall effect of SarZ is decreased expression of surface proteins and increased synthesis of secreted virulence factors; therefore, it was proposed that SarZ is important in facilitating the dissemination of *S. aureus* infections (Tamber and Cheung 2009). The *sarZ* locus also represses biofilm formation, possibly by repressing surface adhesins and enhancing protease expression (Tamber and Cheung 2009).

Redox-responsive regulator

In addition to SrrAB, a redox-dependent transcription repressor (Rex) was found to play a key role in the regulation of anaerobic metabolism in *S. aureus* (Hecker, Reder et al. 2009; Pagels, Fuchs et al. 2010). In comparison to the regulators directly sensing and responding to oxygen changes, Rex employed a unique mechanism by sensing redox status through responding to $NADH/NAD^+$ ratio (Brekasis and Paget 2003). Rex was originally identified in *Thermus aquaticus* (Du and Pene 1999). Later Rex homologues have been found in other bacteria such as *Streptomyces coelicolor* (Brekasis and Paget 2003), *B. subtilis* (Gyan, Shiohira et al. 2006) and *L. monocytogenes* (Brekasis and Paget 2003). Rex contains two conserved domains: N-terminal winged-helix DNA binding domain and C-terminal $NADH/NAD^+$ binding domain (Wang, Bauer et al. 2008). Rex can bind a highly conserved sequence ($TTGTGAAW_4TTCACAA$) in *S. aureus* (Pagels, Fuchs et al. 2010) and represses downstream genes. The binding activity of Rex is

regulated by NADH/NAD^+ ratio. NAD^+ enhances the DNA binding ability of Rex, whereas NADH inhibits this ability (Sickmier, Brekasis et al. 2005). That is to say, an increase in NADH/NAD ratio causes dissociation of Rex-DNA binding and consequently to derepress downstream genes.

A recent study demonstrated that at least 19 genes are direct targets of Rex including lactate fermentation gene such as *ldh1* (lactate dehydrogenase) and 25 genes are indirect target (Pagels, Fuchs et al. 2010). It is no surprise that Rex is the major regulator of lactate dehydrogenase as this is the one-step pathway leading to rapid NAD regeneration to achieve redox rebalance. Rex also regulates the genes related to anaerobic metabolism and genes aiming to decrease the intracellular NADH concentration, which include a series of mixed acids fermentation, nitrite/nitrate respiration and electron transport chain genes. Rex also regulates some virulence genes. Rex represses α-toxins expression and regulates *lukM* encoding leukocidin by suppressing the SrrAB two-component system et al. (Pragman, Herron-Olson et al. 2007; Ulrich, Bastian et al. 2007; Somerville and Proctor 2009).

TCA cycle-responsive regulator

The TCA cycle has three major functions in metabolism, first it provides intermediates for macromolecular biosynthesis; second it provides reducing potential in the form of NADH and $FADH_2$; and third it provides energy in the form of ATP. The TCA cycle participates in the regulation of staphylococcal virulence factor synthesis directly and indirectly (Somerville, Chaussee et al. 2002; Somerville, Cockayne et al. 2003; Vuong, Kidder et al. 2005; Sadykov, Olson et al. 2008; Sadykov, Mattes et al. 2010).

Since one of the TCA cycle functions is providing intermediates, TCA cycle-dependent virulence regulation may be carried out by the supply of metabolites (Somerville, Cockayne et al. 2003). For instance, the expression of delta toxin is inhibited by glutamate auxotrophy that is caused by TCA cycle inactivation (Somerville, Cockayne et al. 2003). Lacking glutamate limits delta toxin synthesis even though its template RNA is abundantly present. This repression can be reversed by adding glutamate to the culture media. Another example of metabolic regulation is that TCA cycle mutants fail to synthesize capsule due to decreased carbon flow into gluconeogenesis, which provides the biosynthetic precursors of capsule (Sadykov, Mattes et al. 2010).

Genetic regulation may also be used in TCA cycle-dependent regulation of virulence factors. A good example is its effect on the *icaADBC* operon which is responsible for PIA biosynthesis (Vuong, Kidder et al. 2005; Sadykov, Olson et al. 2008). There is no evidence that an enzyme of the TCA cycle directly regulates the *ica* operon; therefore, this regulation is likely mediated by a response regulator that can respond to metabolic changes caused by fluctuation in TCA cycle activity. As mentioned above, three major functions of TCA cycle is to provide the intermediates for biosynthesis, to generate reducing potential, and also to provide energy. So an indirect regulation is likely to occur when a metabolite-responsive regulator (e.g., CcpA) responds to changes in TCA cycle associated biosynthetic intermediates, the redox status, and/or ATP.

GlnR, a regulator for nitrogen metabolism, is a good candidate of metabolite-responsive regulator. It can sense the concentration of glutamate, glutamine and ammonium level. Glutamate and glutamine are closely associated with the TCA cycle

because the TCA cycle provides the carbon skeleton α-ketoglutarate for those metabolites. GlnR affects many virulence factors expression and methicillin resistance. CodY and CcpA also can respond the TCA cycle induced metabolic change. As stated earlier, the affinity of CodY to DNA is increased while binding to BCAA and GTP. The concentration of BCAA such as valine, leucine, and isoleucine is associated with TCA cycle activity. It was reported that the TCA cycle mutant has a higher concentration of BCAA compared with wild type (Sadykov, Olson et al. 2008). The regulation of CcpA is modulated by the level of fructose-1,6-bisphosphate or glucose-6-phosphate which is increased by the inactivation of the TCA cycle. So it is reasonable to hypothesize that CcpA may respond to the TCA cycle-induced changes. In addition to the biosynthetic intermediate-responsive regulators, Cys-based oxidation sensor like MgrA and SarZ may also respond to the changes induced by TCA cycle. When the TCA cycle is more active, more oxidative stress is present inside the cells, and the DNA binding ability of MgrA and SarZ decreases. In this way, the downstream gene expression is affected. As we stated earlier, MgrA and SarZ are involved in autolysis of the cell. The TCA cycle mutant cannot perform cell autolysis (Somerville, Chaussee et al. 2002).

V. Conclusions

S. aureus elaborates many virulence factors that allow the organism to establish and spread infections under various conditions. The regulation of these virulence factors

is intimately linked with metabolism by the metabolic regulators. Understanding the linkage may help find a new and effective target of antimicrobial therapy.

CHAPTER II

STAPHYLOCOCCUS AUREUS BIOFILM METABOLISM AND THE INFLUENCE OF ARGININE ON POLYSACCHARIDE INTERCELLULAR ADHESIN SYNTHESIS, BIOFILM FORMATION, AND PATHOGENESIS

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ABSTRACT

Staphylococcus aureus and *S. epidermidis* are the leading causes of nosocomial infections in the USA and often associated with biofilms attached to indwelling medical devices. Despite the importance of biofilms, there is very little consensus about the metabolic requirements of *S. aureus* during biofilm growth. To assess the metabolic requirements of *S. aureus* growing in a biofilm, we grew USA200 and USA300 clonal types in biofilm flow cells, and measured the extraction and accumulation of metabolites. In spite of the genetic differences, both clonal types extracted glucose and accumulated lactate, acetate, formate, and acetoin, suggesting glucose was catabolized to pyruvate that was then catabolized via the lactate dehydrogenase, pyruvate formate-lyase, and butanediol pathways. Additionally, both clonal types selectively extracted the same six amino acids from the culture medium (serine, proline, arginine, glutamine, glycine, and threonine). These data and recent speculation about the importance of arginine in biofilm growth and the function of arginine deiminase in USA300 clones, led us to genetically inactivate the sole copy of the arginine deiminase operon by deleting the arginine/ornithine antiporter (*arcD*) in the USA200 clonal type and assess the effect on biofilm development and pathogenesis. Although inactivation of *arcD* did completely inhibit arginine transport and did reduce polysaccharide intercellular adhesin accumulation, *arcD* mutants formed biofilms and achieved cell densities in catheter infection studies equivalent to isogenic wild-type strains.

INTRODUCTION

Staphylococcus aureus is a leading cause of nosocomial and community-acquired infections. Although the types and severity of diseases produced by this opportunistic pathogen vary, it is a frequent cause of infections associated with indwelling medical devices (*e.g.*, catheters and artificial heart valves). Indwelling device-associated infections commonly involve the formation of a bacterial biofilm on an uncoated plastic surface, or a plastic surface coated with host proteins (Vuong and Otto 2002). Due to the importance of *S. aureus* biofilms in medical device-associated infections, a considerable amount of research has been directed at understanding the mechanisms of biofilm formation. Much of this research has focused on the bacterial mediators of biofilm formation [*e.g.*, (Rachid, Ohlsen et al. 2000; Vuong, Saenz et al. 2000; Trotonda, Manna et al. 2005; Pamp, Frees et al. 2006)], the environmental effectors of biofilm formation [*e.g.*, (Karaolis, Rashid et al. 2005; Jones, Muller et al. 2006; Shanks, Sargent et al. 2006)], and more recently the global changes that occur during biofilm development (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005; Yao, Sturdevant et al. 2005; Resch, Leicht et al. 2006). The consensus from transcriptional profiling studies of *S. aureus* biofilms is that bacteria are growing microaerobically or anaerobically relative to planktonic cultures (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005). This is exemplified by increased expression of genes of the arginine deiminase and mixed acid fermentation pathways and pyruvate formate lyase. Support for the idea that staphylococci growing in a biofilm are growing microaerobically can be found in the observations that anaerobiosis increases biofilm formation and polysaccharide

intercellular adhesin (PIA) synthesis (Cramton, Ulrich et al. 2001; Stepanovic, Djukic et al. 2003).

Although the number of known requirements for *S. aureus* biofilm formation is low, a considerable amount of research has provided important information regarding potential mediators of biofilm formation. As examples, *S. aureus* regulates the formation of biofilms in response to nutrient availability, oxygen tension, and a variety of stresses (Rachid, Ohlsen et al. 2000; Cramton, Ulrich et al. 2001; Götz 2002; Lim, Jana et al. 2004; Johnson, Cockayne et al. 2005). Importantly, these observations highlight a recurrent regulatory theme in pathogenesis; environmental factors alter the metabolic status of the bacteria, resulting in an alteration of virulence (Mekalanos 1992) or, in this incidence, biofilm-forming capacity (Bowden and Li 1997). Despite this being a common regulatory theme, the relationship between environmental factors and pathogenesis is poorly defined. Addressing this relationship is particularly important in the era of "omics", when genomics, proteomics, and high throughput mutagenesis screens consistently identify the genes of bacterial physiology and metabolism as being important, or essential, for pathogenesis (Dunman, Murphy et al. 2001; Kohler, von Eiff et al. 2003; Kuroda, Kuroda et al. 2003; Mongodin, Finan et al. 2003; Beenken, Dunman et al. 2004; Bischoff, Dunman et al. 2004).

To enhance our understanding of the metabolic requirements of *S. aureus* biofilm development, two *S. aureus* strains (UAMS-1 and UAMS-1182) were grown in flow cells and the culture medium effluent was collected and analyzed for nutrient extraction, secondary metabolite accumulation, and oxygen concentration. These two strains were chosen because they represent both methicillin susceptible and methicillin resistant *S.*

aureus (MSSA and MRSA) phenotypes and are from distinct genetic backgrounds (USA200 and USA300 clonal types).

MATERIALS AND METHODS

Bacterial strains and growth conditions. By pulsed-field gel electrophoresis (PFGE), strain UAMS-1 is an MSSA strain of the USA200 clonal group, while UAMS-1182 is an MRSA strain indistinguishable from the USA300 clonal group (data not shown) (McDougal, Steward et al. 2003). PFGE control strains (i.e., known USA clonal groups) were provided by NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*; www.narsa.net). In addition, UAMS-1182 was identified as belonging to the USA300 clonal group based on the presence of the arginine catabolic mobile element (Goering, McDougal et al. 2007) as determined by PCR (data not shown). Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in 2xYT broth (Sambrook and Russell 2001) or on 2xYT agar and *S. aureus* strains were grown in tryptic soy broth containing 0.25% glucose (TSB) (BD Biosciences) or on TSB containing 1.5% agar. Unless otherwise stated, all bacterial cultures were inoculated 1:200 from an overnight culture (normalized for growth) into TSB, incubated at 37ºC, and aerated at 225 rpm with a flask-to-medium ratio of 10:1. Antibiotics were purchased from Fisher Scientific or Sigma Chemical and, when used, were used at the following concentrations: ampicillin, 100 µg/ml (*E. coli*); erythromycin, 8 µg/ml and chloramphenicol, 8 µg/ml (*S. aureus*).

Construction of *S. aureus arc* mutants. A 1.8-kb internal fragment of *arcD* gene was amplified by PCR and cloned into the *Sma*I site of pBluescript II K/S (+) (Stratagene) to construct the plasmid pYF-1. The *ermB* cassette of pEC4 (Bruckner 1997) was inserted

into the *NdeI* site within the *arcD* fragment of pYF-1 to generate the plasmid pYF-2. Next, the *arcD*::*ermB* fragment was cloned into the *KpnI* and *EcoRI* site of the temperature-sensitive shuttle plasmid pTS1 (Greene, McDevitt et al. 1995) to generate the plasmid pYF-3. The temperature-sensitive plasmid pYF-3 was electroporated into *S. aureus* strain RN4220 (Schenk and Laddaga 1992) and was then introduced into strain UAMS-1 by Φ85 phage transduction (Novick 1991). Strain UAMS-1 containing pYF-3 was used to construct an *arcD* mutant by the method described by Foster (Foster 1998). Putative mutants were confirmed by PCR, Southern blotting (Southern 1975), and enzymatic assays.

To facilitate monitoring the course of the catheter-associated infections, we generated an *arcD* mutation in *S. aureus* strain Xen40 (Caliper Life Sciences, Hopkinton, MA). Xen40 is strain UAMS-1 with a chromosomal insertion of the *luxABCDE* operon modified for expression in gram-positive bacteria (Kadurugamuwa, Sin et al. 2004). The mutant was made using the pKOR1 mutagenesis vector as previously described (Bae and Schneewind 2006) using upstream primers ArcD1-attB2F and ArcD1-SacIIR and downstream primers ArcD2-SacIIF and ArcD2-attB1R (Table 2). The PCR products were digested with SacII, ligated together, amplified using the two outside primers (ArcD1-attB2F and ArcD2-attB1R), and ligated into pKOR1. The recombinant pKOR1 *arcD* construct was transformed into strain Xen40 and the *arcD* mutant was constructed as previously described (Bae and Schneewind 2006). Inactivation of *arcD* was verified by PCR using the ArcD1-attB2F and ArcD2-attB1R primers, by confirming bioluminescence using the IVIS-200 imaging system (Caliper Life Sciences), and enzymatic assays. A

Xen40 *arcD* mutant strain (UAMS-1272) with growth characteristics indistinguishable from UAMS-1-*arcD*::*ermB* was chosen for the animal experiments.

PIA Immunoblot assay. PIA accumulation was determined essentially as described (Vuong, Kidder et al. 2005). Briefly, bacterial cultures were grown for 16 h at 37° C with a flask-to-medium ratio of 5:1 and aerated at 160 rpm. Equivalent cell densities of *S. aureus* were harvested by centrifugation and PIA was extracted with 0.5 M EDTA (pH 8.0) by boiling for 5 min. After boiling, the samples were centrifuged and the supernatants were harvested and incubated with Proteinase K (20 mg/ml; Sigma-Aldrich) for 30 min. 100 µl aliquots of PIA samples were applied to Nytran nylon membranes (Waterman Inc.) in triplicate, air-dried, and blocked overnight with 5% skim milk. Membranes were incubated for 2h with anti-PIA antiserum and for 2 h with an anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate. The presence of PIA was detected by the addition of Sigma Fast BCIP/NBT substrate (Sigma-Aldrich). The integrated density values of spots on autoradiographs were determined with the TotalLab software (Nonlinear Dynamics Ltd.).

Flow cell biofilm formation. *S. aureus* strains were grown in flow-cell apparatuses (Stovall Life Sciences, Greensboro, NC) coated with 20% human serum. To initiate the biofilm, 500 µl of dilute overnight cultures ($O.D._{600}$ = 0.15) were injected into each chamber and the flow-cell was incubated upside down at 37º C for 1 hour. Following the incubation period, a continuous flow of TSB containing 0.5% glucose and 3% NaCl was delivered at a flow rate of 0.5 ml per minute per chamber. 12 hours post-inoculation,

effluent samples were collected at 4 h intervals and analyzed for pH and dissolved oxygen concentration or stored at -20º C until required.

Measurement of dissolved oxygen. The dissolved oxygen (DO) concentration was monitored using an Accumet AR60 meter equipped with YSI 5905 oxygen sensor (Fisher Scientific).

Measurement of glucose, lactate, acetate, formate, ammonia and acetoin concentrations. Glucose, lactate, acetate, formate and ammonia concentrations were determined with kits purchased from R-Biopharm, Inc. and used according to the manufacturer's directions.

Acetoin concentrations were determined essentially as described (Grundy, Waters et al. 1993). Briefly, 1 ml of effluent was mixed sequentially with 0.2 ml 0.5% creatine, 0.2 ml 5% α-naphthol, and 0.2 ml 40% potassium hydroxide. The mixture was vortexed for 30 seconds and incubated at room temperature for 1h. The absorbance at 540 nm was measured and the unknown acetoin concentrations were determined with a standard curve generated using known acetoin concentrations.

Enzymatic activity assays. Bacteria were grown without agitation overnight in TSB supplemented with 20 mM arginine. Cell-free lysates of *S. aureus* were prepared as follows: Aliquots (3 ml) were harvested by centrifugation, washed with cold 0.01 M potassium phosphate buffer (pH 7.0), and suspended in 3.0 ml of lysis buffer containing 0.1 M potassium phosphate buffer and 25 μg/ml lysostaphin (AMBI). The samples were

incubated at 37º C for 15 min and passed through a French press (2 x at 15,000 psi). The lysate was centrifuged for 5 min at 20,800 x *g* at 4º C. Arginine deiminase activity assays were performed essentially as described (34). In brief, cell-free lysates (100 µl) were added to Eppendorf tubes containing pre-warmed (37° C) reaction buffer (400 µl) [5.8 mM L-arginine and 131 mM potassium phosphate (pH 5.8)] and incubated at 37° C for 15 min. After 15 min, the reaction was stopped by the addition of 50 µl of ice-cold 70% perchloric acid and the samples were clarified by centrifugation. The supernatant (400 µl) was analyzed for citrulline production as described by Sugawara (Sugawara, Yoshizawa et al. 1998). One unit of ADI activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol of citrulline per min. Protein concentrations were determined by the Lowry method (Lowry, Rosebrough et al. 1951).

Assay of amino acids. The concentrations of free amino acids in TSB were determined with a Beckman Model 6300 amino acid analyzer (Scientific Research Consortium, Inc., St. Paul, MN).

In vivo biofilm formation. A catheter-based murine model was used to assess biofilm formation in vivo (Christensen, Simpson et al. 1983; Rupp, Ulphani et al. 1999). Briefly, anesthesia was induced by intraperitoneal injection of 0.75 mg of a 2,2,2 tribromoethanol in tert-amyl alcohol (Sigma-Aldrich) per gram of body weight. Sterile 1 cm Teflon intravenous catheter segments (B. Braun, Bethlehem, PA) were then implanted subcutaneously in each flank of 6-8 week old NIH-Swiss mice (Harlan Industries, Inc., Indianapolis, IN). After wound closure with Vetbond surgical adhesive (3M, St. Paul,

MN), each catheter lumen was inoculated with 100 μl of phosphate-buffered saline (PBS) containing $5x10^5$ colony forming units (cfu). Groups of 10 mice (20 catheters total) were infected with each strain. Mice were imaged immediately after inoculation for 5 min using the IVIS-200 imaging system. All mice from each group were humanely sacrificed 2 days post-infection and imaged a second time immediately following sacrifice. To quantify bioluminescence, a defined region of interest corresponding to the size of the implanted catheter was created and used to measure total flux (photons/sec) from each catheter. This was done both immediately after inoculation and at the time of sacrifice. Statistical significance was assessed using the One Way Analysis of Variance with All Pairwise Multiple Comparison Procedure (Tukey Test) as formatted in SigmaStat software (SPSS Inc., Chicago, IL). To quantify biofilm formation by each strain, infected catheters were removed immediately after sacrifice, gently washed three times in sterile PBS to remove non-adherent bacteria, and subsequently placed in 5 ml of sterile PBS. Adherent bacteria were removed from the catheters by sonication. The number of recovered bacteria was then quantified by serial dilution and plate counting on the appropriate selective media. Statistical significance of quantitative plate counts was assessed using the Mann Whitney Rank Sum test as formatted in SigmaStat software. All mouse infection experiments were reviewed and approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee, and comply with Animal Welfare Legislation and NIH guidelines and policies.

RESULTS

***Staphylococcus aureus* carbohydrate metabolism during biofilm growth.** Transcriptional profiling experiments suggest that *S. aureus* growing in a biofilm are growing microaerobically or anaerobically (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005). To biochemically test this possibility, we grew strains UAMS-1 and UAMS-1182 in a flow cell apparatus with a continuous flow of TSB culture medium and analyzed the effluent for the extraction of glucose and accumulation of common fermentation products (Fig. 1 and 2). Consistent with the transcriptional profiling data, *S. aureus* growing in a biofilm extracted glucose from the culture medium and accumulated the fermentation products lactic acid, acetic acid, and formic acid (Fig. 2). The accumulation of organic acids in the culture medium occurred coincident with a decreased pH and induction of the butanediol pathway, as suggested by the accumulation of acetoin (Fig. 2). Bacteria induce the butanediol pathway in response to a decrease in the environmental pH (Booth and Kroll 1983) and as a means to prevent a redox imbalance by oxidizing NADH to NAD^+. The accumulation of organic acids and alcohols in the culture medium effluent is strongly indicative of oxygen-limited growth. To determine if the biofilm was oxygen-limited, we measured the oxygen concentration of the culture medium effluent for strains UAMS-1 and UAMS-1182 (Fig. 3). As expected, as the biofilm matured, it depleted oxygen from the culture medium (Fig. 3). Taken together, these data suggest that reduced oxygen tension induced a fermentative metabolism resulting in increased pH stress.

***S. aureus* growing in a biofilm selectively extract amino acids.** Bacteria regulate the selective transport of amino acids into the cytosol; that is to say, they take what they need when they need it (Dunman, Murphy et al. 2001; Somerville, Chaussee et al. 2002; Beenken, Dunman et al. 2004; Horsburgh, Wiltshire et al. 2004; Schwan, Wetzel et al. 2004). This is an advantageous adaptation allowing bacteria to acquire necessary amino acids from their environment and redirect energy from amino acid biosynthesis into growth. It has been postulated that amino acid catabolism is important for staphylococcal biofilm formation and pH homeostasis (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005; Resch, Leicht et al. 2006). To determine which amino acids *S. aureus* extracted from the culture medium during biofilm growth, we measured the free amino acid concentrations of culture medium effluent and expressed this as function of the free amino acid concentration in sterile culture medium (Fig. 2E and 2F). Although the extent to which amino acids were extracted varied, strains UAMS-1 and UAMS-1182 immediately began selectively extracting the free amino acids glutamine, serine, proline, glycine, threonine, and arginine, while other amino acids remained in the medium (e.g., valine, tryptophan). The extraction of these six amino acids differs somewhat from that seen in planktonic growth, where during exponential growth serine, glycine, glutamine, glutamate, alanine, and threonine are selectively extracted from the culture medium and proline and arginine are not extracted until carbon becomes limiting [(Somerville, Chaussee et al. 2002) and Somerville unpublished data]. The selective extraction of amino acids from the culture medium during biofilm growth, suggested the amino acids were being used for carbon and/or energy production, and not just protein synthesis. Amino acid catabolism usually involves the release of ammonia through a deamination

step; thus, it is possible to determine the fate of amino acids by measuring the concentration of ammonia in the culture medium effluent (Fig 2B). Concomitant with the extraction of amino acids from the culture medium was the accumulation of ammonia in the effluent, indicating amino acid catabolism. These data led us to speculate that interfering with amino acid transport and/or catabolism might limit biofilm formation, PIA synthesis, or virulence. To test these possibilities, we chose to genetically inactivated the *arcD* gene (*arcD* encodes the arginine/ornithine antiporter) of strain UAMS-1 and assessed its ability to synthesize PIA and form a biofilm. The choice to inactivate the arginine transporter, as opposed to a transporter of one of the other selectively extracted amino acids, was based on previous transcriptional profiling data (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005; Yao, Sturdevant et al. 2005), proteomic data (Resch, Leicht et al. 2006), and speculation about the potential importance of the arginine deiminase pathway in staphylococcal pathogenesis (Diep, Gill et al. 2006).

Growth of UAMS-1 arcD mutant strain. In *S. aureus*, the genes coding for the anaerobic catabolism of arginine are contained within the arginine deiminase (*arc*) operon. The *arc* operon is comprised of arginine deiminase (*arcA*), ornithine transcarbamylase (*arcB*), the arginine/ornithine antiporter (*arcD*), and carbamate kinase (*arcC*). The arginine/ornithine antiporter is a cationic exchange system that catalyzes a one-to-one stoichiometric exchange of arginine and ornithine (Crow and Thomas 1982; Driessen, Poolman et al. 1987; Poolman, Driessen et al. 1987); hence, as the bacteria extract arginine from the culture medium, ornithine accumulates in the medium. As expected, inactivation of the *arcD* gene prevented the extraction of arginine from the culture

medium and the accumulation of ornithine in the medium during biofilm growth (Fig. 4A). Inactivation of *arcD* also reduced the accumulation of ammonia in the culture medium (after 48 h growth, the concentration of ammonia in the culture medium effluent was 3.31 mM in UAMS-1 and 1.38 mM in UAMS-1-*arcD*::*ermB*.) Additionally, under microaerobic growth conditions (5:1 flask-to-medium ratio and 100 rpm aeration), the *arcD* mutant strain (UAMS-1-*arcD*::*ermB*) accumulated a significantly lower concentration of ammonia in the culture medium relative to the isogenic wild-type strain (Fig. 4D), suggesting that arginine catabolism was inhibited. Taken together, these data confirm the correct gene was inactivated and that it was the sole arginine transporter.

Arginine transport and/or catabolism enhance the accumulation of PIA. Two different *S. aureus* clonal types selectively extracted six amino acids, including arginine, from the culture medium during growth in a biofilm (Fig. 2E and 2F). These data and the fact that *arcD* inactivation does not cause a growth defect during planktonic growth (Fig. 4B) suggest that arginine may be important for one or more aspects of biofilm maturation. To test this possibility, we assessed the ability of UAMS-1 and UAMS-1-*arcD*::*ermB* strains to grow in biofilm flow-cells. The gross morphology of biofilms formed by strain UAMS-1-*arcD*::*ermB* and strains UAMS-1 and UAMS-1182 was very similar (Fig. 1), demonstrating that bacterial attachment and biofilm maturation can occur independent of arginine transport or catabolism. To determine if *arcD* inactivation affected PIA accumulation, we determined the amount of cell-associated PIA during the exponential and stationary phases of planktonic growth. Interestingly, the accumulation of PIA was lower in strain UAMS-1-*arcD*::*ermB* relative to the isogenic strain UAMS-1 (46-51%

reduction, *P* = 0.0015), suggesting the biofilm formed by the *arcD* mutant strain (Fig. 1) might be PIA-limited.

The affect of arginine transport on catheter-associated infections. To facilitate monitoring the course of the catheter-associated infections, we deleted the *arcD* gene from a strain of UAMS-1 carrying the *luxABCDE* operon (strain Xen40) and measured in vivo bioluminescence. Consistent with strain UAMS-1-*arcD*::*ermB*, the Xen40 *arcD* deletion mutant (strain UAMS-1272) had reduced PIA accumulation (46-51% reduction, *P* = 0.0015) and formed a biofilm in a flow cell equivalent to the isogenic wild-type strain (data not shown). Additionally, genetic inactivation of *arcD* in both UAMS-1 and Xen40 inhibited arginine deiminase activity (data not shown). The in vivo growth of the *arc* operon mutant and the wild-type strains was statistically equivalent using the Mann Whitney rank sum test (p = 0.218). Similarly, bioluminescence on day 2 was equivalent between the wild-type and *arcD* mutant strains (data not shown). Taken together, these demonstrate that in vitro and in vivo biofilms can mature in the absence of the arginine deiminase/*arc* operon.

DISCUSSION

Indwelling medical device-associated infections caused by bacterial biofilms are a significant source of morbidity in the United States, with two of the more common causative agents of these infections being *S. aureus* and *S. epidermidis* (Trampuz and Widmer 2006). In spite of the prevalence of biofilm-associated staphylococcal infections, there is little consensus about the requirements necessary to form a biofilm. To address the metabolic aspects of this deficiency, we grew two genetically distinct *S. aureus* strains in biofilm flow cells, measured the extraction and accumulation of metabolites, and assessed the importance of one metabolite, arginine, on biofilm development and pathogenesis.

Consistent with previous observations (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005), our data demonstrate that *S. aureus* growing in a biofilm are growing microaerobically (Fig. 2 and 3). We found that mature biofilms maintain a dynamic metabolic flux of carbon and amino acid uptake and excretion (Fig. 2), a situation similar to that proposed for static *S. aureus* biofilms (Resch, Rosenstein et al. 2005). This dynamic metabolic flux contrasts with that observed in *S. epidermidis* biofilms where it was determined that growth in a biofilm leads to "low metabolic" activity (Yao, Sturdevant et al. 2005). Specifically, our data strongly suggest that *S. aureus* catabolize glucose to pyruvate by glycolysis and then catabolize pyruvate via lactate dehydrogenase (EC 1.1.1.27) and pyruvate formate-lyase (EC 2.3.1.54) (Fig. 2C and D). Glycolysis generates two molecules of pyruvate for every molecule of glucose consumed; however, in the process it reduces two molecules of NAD^+ to NADH.

Reduction of NAD^+ to NADH, without an equivalent means to oxidize NADH, can create a redox imbalance and inhibit growth. Under microaerobic or anaerobic growth conditions, the majority of pyruvate is reduced to lactic acid (Kendall, Friedmann et al. 1930; Krebs 1937), concomitant with a 1:1 stoichiometric oxidation of NADH back to NAD^+. To compensate for the diversion of pyruvate into pyruvate formate-lyase, *S. aureus* also shunt pyruvate into the butanediol pathway via acetolactate synthase (EC 2.2.1.6) (Fig. 2C and D). Shunting pyruvate into the butanediol pathway will facilitate oxidation of NADH and decrease the accumulation of organic acids. These data and deductions suggest that a tenuous redox balance exists during biofilm growth, a potentially exploitable weakness for therapeutic intervention. This suggestion is supported by the observation that inactivation of the butanediol pathway at acetolactate decarboxylase (EC 4.1.1.5) inhibits biofilm formation (Cassat, Dunman et al. 2006). In addition, this suggestion is a reasonable explanation for the absence of dramatic effects on in vivo or in vitro biofilm formation in arginine transport and/or catabolism deficient strains; specifically, the arginine deiminase pathway is not involved in the reduction or oxidation of NAD^+ or NADH.

In a complex medium containing glucose, *S. aureus* will preferentially catabolize glucose for carbon and energy through the glycolytic and pentose phosphate pathways (Kendall, Friedmann et al. 1930; Goldschmidt and Powelson 1953; Collins and Lascelles 1962; Mah, Fung et al. 1967; Cohen 1972). Despite a preference for glucose and a high concentration of glucose in the medium (>15 mM; Fig. 2A), *S. aureus* strains growing in a biofilm selectively extract and catabolize six amino acids, including arginine, from the culture medium (Fig. 2 and 4). These data lend credence to the speculation that arginine

metabolism may be important for staphylococcal survival and/or pathogenesis (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005; Diep, Gill et al. 2006; Resch, Leicht et al. 2006). This speculation generated two very reasonable hypotheses regarding the role of arginine catabolism in staphylococcal host-pathogen interactions: First, the deamination of arginine by the arginine deiminase pathway results in the extracellular accumulation of ammonia, facilitating bacterial pH homeostasis (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005; Resch, Leicht et al. 2006). Second, the staphylococcal arginine deiminase may function in a capacity similar to that in *Streptococcus pyogenes* (Diep, Gill et al. 2006); specifically, arginine deiminase may aid in evasion of the host immune response by inhibiting peripheral blood mononuclear cell proliferation (Degnan, Palmer et al. 1998; Degnan, Fontaine et al. 2000). Hence, one function of arginine may be to induce transcription of the arginine deiminase (*arc*) operon. To test the first hypothesis, we genetically inactivated the *arcD* gene, eliminating arginine transport (Fig. 4A), and assessed the affect on ammonia accumulation and the extracellular pH (Fig. 4). Inactivation of *arcD* completely inhibited the transport of arginine, the accumulation of ornithine (Fig. 4A), and significantly decreased the accumulation of ammonia; however, the pH of the biofilm effluent was unchanged (Fig. 4B). Therefore, in contrast to previous speculation (Beenken, Dunman et al. 2004; Resch, Rosenstein et al. 2005; Diep, Gill et al. 2006; Resch, Leicht et al. 2006), our results demonstrate that the ammonia generated by the arginine deiminase pathway is insufficient to offset the pH decrease due to the accumulation of organic acids (Fig. 4B and C). To test the second hypothesis, we assessed the affect *arcD* inactivation in a mouse indwelling device infection model. Mutants lacking *arcD* have dramatically reduced

arginine deiminase activity (data not shown), yet they achieved in vivo cell densities equivalent to the isogenic wild-type strain, suggesting that arginine deiminase is not required for biofilm formation and survival in vivo at least as defined by this model. It is possible that arginine deiminase is important in certain types of infections, such as when preferred nutrients are limiting or during a more active and prolonged immune response; however, additional work will be needed to address these possibilities.

Table 1. Strains and plasmids used in this study.

Plasmid or strain	**Relevant genotype and/or characteristic(s)**	**Source or Reference**
pBluescript II KS(+)	*E. coli* phagemid cloning vector	Stratagene
pTS1	*S. aureus* - *E. coli* temperature-sensitive shuttle vector	(Greene, McDevitt et al. 1995)
pEC4	pBluescript II KS(+) with *ermB* inserted into *Cla*I site	(Bruckner 1997)
pYF-1	pBluescript II KS(+) containing an internal fragment of the *arcD* gene of UAMS-1 inserted into *Sma*I site	This study
pYF-2	pYF-1 containing the *ermB* cassette of pEC4 inserted into the *Nde*I site of the *arcD* fragment	This study
pYF-3	The *arcD*::*ermB* PCR product pYF-2 inserted into the *Kpn*I and *EcoR*I site of pTS1	This study
RN4220	Restriction-negative *S. aureus*	(Novick 1991)
UAMS-1	*S. aureus* clinical isolate	Mark Smeltzer strain collection
UAMS-1182	*S. aureus* clinical isolate	Mark Smeltzer strain collection
UAMS-1-*arcD::ermB*	UAMS-1 containing an *ermB* insertion into *arcD*	This study
Xen40	Strain UAMS-1 containing *luxABCDE*	Caliper Life Sciences
UAMS-1272	Xen40 *arcD* deletion strain	This study

Table 2. Primers used in this study.

Primer	Nucleotide sequence (5' - 3')
arcDf	GCGAGTCAATATGGTGGTTCAG
arcDr	ACCCGATAACTGCAAGTACG
ermBNdeIf	CCGATTCATATGGTGATTACGCAGATAAATAAATACG
ermBNdeIr	CCGATTCATATGCAAAAGCGACTCATAGAATTATTTCC
arcDKpnIf	GCGGGTACCGCGAGTCAATATGGTGGTTCAG
arcDEcoRIr	GCGGAATTCACCCGATAACTGCAAGTACG
arcDVf	GAAGGAATAAACTTAACTTACGTTGGA
arcDVr	GCTTCTGTTGTCTGTATCGCATT
arcAf	ACACGGTATAACAATCAATCGG
arcAr	TCACTACAACGCCTGGTC
arcBf	GTAGAGGATACTGCGAAAG
arcBr	TGGAATGCTGGTAAACAA
arcCf	TGCGTCACCACTACCTCA
arcCr	GCTTCGTATGCCTGCTCT
icaAf	CTTGCCTGTTATAACGAAAGTGAAAC
icaAr	GAGTGAAGACACCCGAAATAGTATTG
ArcD1-attB2F	GGGGACCACTTTGTACAAGAAAGCTGGGTTTGTTGTGCTTC AGCTGTTGCTTCTTT
ArcD1-SacIIR	ATATTTCCCGCGGTCAATTAGATTCGCCAAGTATGGCACA
ArcD2-SacIIF	TATAACCCGCGGAT TATATTGAACGCACCGCCACCAAT
ArcD2-attB1R	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACA GACGATTGGCATCCTACACAAA

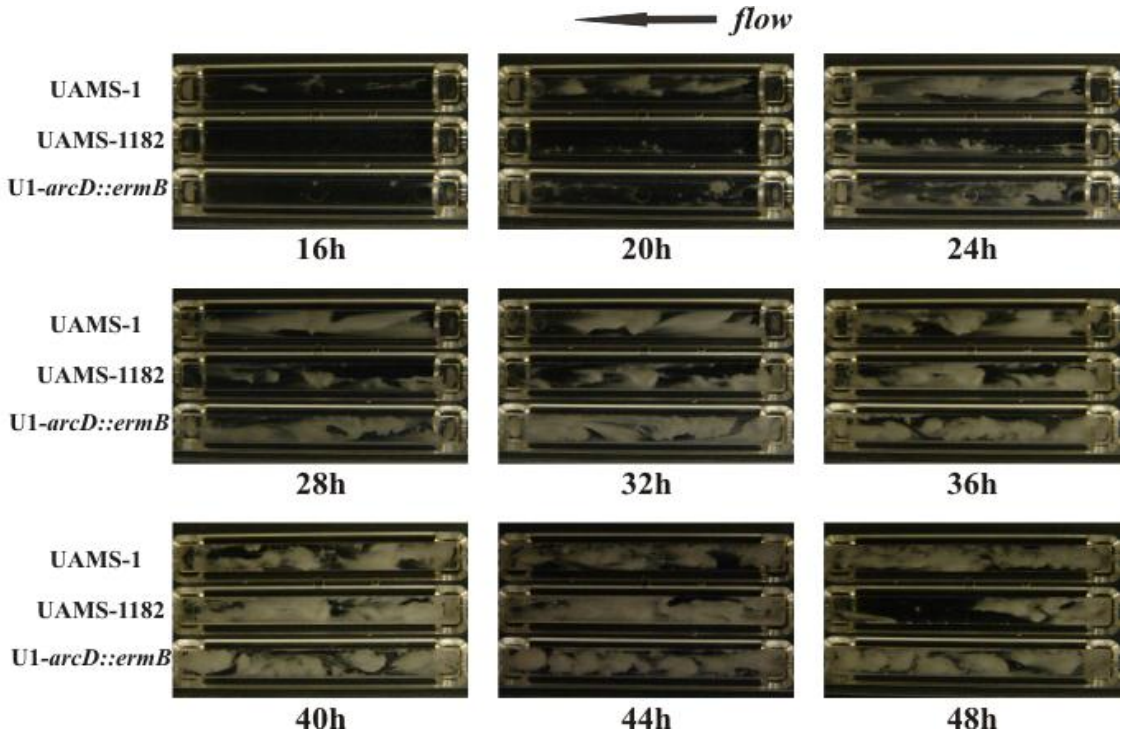

Fig. 1. Growth of *S. aureus* strains UAMS-1, UAMS-1182, and UAMS-1-*arcD*::*ermB* in a 3-chamber flow cell. Bacterial strains were grown at 37° C with a continuous flow (0.5 ml per minute per chamber) of TSB containing 0.5% glucose and 3% NaCl. The results are representative of multiple (>3) independent experiments. In the figure, strain UAMS-1-*arcD*::*ermB* is abbreviated as U1-*arcD*::*ermB.*

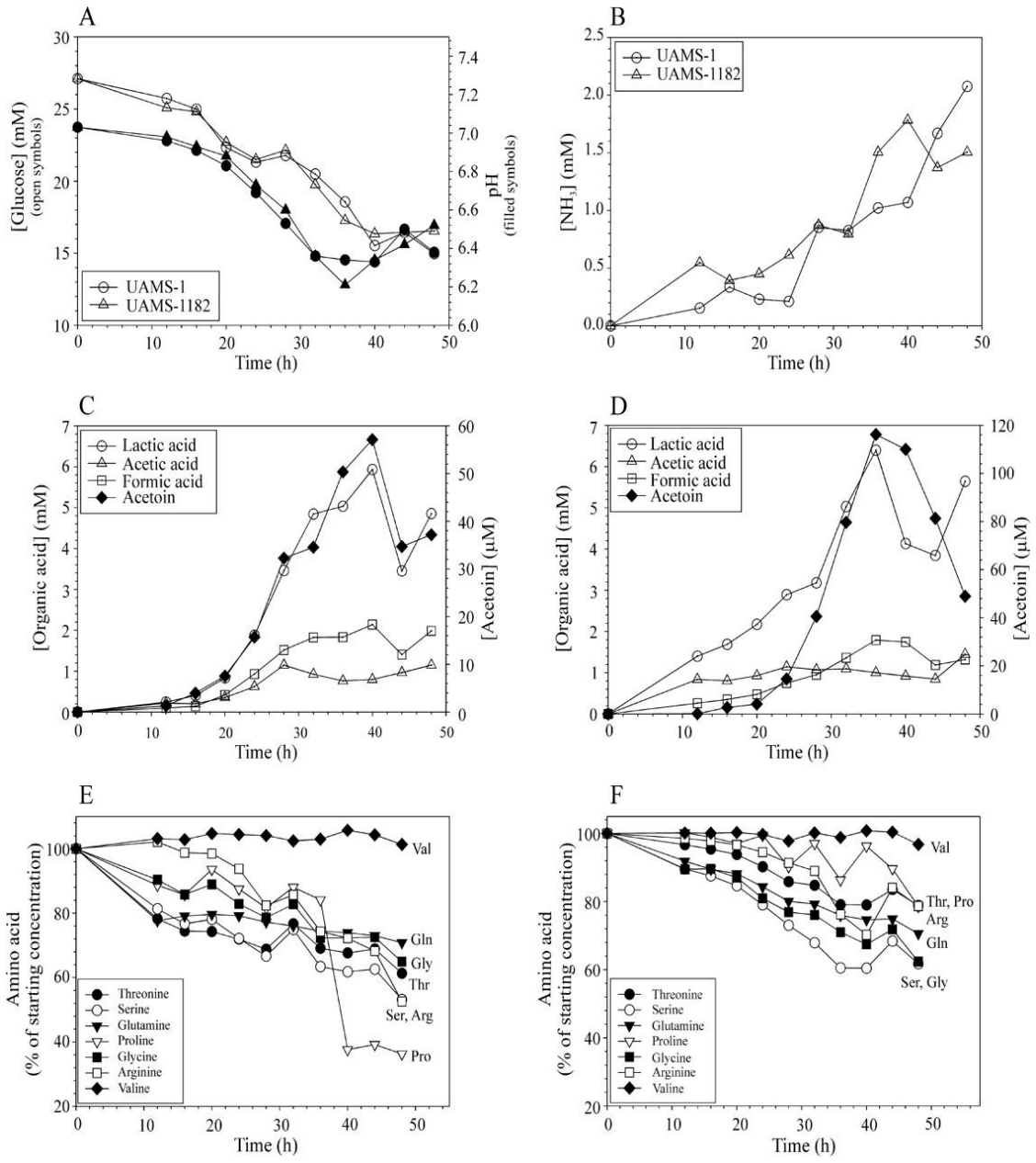
A
[Glucose] (mM)
(open symbols)
pH
(filled symbols)
Time (h)
UAMS-1
UAMS-1182
B
[NH3] (mM)
Time (h)
UAMS-1
UAMS-1182
C
[Organic acid] (mM)
[Acetoin] (μM)
Time (h)
Lactic acid
Acetic acid
Formic acid
Acetoin
D
[Organic acid] (mM)
[Acetoin] (μM)
Time (h)
Lactic acid
Acetic acid
Formic acid
Acetoin
E
Amino acid
(% of starting concentration)
Time (h)
Val
Gln
Gly
Thr
Ser, Arg
Pro
Threonine
Serine
Glutamine
Proline
Glycine
Arginine
Valine
F
Amino acid
(% of starting concentration)
Time (h)
Val
Thr, Pro
Arg
Gln
Ser, Gly
Threonine
Serine
Glutamine
Proline
Glycine
Arginine
Valine

Fig. 2. Metabolite extraction and accumulation by *S. aureus* strains UAMS-1 and UAMS-1182 during biofilm growth in flow cells. (A) The decreased pH of the culture medium corresponds to decreasing glucose concentrations. (B) Ammonia accumulation in the culture medium effluent. (C) Accumulation of organic acids and acetoin in the culture medium effluent of strains UAMS-1 and (D) UAMS-1182. (E) Free amino acid extraction from the culture medium by strains UAMS-1 and (F) UAMS-1182. The results presented are representative of at least two independent experiments. Symbols are defined in the figure insets.

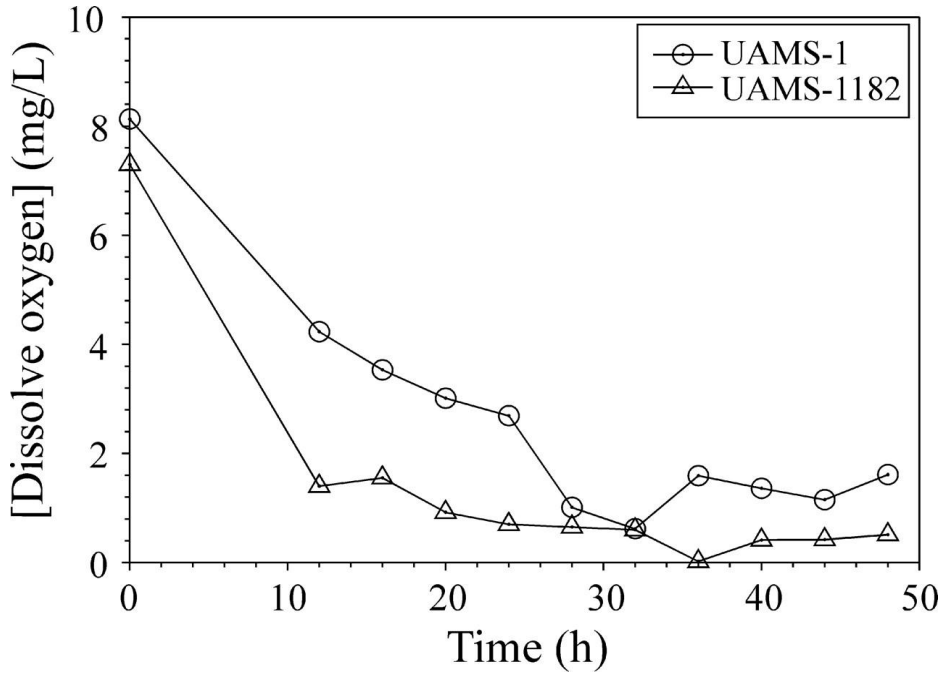

Fig. 3. Dissolved oxygen concentration in the culture medium effluent of strains UAMS-1 and UAMS-1182 grown in flow cells. Symbols are defined in the figure insets. The results presented are representative of at least three independent experiments.

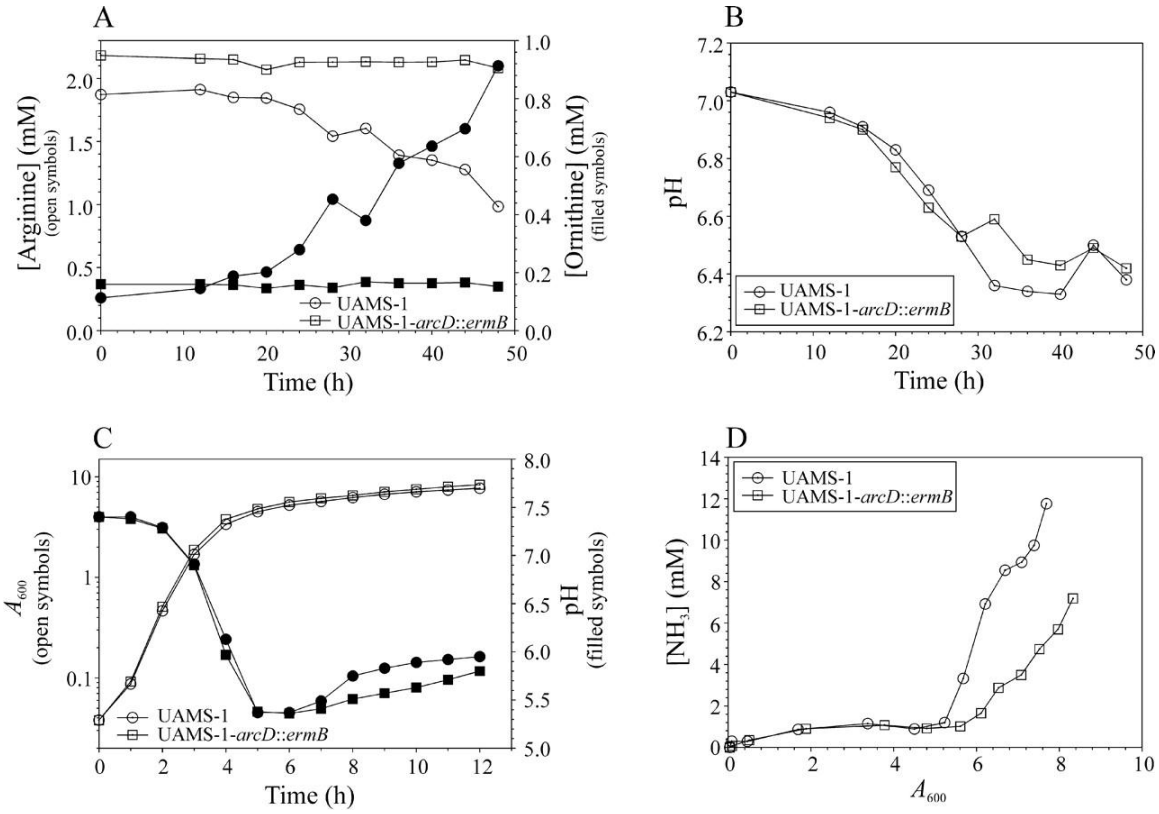

Fig. 4. Growth characteristics of strains UAMS-1 and UAMS-1-*arcD*::*ermB* grown under biofilm and planktonic conditions. (A) Arginine extraction and ornithine accumulation in culture medium of strains UAMS-1 and UAMS-1-*arcD*::*ermB* grown in biofilm flow cells. (B) pH of the biofilm culture medium effluent from panel A. (C and D) Planktonic growth of strains UAMS-1 and UAMS-1-*arcD*::*ermB* under microaerobic conditions (5:1 flask-to-medium ratio, TSB, and 100 rpm aeration.) (C) Growth and pH of the culture medium of strains UAMS-1 and UAMS-1-*arcD*::*ermB*. (D) Ammonia accumulation in the culture medium as a function of growth (A_{600}) for the cultures in panel C. The results presented are representative of at least two independent experiments.

CHAPTER III

TRICARBOXYLIC ACID CYCLE-DEPENDENT ATTENUATION OF STAPHYLOCOCCUS AUREUS IN VIVO VIRULENCE BY SELECTIVE INHIBITION OF AMINO ACID TRANSPORT

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ABSTRACT

Staphylococci are the leading causes of endovascular infections worldwide. Commonly, these infections involve the formation of biofilms on the surface of biomaterials. Biofilms are a complex aggregation of bacteria commonly encapsulated by an adhesive exopolysaccharide matrix. In staphylococci, this exopolysaccharide matrix is comprised of polysaccharide intercellular adhesin (PIA). PIA is synthesized when the tricarboxylic acid (TCA) cycle is repressed. The inverse correlation between PIA synthesis and TCA cycle activity led us to hypothesize that increasing TCA cycle activity would decrease PIA synthesis, biofilm formation, and reduce virulence in a rabbit catheter-induced model of biofilm infection. TCA cycle activity can be induced by preventing staphylococci from exogenously acquiring a TCA cycle-derived amino acid necessary for growth. To determine if TCA cycle induction would decrease PIA synthesis in *Staphylococcus aureus*, the glutamine permease gene (*glnP*) was inactivated and TCA cycle activity, PIA accumulation, biofilm forming ability, and virulence in an experimental catheter-induced endovascular biofilm (endocarditis) model were determined. Inactivation of this major glutamine transporter increased TCA cycle activity, transiently decreased PIA synthesis, and significantly reduced in *vivo* virulence in the endocarditis model in terms of achievable bacterial densities in biofilm-associated cardiac vegetations, kidneys and spleen. These data confirm the close linkage of TCA cycle activity and virulence factor production and establish that this metabolic linkage can be manipulated to alter infectious outcomes.

INTRODUCTION

The number of patients in whom indwelling biomaterials are implanted annually (*e.g.* vascular access catheters) is steadily increasing (Eltchaninoff, Zajarias et al. 2008). Moreover, the implantation of such biomaterials predisposes an individual to life-long, device-related infective endocarditis (IE), an extremely serious complication with a high incidence of mortality (Piper, Korfer et al. 2001; El-Ahdab, Benjamin et al. 2005). The most common causative agents of IE are low-G+C Gram-positive bacteria, with the predominant bacterial etiological agents being *Staphylococcus aureus* and coagulase-negative staphylococci (*e.g.*, *Staphylococcus epidermidis*) (Fowler, Miro et al. 2005). These biomaterial-associated infections frequently involve the formation of a bacterial biofilm, featuring an aggregation of bacteria usually encapsulated in an exopolysaccharide matrix (Donlan 2001). The exopolysaccharide provides structural stability to biofilms, enhanced adhesion to surfaces, and protection from host defenses and antibiotics (Vuong, Kocianova et al. 2004; Olson, Garvin et al. 2006; Begun, Gaiani et al. 2007). In staphylococci, this exopolysaccharide matrix is composed of polysaccharide intercellular adhesin (PIA) (Mack, Fischer et al. 1996).

PIA is an *N*-acetyl-glucosamine polymer (Mack, Fischer et al. 1996) whose biosynthesis requires the enzymes encoded within the intercellular adhesin (*ica*) operon (*icaADBC*) (Heilmann, Schweitzer et al. 1996). Regulation of the *ica* operon is complex, involving at least two DNA binding proteins (IcaR and SarA) (Conlon, Humphreys et al. 2002; Beenken, Blevins et al. 2003; Valle, Toledo-Arana et al. 2003; Tormo, Marti et al. 2005; Handke, Slater et al. 2007), the alternative sigma factor σ^B, and the tricarboxylic

acid (TCA) cycle (Vuong, Kocianova et al. 2004; Majerczyk, Sadykov et al. 2008). IcaR is homologous to the TetR family of transcriptional regulatory proteins and is a transcriptional repressor of *icaADBC* (Conlon, Humphreys et al. 2002). SarA is a DNA binding protein (Cheung, Koomey et al. 1992) that affects the expression of many genes in *S. aureus* and *S. epidermidis* (Blevins, Gillaspy et al. 1999). σ^B affects PIA synthesis by controlling the expression of *icaR* (Knobloch, Jager et al. 2004). Inhibition of TCA cycle activity de-represses transcription of the *ica* operon, linking central metabolism to PIA synthesis (Vuong, Kidder et al. 2005; Sadykov, Olson et al. 2008).

One function of the TCA cycle is to supply intermediates (*i.e.*, oxaloacetate, α-ketoglutarate, and succinyl-CoA) for biosynthesis. For this reason, transcription of genes encoding the first three enzymes of the TCA cycle is regulated by the availability of amino acids (Goldschmidt and Powelson 1953; Somerville, Said-Salim et al. 2003). That is to say, during exponential growth when amino acids are exogenously available to staphylococci, TCA cycle activity is repressed. De-repression of TCA cycle activity occurs during the post-exponential growth phase when the availability of exogenous amino acids becomes growth limiting. This amino acid-dependent repression of TCA cycle activity and the reciprocal relationship between TCA cycle activity and PIA biosynthesis led us to hypothesize that hindering exogenous amino acid uptake would increase TCA cycle activity resulting in reduced PIA synthesis, limiting biofilm formation, and attenuating virulence in an in *vivo* biofilm model, catheter-induced experimental IE. To test this hypothesis, the major glutamine transport gene, glutamine permease (*glnP*), in *S. aureus* strain UAMS-1 was inactivated and the effects on TCA cycle activity, PIA accumulation, the ability to form a biofilm, and the capacity to initiate

and propagate IE were determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in 2xYT broth (Sambrook and Russell 2001) or on 2xYT agar and *S. aureus* strains were grown in tryptic soy broth (TSB) (BD Biosciences) or on TSB-containing 1.5% agar (TSA). Unless otherwise stated, all bacterial cultures were inoculated 1:200 from an overnight culture (normalized for growth) into TSB, incubated at 37°C, and aerated at 225 rpm with a flask-to-medium ratio of 10:1. Antibiotics were purchased from Fisher Scientific or Sigma Chemical and, when used, were used at the following concentration: *E. coli*: ampicillin (100µg/ml); *S. aureus*: erythromycin (5-8µg/ml), chloramphenicol (8µg/ml), minocycline (2µg/ml), and tetracycline (10µg/ml).

Selection of amino acid transporter to inactivate. The amino acid transporter to be inactivated was selected based on three inclusion criteria: i) the amino acid must be extensively extracted by *S. aureus* during growth in a biofilm (Zhu, Weiss et al. 2007); ii) there must be a complete biosynthetic pathway for the amino acid identified as being extracted; and iii) the amino acid must be derived from one or more TCA cycle biosynthetic intermediates. These three criteria define an amino acid that is important during growth in a biofilm but that can be synthesized from the coordinated action of the TCA cycle and an amino acid biosynthetic pathway. Previously we determined that Gln, Ser, Pro, Gly, Thr, and Arg were the most extensively extracted amino acids by *S. aureus* strains UAMS-1 and UAMS-1182 during growth in biofilm flow cells (Zhu, Weiss et al.

2007). Of these amino acids, Ser and Gly are not synthesized from TCA cycle intermediates; therefore, they do not fulfill our inclusion criteria. Although Thr fulfills all inclusion criteria, it is transported by a multiple amino acid transporter; hence, inactivating this transporter would affect the transport of other amino acids. This left the transporters of Gln, Pro, and Arg as possible candidates for genetic inactivation. Mutational studies involving the Pro transporter (*putP*) and the Arg transporter (*arcD*) have been reported (Schwan, Coulter et al. 1998; Bayer, Coulter et al. 1999; Zhu, Weiss et al. 2007); hence, inactivating *putP* or *arcD* would be unnecessarily duplicative of prior research. In addition, the TCA cycle was not significantly induced in an *arcD* mutant strain ((Zhu, Weiss et al. 2007) and data not shown), presumably due to the bacteria's ability to generate arginine from ornithine by acquiring exogenous glutamate. Taken together, the Gln permease (*glnP*) was selected for inactivation and testing of our central hypothesis.

Construction of *S. aureus* glutamine permease mutant. PCR primers (Table 2) were designed primarily using the *S. aureus* COL genome sequence (GenBank # NC_002951). A 1.4-kb PCR product encompassing *orf1916* (putative glutamine permease, *glnP*) was amplified using primers COL1916-f and COL1916-r and cloned into the *Sma*I site of pBluescript II K/S (+) (Stratagene) to generate plasmid pYF-4. The *ermB* cassette of pEC4 (Bruckner 1997)was amplified using primers ErmBNsiI-f and ErmBBbvI-r and ligated into pYF-4 digested with *Nsi*I and *Bbv*CI (this deletes 576 bp of *glnP*) to generate the plasmid pYF-5. The *glnP*::*ermB* fragment of pYF-5 was inserted into the *Sma*I site of the temperature-sensitive shuttle vector pTS1 (Foster 1998) to create pYF-6. The

temperature-sensitive plasmid pYF-6 was electroporated into *S. aureus* strain RN4220 and then introduced into strain UAMS-1 by Φ85 phage transduction. Strain UAMS-1 containing pYF-6 was used to construct the *glnP* mutant by the method of Foster (Foster 1998). To minimize the possibility that any phenotypes were the result of random mutations occurring during the temperature shifts, the resulting *glnP*::*ermB* mutation was backcrossed into wild-type strain UAMS-1 using transducing phage 85. Mutants were confirmed by PCR and Southern blotting.

Construction of *glnP* complementing plasmid. Plasmid pCL15 (Table 1) containing a P_{spac} promoter, was used to construct the *glnP* complementation plasmid pYM6. A 1.5 kb promoterless *glnP* gene from *S. aureus* strain UAMS-1 was PCR amplified using the primers HindIII-SD-glnP-f and EcoRI-glnP-r and ligated into plasmid pCL15 digested with *Hind*III and *Eco*RI. The ligation mixture was used to transform *S. aureus* strain RN4220. After isolation of pYM6 from RN4220 it was electroporated into *S. aureus* strain UAMS-1-*glnP::ermB*.

γ-L-Glutamyl hydrazide (GGH) susceptibility assay. To determine if the *glnP* gene codes for a glutamine transporter, *S. aureus* strains were grown overnight in a chemically defined media lacking glutamate and glutamine (Horsburgh, Ingham et al. 2001) and diluted into sterile medium to an OD_{600} of 0.005 containing different concentrations of the glutamine analogue GGH (Sigma Chemical). Cultures were grown at 37°C with shaking (225 rpm) for 12 h. Bacterial densities were determined by measuring the optical density at 600 nm.

Enzymatic activity assays. To confirm activation of the TCA cycle, we performed two enzymatic assays querying the oxidative and reductive branches of the TCA cycle. Isocitrate dehydrogenase (oxidative branch) enzymatic activity assays were performed as described (Somerville, Chaussee et al. 2002). Fumarase (reductive branch) activity was measured as described (Hill and Bradshaw 1969). Protein concentrations were determined by the modified Lowry assay (Pierce Chemical).

Determination of NAD^+ and NADH concentrations. TCA cycle activity requires oxidized nicotinamide adenine dinucleotide (NAD); therefore, an increase in TCA cycle activity can alter the NAD^+/NADH redox balance. The intracellular concentrations of NAD^+ and NADH were determined using an enzymatic cycling assay kit (Biovision). Total NAD^+ and NADH concentrations were normalized to the bacterial density and were performed in quadruplicate for three independent experiments.

PIA Immunoblot assay. PIA accumulation was determined as described (Zhu, Weiss et al. 2007). The data are presented as the percentage difference in PIA accumulation relative to UAMS-1 at 2 h.

Northern blot analysis. Decreasing TCA cycle activity increases the transcription or message stability of the global regulator RNAIII (Somerville, Chaussee et al. 2002; Shaw, Golonka et al. 2004) and RNAIII regulates the expression of secreted (*e.g.*, serine protease, *sspA*) and cell-associated (*e.g.*, protein A, *spa*) virulence determinants. To

determine if increasing TCA cycle activity affected RNAIII message levels northern blot analysis of transcripts was performed as described (Sambrook and Russell 2001), except that total RNA was isolated using the FastRNA Pro Blue kit (Qbiogene) and purified using an RNeasy kit (Qiagen). Probes for northern blotting were generated by PCR amplification of unique internal regions of RNAIII, *spa*, *cna*, and *fnbA* (Table 2) and labeled using the North2South random prime labeling kit (Pierce). Detection was performed using the chemiluminescent nucleic acid detection module (Pierce).

Construction of *sspA-xylE* and *icaA-xylE* reporter plasmids. To assess the effect of TCA cycle mediated RNAIII changes on serine protease transcription, a reporter plasmid was constructed. Similarly, to validate changes in PIA biosynthesis an *icaA* reporter plasmid was constructed. To construct the *sspA* reporter plasmid, an 822-bp region of strain UAMS-1 genomic DNA, containing the *sspA* promoter and the first 189 nucleotides of *sspA*, was amplified by PCR using primers sspA(SA)-PstI-f and sspA(SA)-BamHI-r (Table 2). The PCR product was ligated to the *xylE* gene of pLL38 (Chen, Luong et al. 2007) after digestion of both with PstI and BamHI to generate the reporter plasmid pMRS13. The *icaA-xylE* transcriptional fusion was made by PCR amplification of a 394 bp region containing the promoter and the first 216 nucleotides of *icaA* using primers icaA-P1 and icaA-P2 (Table 2). The resulting PCR product was ligated to the *xylE* gene in pLL38 at the PstI-BamHI sites to generate pML3783. Reporter plasmids were introduced into strains UAMS-1 and UAMS1-*glnP*::*ermB* by phage transduction. Catechol 2,3-dioxygenase activity was assayed as described (Chen, Luong et al. 2007). Promoter activity was defined as the absorbance at 375 nm for one OD_{660} unit and

expressed relative to the mean activity present in strain UAMS-1 at 2 h post-inoculation.

Western blot analysis. As mentioned, RNAIII regulates protein A expression. To determine if TCA cycle mediated changes in RNAIII levels effected protein A biosynthesis, protein A was collected as described (Vytvytska, Nagy et al. 2002) and western blot analysis was performed as described (Towbin, Staehelin et al. 1979).

Polystyrene primary attachment assay. The primary attachment assay was performed as described by Lim *et al*. (Lim, Jana et al. 2004). Briefly, bacterial cultures (2 h post-inoculation) were diluted into TSB to yield approximately 300 CFU. Bacteria were poured onto polystyrene Petri dishes (Fisher Scientific) and incubated at 37ºC for 30 min. Following incubation, the Petri dishes were rinsed three times with sterile phosphate-buffered saline (pH 7.5) and covered with 15 ml of TSB containing 0.8% agar maintained at 48°C. The percentage of bacterial attached to the polystyrene was defined as the number of CFU remaining in Petri dishes after washing compared to the number of CFU in control plates. The experiment was repeated three times.

Flow cell biofilm formation. *S. aureus* were grown in flow-cell chambers (Stovall Life Sciences) as described (Zhu, Weiss et al. 2007). To assess bacterial growth, 12 h post-inoculation and every 4 h thereafter, effluent samples were collected and the pH and lactic acid accumulation were measured.

Experimental infective endocarditis (IE). A well-characterized rabbit model of IE

(Dhawan, Yeaman et al. 1997; Xiong, Bayer et al. 2004) was used to study three infection outcomes of *S. aureus* UAMS-1 parental vs. UAMS-1-*glnP*::*ermB*: 1) early bacteremia clearance; 2) initial vegetation colonization; and 3) intrinsic virulence in a rabbit model of IE. In brief, female New Zealand white rabbits (Irish Farms Products and Services) underwent transcarotid-transaortic valve catheterization (Dhawan, Yeaman et al. 1997; Xiong, Kupferwasser et al. 1999; Xiong, Bayer et al. 2004). Subsequent infection challenge studies are described below:

i) Early clearance of bacteremia and initial colonization of vegetations. Animals were challenged by the intravenous (iv) injection of *S. aureus* strains UAMS-1 parental or UAMS-1-*glnP*::*ermB* (10^9 CFU/animal) at 24 hr post-catheterization. At 1 and 30 minutes post-challenge, blood samples were obtained for quantitative culture. Previous studies in this model suggest that initial clearance of the bloodstream maximally occurs between 30-60 min post-challenge in this model (Dhawan, Yeaman et al. 1997). In addition, to assess vegetation colonization, animals were sacrificed by a rapid iv injection of sodium pentobarbital (200 mg/kg; Abbott Laboratories) at 30 minutes post-challenge: all vegetations were removed and processed for quantitative culture onto TSA plates and incubated at 37^{o}C for 24 hr. *S. aureus* densities were expressed as mean ± SD $\log_{10}$ colony-forming units per gram of vegetation.

ii) Intrinsic virulence. The intrinsic virulence of IE can be measured as a composite of: i) induction rates of IE over an inoculum challenge range; and ii) target tissue bacterial densities at 48 hr after infection at the ID_{95} inoculum. Thus, animals were challenged at 10^4 or 10^5 CFU iv with strains UAMS-1 or UAMS-1-*glnP*::*ermB* at 24 hr post-catheterization (the inoculum range encompassing the ID_{95} for most *S. aureus*

strains in this model (Bayer, Coulter et al. 1999)). At 48 hr after infection, all animals were euthanized, their cardiac vegetations, kidneys and spleen removed and quantitatively cultured as detailed above.

All experiments involving animals were reviewed and approved by the LA Biomedical Research Institute's Institutional Animal Care and Use Committee, and comply with Animal Welfare Legislation and NIH guidelines and policies.

Statistical analysis. The statistical significance of changes between wild-type and mutant strains (*e.g.*, NADH concentrations) was assessed with Student's *t* test. *P* values less than 0.05 were considered significant.

RESULTS

***Staphylococcus aureus glnP* mutant physiology.** The TCA cycle can be induced by inhibiting the ability of staphylococci to acquire a TCA cycle-derived amino acid, thus forcing the bacteria to synthesize that amino acid for growth. To do this, a 576 bp portion of the putative Gln permease (*glnP*, orf SACOL1916) was deleted in *S. aureus* strain UAMS-1 and replaced with an *ermB* cassette and TCA cycle activity was assessed. Inactivation of *glnP* did not alter the growth rate, growth yield, or the pH profile of the culture medium (Fig. 1A), demonstrating the absence of any growth defects in the *glnP* mutant strain relative to strain UAMS-1. It is possible that phenotypic differences could occur in different media and growth conditions. To confirm that orf SACOL1916 encodes for the glutamine permease *glnP*, the susceptibility of strains UAMS-1 and UAMS-1-*glnP*::*ermB* to the toxic glutamine analog γ-L-glutamyl hydrazide was determined (Fig. 1B). As expected, *glnP* inactivation significantly ($P < 0.01$) decreased the susceptibility of strain UAMS-1-*glnP*::*ermB* to γ-L-glutamyl hydrazide, strongly suggesting that orf SACOL1916 encodes a Gln transporter. Complementation of strain UAMS-1-*glnP*::*ermB* with plasmid pYM6 restores susceptibility to γ-L-glutamyl hydrazide equivalent to parental strain UAMS-1 (Fig. 1B). Of note, although the *glnP* mutant strain is less susceptible to γ-L-glutamyl hydrazide than is the parental strain, it does remain susceptible at higher concentrations of γ-L-glutamyl hydrazide, suggesting there is lower affinity transporter capable of transporting glutamine.

As stated, the TCA cycle is repressed during growth in nutrient rich conditions.

The carbon backbone of glutamate is the TCA cycle intermediate α-ketoglutarate; therefore, if Gln transport is hindered, then bacteria will increase synthesis of α-ketoglutarate to offset the decrease in Gln transport. This increase in synthesis of α-ketoglutarate means that there will be an increase in TCA cycle activity at a time when the TCA is normally repressed, which is analogous to the TCA cycle repression in biofilm growth under nutrient rich conditions. To assess the effect of *glnP* inactivation on TCA cycle activity under nutrient rich conditions that are most similar to the nutrient rich conditions of a biofilm, the exponential to early post-exponential growth phase TCA cycle activity was assessed in strains UAMS-1 and UAMS-1-*glnP*::*ermB* by measuring the activities of one enzyme in the oxidative branch (isocitrate dehydrogenase) and one enzyme in the reductive branch (fumarase) and by measuring the intracellular concentrations of NADH (Fig. 1C, 1D, and1F). Consistent with the central hypothesis, *glnP* inactivation increased TCA cycle activity relative to the isogenic parental strain UAMS-1 (Fig. 1C) under conditions that are similar to the nutrient rich conditions encountered in a biofilm flow cell. Complementation of strain UAMS-1-*glnP*::*ermB* with a plasmid encoded copy of *glnP* restored TCA cycle activity to levels consistent with the wild-type strain UAMS-1 (Fig. 1D). As expected, increased TCA cycle activity was accompanied by a transient increase in the intracellular concentration of NADH (Fig. 1F). Overall, these data confirm that selective inhibition of amino acid transport increased TCA cycle activity and altered the metabolic status of the bacteria and that this altered metabolic status was due to *glnP* inactivation.

Inactivation of *glnP* alters the temporal pattern of virulence factor synthesis. Our central hypothesis predicts that increased TCA cycle activity will decrease PIA

biosynthesis. To determine if increased TCA cycle activity altered transcription of *icaADBC* and/or synthesis of PIA, the relative amounts of cell-associated PIA produced by strains UAMS-1 and UAMS-1-*glnP*::*ermB* were determined using a PIA immunoblot assay, while *icaADBC* transcription was assessed using a P_{icaA}-*xylE* reporter plasmid (Fig. 2A-C). As hypothesized, increased TCA cycle activity correlated with decreased *icaADBC* transcription (Fig. 2A) and PIA accumulation (Fig. 2B and C); however, these results persisted only while the intracellular NADH concentration in strain UAMS-1-*glnP*::*ermB* was greatest (2 h; Fig. 1D). To assess if *glnP* inactivation would affect biofilm formation, the gross morphology of biofilms was assessed by growth of strains UAMS-1 and UAMS-1-*glnP*::*ermB* in biofilm flow cells under nutrient rich conditions (Fig. 3). These nutrient rich conditions are similar to the exponential growth phase of planktonic cultures (Fig. 1C and 1D). Inactivation of *glnP* delayed biofilm maturation; however, the morphology of the biofilms formed by the wild-type and *glnP* mutant strains was similar after approximately 40-48 h of growth (Fig. 3).

TCA cycle activity is linked with the synthesis of virulence factors and virulence factor regulators (Recsei, Kreiswirth et al. 1986; Somerville, Chaussee et al. 2002; Somerville, Cockayne et al. 2003), including RNAIII, part of the *agr* quorum sensing system (Recsei, Kreiswirth et al. 1986). The transcription and/or stability of RNAIII is increased in an *S. aureus* TCA cycle mutant (Somerville, Chaussee et al. 2002), raising the possibility that increased TCA cycle activity (Fig. 1C) could decrease RNAIII transcript levels and alter the temporal pattern of virulence factor synthesis. To determine if increased TCA cycle activity altered RNAIII levels in strain UAMS-1-*glnP*::*ermB* relative to the parental strain UAMS-1, northern blot analysis of RNAIII transcript levels

was performed (Fig. 4A). As expected, increased TCA cycle activity correlated with decreased RNAIII transcript levels relative to the parental strain (Fig. 4A). RNAIII is a negative regulator of adhesin biosynthesis (Recsei, Kreiswirth et al. 1986) and a positive regulator of secreted virulence determinants (Shaw, Golonka et al. 2004); hence, *glnP* inactivation should increase adhesin synthesis such as protein A (*spa*), collagen adhesin (*cna*), and fibronectin binding protein A (*fnbA*) and decrease transcription of secreted proteins like serine protease (*sspA*). Consistent with decreased RNAIII levels, *glnP* inactivation increased the exponential phase synthesis of protein A (Fig. 4B) and moderately increased transcription of *fnbA* and *cna* (data not shown) and decreased transcription of *sspA* (Fig. 4C). These data are consistent with strain UAMS-1-*glnP*::*ermB* having an increased ability to adhere to surfaces. To determine if *glnP* inactivation enhanced bacterial adherence to polystyrene, a polystyrene primary attachment assay was performed with strains UAMS-1 and UAMS-1-*glnP*::*ermB*. Primary attachment of strains UAMS-1 and UAMS-1-*glnP*::*ermB* to polystyrene was equivalent (data not shown). These data demonstrate the altered bacterial metabolic status changed the temporal pattern of virulence factor synthesis.

Experimental infective endocarditis (IE). i) Clearance of early bacteremia and initial colonization of vegetations. No significant differences in the rates of early bacteremia clearance were observed in rabbits challenged with the parental strain UAMS-1 or its *glnP* mutant at either 1 or 30 minutes post-infection (data not shown). Similarly, no significant differences were observed in the extent of initial colonization of vegetations between strains UAMS-1 and UAMS-1-*glnP*::*ermB* (5.60 ± 0.4 and 5.99 ± 0.36 $\log_{10}$CFU/g. veg., respectively).

ii) Intrinsic virulence in the IE model. At the 10^4 and 10^5 CFU challenge inocula of *S. aureus* strains UAMS-1 or the isogenic *glnP* mutant, all catheterized animals developed IE. Importantly, animals individually infected with strain UAMS-1 had significantly higher bacterial densities in all three target tissues vs. animals infected with strain UAMS-1-*glnP*::*ermB* (Table 3; $P < 0.05$).

DISCUSSION

Inactivation of *glnP* in strain UAMS-1 increased TCA cycle activity at a time when it is normally repressed (Fig. 1C). Consistent with our central hypothesis, inactivation of *glnP* also correlated with transient decreases in PIA synthesis (Fig. 1C) and RNAIII levels (Fig. 4A), delayed maturation of biofilms (Fig. 3), and significantly reduced bacterial densities in cardiac vegetations, kidneys and spleen in an endocarditis model. Precedence for amino acid permease inactivation attenuating *S. aureus* virulence was shown for the high affinity proline permease (*putP*) (Bayer, Coulter et al. 1999). Inactivation of *putP* significantly reduced bacterial densities in cardiac vegetations in an experimental endocarditis model; however, no data are available regarding the effects on virulence factor synthesis. Similar to *glnP* inactivation, inhibition of arginine transport by deletion of the arginine/ornithine antiporter (*arcD*) decreased PIA accumulation; however, deletion of *arcD* did not affect *S. aureus* virulence in a catheter-based murine infection model (Zhu, Weiss et al. 2007). These data demonstrate that *S. aureus* amino acid transporter inactivation does not produce a uniform effect on virulence and this may be due to the differing metabolic changes necessitated by the specific transporter inactivated.

The TCA cycle supplies biosynthetic intermediates, reducing potential, and a small amount of ATP. In nutrient rich growth conditions, the bacterial demand for biosynthetic intermediates is supplied exogenously; hence, TCA cycle activity is very low (Strasters and Winkler 1963; Collins, Kristian et al. 2002; Somerville, Cockayne et al. 2003). These same nutrient rich growth conditions occur during growth in a biofilm flow cell. When environmental conditions change and nutrients become growth limiting,

staphylococci increase TCA cycle activity and catabolize non-preferred carbon sources such as acetate (Somerville, Chaussee et al. 2002). In *vitro*, the transition from nutrient rich conditions to nutrient limited conditions usually occurs concomitant with the transition from exponential growth to post-exponential growth. As stated, PIA is synthesized when nutrients are abundant (Dobinsky, Kiel et al. 2003); in contrast, TCA cycle activity is repressed under these same conditions (Goldschmidt and Powelson 1953; Gardner and Lascelles 1962; Somerville, Cockayne et al. 2003). In addition to this inverse correlation between PIA synthesis and TCA cycle activity, TCA cycle activity actually represses *icaADBC* transcription and PIA biosynthesis (Vuong, Kidder et al. 2005; Sadykov, Olson et al. 2008). This causal relationship suggested that de-repressing TCA cycle activity would decrease PIA synthesis. Normally, staphylococci de-repress TCA cycle activity when nutrients become growth limiting; however, TCA cycle activity can be induced by withdrawing a nutrient from the culture medium or hindering the transport of that nutrient. Using the latter strategy in the current study, inactivation of the glutamine permease resulted in increased TCA cycle activity relative to the parental strain UAMS-1 (Fig. 1C and 1D). The increased TCA cycle activity altered the bacterial metabolic status (Fig. 1F), the temporal pattern of virulence factor synthesis, and transiently decreased PIA synthesis (Fig. 2A-C and 4A-C). These data suggested that manipulating the bacterial metabolic status would decrease virulence. To test this suggestion, we chose to use a rabbit IE model. This model is thought to be the "gold standard" for experimental endovascular infections because it closely mimics its human counterpart microbiologically, immunologically, histopathologically, pathogenetically and anatomically (in terms of organ involvements) (Bayer and Norman 1990).

There are at least 4 important phases in the pathogenesis of endovascular infections: i) initial seeding of the bloodstream and survival therein; ii) colonization of damaged endothelial surfaces; iii) survival and progression of infection at damaged endothelial sites, and subsequent hematogenous dissemination to other target organs; and iv) reseeding of damaged endothelial sites. The experimental IE model allows one to dissect most of these phases and compare the capacity of parental bacterial strains with their relevant isogenic mutants defective in specific phenotypes-of-interest. Thus, we were able to compare the abilities of the parental UAMS-1 and its *glnP* mutant strain to execute the above endovascular pathogenetic stages. Since the *glnP* mutation causes a delay in biofilm formation secondary to TCA cycle activation and PIA suppression in *vitro*, and the catheter-induced IE model above is dependent at least in part on biofilm-related vegetation formation, this provided an ideal experimental milieu. Several interesting observations emerged from these in *vivo* studies. Firstly, the bacteremic clearance and initial endothelial site colonization phases were not impacted by the *glnP* mutation. As bloodstream clearance is probably most dependent on susceptibility or resistance to innate host defenses such as opsonophagocytosis and antimicrobial peptide-induced killing (Verdrengh and Tarkowski 1997; Yeaman and Bayer 1999; Ganz 2003; Clarke and Foster 2006; Nathan 2006), this outcome is not unexpected. Moreover, since the colonization phase is related to expression of a cadre of adhesins (Xiong, Bayer et al. 2004; Clarke and Foster 2006), the non-effect of *glnP* inactivation on this pathogenetic stage is also not surprising. Of note, in the intrinsic virulence experiments by the parental or *glnP* mutant challenge, a differential fitness advantage of the parental strain vs. the *glnP* mutant within catheter-related cardiac vegetations was observed (Table 3). With a

reduction in glutamine uptake and activation of the TCA cycle, the delay in biofilm formation within vegetations should enable increased access of both cellular and secreted host defenses (*e.g.*, platelet microbicidal proteins (Yeaman and Bayer 1999)) at this site, translating into enhanced clearance of the *glnP* mutant from this location. Finally, in the two major hematogenous dissemination target organs in this model (*i.e.*, kidneys and spleen), achievable bacterial densities in animals infected with the parental strain significantly exceeded those of the *glnP* mutant. This outcome undoubtedly reflects the larger bacterial burden in biofilm-associated vegetations of parental-infected animals available for subsequent septic embolization to such target organs.

Conclusion. The close linkage of the TCA cycle and virulence determinant synthesis (Somerville, Chaussee et al. 2002; Somerville, Cockayne et al. 2003; Vuong, Kidder et al. 2005; Zhu, Weiss et al. 2007) can be exploited to modify the temporal pattern of virulence factor synthesis (Fig. 2A-C and 4A-C) and attenuate virulence (Table 3). In addition, increasing TCA cycle activity could potentially increase the in *vivo* susceptibility of *S. aureus* to bactericidal antibiotics (Kohanski, Dwyer et al. 2007). Taken together, these data suggest that vaccines directed against unique epitopes of bacterial amino acid transporters, such as GlnP, could provide therapeutic benefits beyond facilitating immune system recognition of the infectious agent. Specifically, vaccines directed against amino acid transporters capable of inducing TCA cycle activity would theoretically facilitate protection/treatment by three different means: i) conventional immune mediated recognition and killing of bacteria; ii) attenuation of virulence due to disruption of the temporal pattern of virulence factor synthesis; and iii)

enhancement of killing by bactericidal antibiotics (Kohanski, Dwyer et al. 2007).

Table 1: Strains and plasmids used in this study

Bacterial strains and plasmids	**Relevant properties**[a]	**Reference or source**
pBluescript II KS(+)	*E.coli* phagemid cloning vector	Stratagene
pTS1	*S. aureus-E. coli* temperature-sensitive shuttle vector	(Greene, McDevitt et al. 1995)
pEC4	pBluescript II KS(+) with *ermB* inserted into ClaI site	(Bruckner 1997)
pYF-4	pBluescript II KS(+) containing a portion of *glnP*	This study
pYF-5	pYF-4 containing the *ermB* cassette of pEC4 inserted into the NsiI and BbvCI sites of the *glnP*	This study
pYF-6	*glnP::ermB* PCR product of pYF-5 inserted into the SmaI site of pTS1	This study
pLL38	*xylE* transcriptional fusion vector	(Chen, Luong et al. 2007)
pML3783	394-bp *icaA* promoter fused to the *xylE* gene of pLL38; Spc^R, Tet^R	This study
pMRS13	822-bp *sspA* promoter fused to the *xylE* gene of pLL38; Spc^R, Tet^R	This study
pCL15	Expression vector; derivative of pSI-1; Cam^R	(Luong, Dunman et al. 2006)
pYM6	pCL15 with *glnP* gene under the control of the P_{spac} promoter; Cam^R	This study
RN4220	Restriction-deficient *S. aureus* cloning strain	(Novick 1991)
UAMS-1	*S. aureus* osteomyelitis isolate	(Zhu, Weiss et al. 2007)
UAMS-1-*glnP::ermB*	*glnP* insertion mutant of UAMS-1	This study

[a] Amp, ampicillin; Cam, chloramphenicol; Erm, erythromycin; Min, minocycline; Tmp, trimethoprim; ts – temperature sensitive origin of replication

Table 2: Primers used in this study.

Primer target	Primer designation	Nucleotide sequence (5′ - 3′)	PCR product use
glnP	COL1916-f	GTATATCAATCCCACAGCACATG	PCR cloning
glnP	COL1916-r	TAATCACTGGCATTCAATCTCC	PCR cloning
glnP	HindIII-SD-glnP-f	GATAAGCTTAGGGAGGGCTGTATAAATGAAG TG	PCR cloning
glnP	EcoRI-glnP-r	CTAGAATTCTTAATCACTGGCATTCAATCTCC CTTC	PCR cloning
ermB	ErmBNsiI-f	CCGATGCATGTGATTACGCAGATAAATAAAT ACG	PCR cloning
ermB	ErmBBbvI-r	CCGATTGCTGAGGCAAAAGCGACTCATAGA ATTATTTCC	PCR cloning
RNAIII	SARNAIII-f	GAAGGAGTGATTTCAATGGCACAAG	Northern blot
RNAIII	SARNAIII-r	GGCTCACGACCATACTTATTATTAAGGG	Northern blot
spa	SPA-f	ACCTGGTGATACAGTAAATGACATTGC	Northern blot
spa	SPA-r	GCACCTAAGGCTAATGATAATCCACC	Northern blot
16S rRNA	SA16SrRNA-f	GGTCTTGCTGTCACTTATAGATGG	Northern blot
16S rRNA	SA16SrRNA-r	GAATTCCACTTTCCTCTTCTGCAC	Northern blot
cna	Cna-f	AGTGACATGGTCTAATCTTCCGG	Northern blot
cna	Cna-r	TCCACTTTTGATGGCTTATCTGG	Northern blot
fnbA	MW2-fnbAN-f	ACTATGAGCGGGCAGTACGATG	Northern blot
fnbA	MW2-fnbAN-r	GATTGGACCTTGAGCTTGACCATG	Northern blot
sspA	sspA(SA)-PstI-f	CGATCTGCAGGTAAAGGATTTGTAAGGAT TTCCTATGC	PCR cloning
sspA	sspA(SA)-BamHI-r	CTAGGATCCCGTTGTTCTAATGGTTTAAGG TTACCG	PCR cloning
icaA	icaA-P1	CTGCAGCTTATCCTTCAATTTTTATAACCC CCTAC	PCR cloning
icaA	icaA-P2	GGATCCGAGTGCAAGAACATTAGACAACG	PCR cloning

Table 3. *S. aureus* target tissue densities in rabbit endocarditis model at 48 h post-infection.

Bacterial strain (no. of animals)	**Challenge dose (CFU/ml)**	***S. aureus* bacterial densities (log_{10} CFU/g of tissue)**		
		Vegetations	**Kidneys**	**Spleen**
UAMS-1 (n=7)	10^4	6.50 ± 1.27	4.50 ± 0.91	5.35 ± 0.90
UAMS-1-*glnP::ermB* (n=7)		4.60 ± 1.19*****	3.29 ± 0.96*****	3.77 ± 1.61*****
UAMS-1 (n=9)	10^5	7.29 ± 1.06	4.98 ± 0.88	5.65 ± 0.32
UAMS-1-*glnP::ermB* (n=9)		5.74 ± 1.48*****	3.53 ± 1.08*****	4.29 ± 1.56*****

***P* < 0.05 versus UAMS-1 parental strain.**

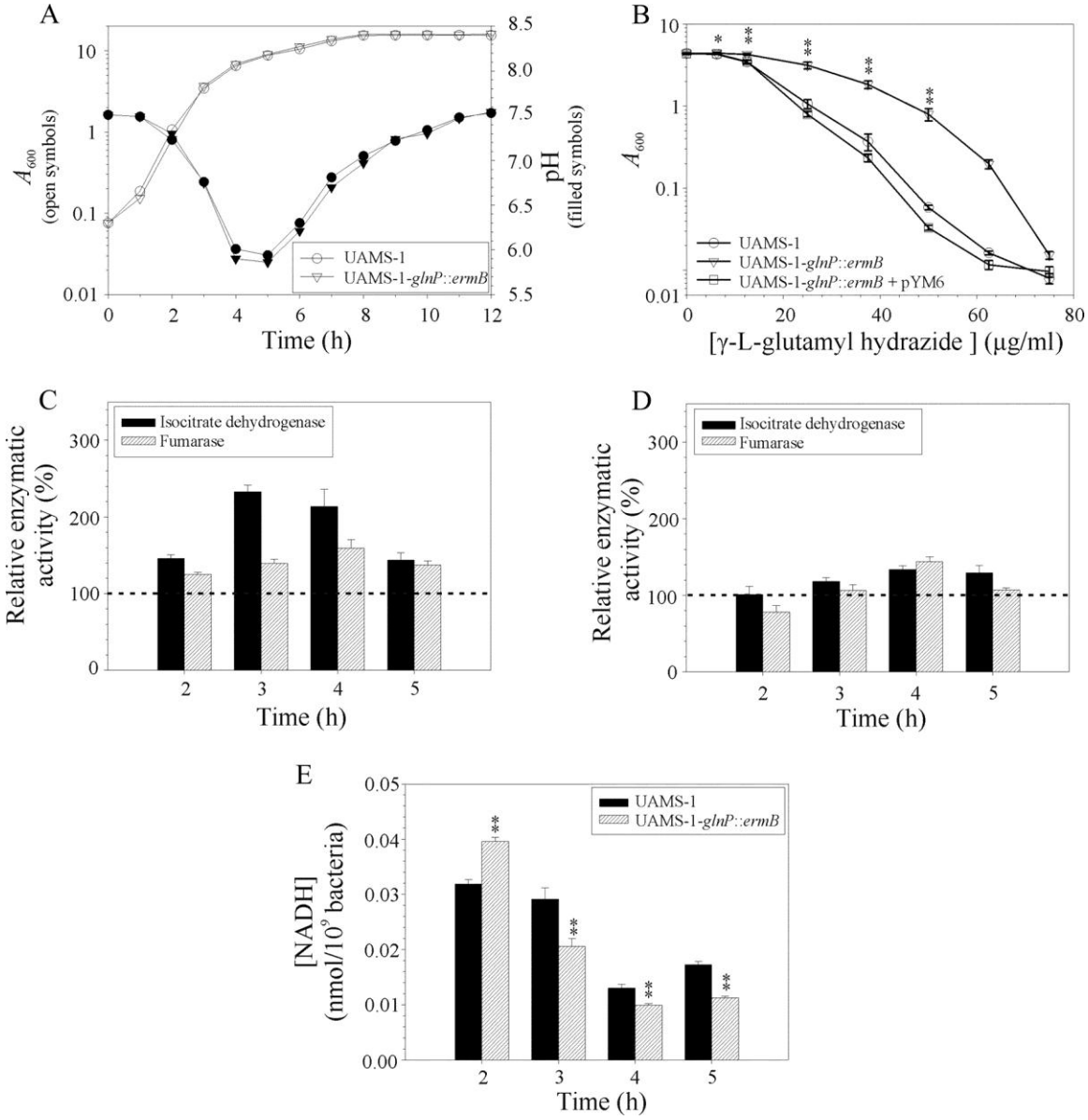
A
A_{600} (open symbols)
pH (filled symbols)
Time (h)
UAMS-1
UAMS-1-glnP::ermB
B
A_{600}
[γ-L-glutamyl hydrazide] (μg/ml)
UAMS-1
UAMS-1-glnP::ermB
UAMS-1-glnP::ermB + pYM6
C
Relative enzymatic activity (%)
Isocitrate dehydrogenase
Fumarase
Time (h)
D
Relative enzymatic activity (%)
Isocitrate dehydrogenase
Fumarase
Time (h)
E
[NADH] (nmol/10^9 bacteria)
UAMS-1
UAMS-1-glnP::ermB
Time (h)

Fig. 1. Physiological and metabolic characteristics of *S. aureus* strains UAMS-1 and UAMS-1-*glnP*::*ermB. A,* Growth curves and pH of culture supernatants for strains UAMS-1 and UAMS-1-*glnP*::*ermB* grown under aerobic conditions. *B,* Susceptibility of *S. aureus* strains to the glutamine analog γ-L-glutamyl hydrazide. Data are presented as the mean and SEM of 3 independent experiments; $P < 0.01$ vs. UAMS-1 parental strain are indicated by ** and $P < 0.05$ indicated by *. *C,* Isocitrate dehydrogenase and fumarase activities of strain UAMS-1-*glnP*::*ermB* expressed relative to the activities present in the parental strain UAMS-1, which was defined as being 100% and is represented by the dashed line. Data are presented as the mean and SEM of 2 independent experiments each determined in triplicate; for all results the $P < 0.01$ vs. UAMS-1 parental strain. *D,* Isocitrate dehydrogenase and fumarase activities of strain UAMS-1-*glnP*::*ermB* complemented with plasmid pYM6 expressed relative to the activities present in the parental strain UAMS-1, which was defined as being 100% and is represented by the dashed line. Data are presented as the mean and SEM of 2 independent experiments each determined in triplicate. *E,* Intracellular NADH concentrations during the transition from the exponential to post-exponential growth. Data are presented as the mean and SEM of three independent experiments each determined in quadruplicate (** $P < 0.01$ and * $P < 0.05$ vs. UAMS-1 parental strain).

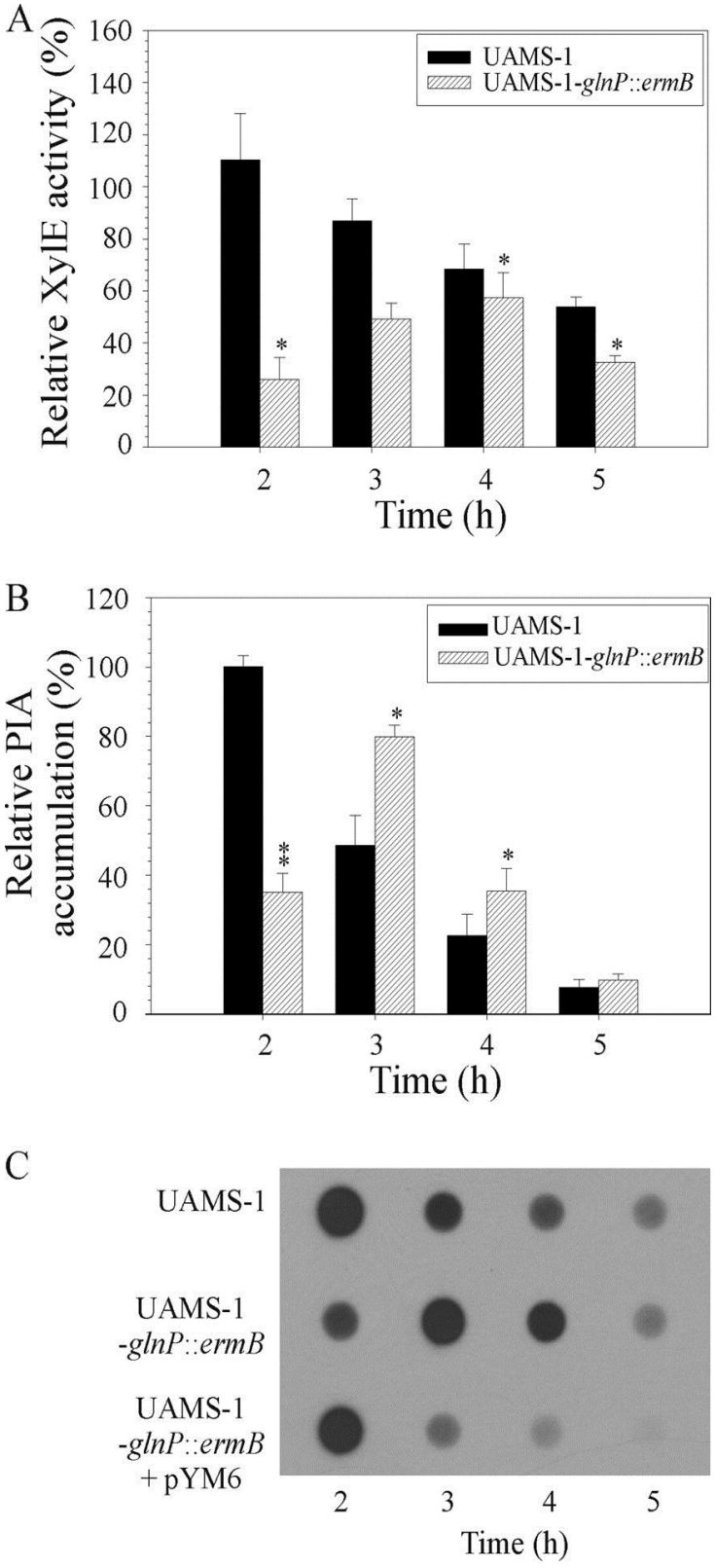
A
Relative XylE activity (%)
160
140
120
100
80
60
40
20
0
UAMS-1
UAMS-1-glnP::ermB
*
*
*
2
3
4
5
Time (h)
B
Relative PIA accumulation (%)
120
100
80
60
40
20
0
UAMS-1
UAMS-1-glnP::ermB
**
*
*
2
3
4
5
Time (h)
C
UAMS-1
UAMS-1 -glnP::ermB
UAMS-1 -glnP::ermB + pYM6
2
3
4
5
Time (h)

Fig. 2. Inactivation of *glnP* transiently decreases *icaADBC* transcription and PIA biosynthesis. *A*, *icaADBC* promoter driven catechol 2,3-dioxygenase activity in strains UAMS-1 and UAMS-1-*glnP*::*ermB*. Data are expressed relative to the activity present in strain UAMS-1 at 2 h post-inoculation and represent the mean and SEM of 3 independent experiments. *B*, Relative PIA accumulation by strains UAMS-1 and UAMS-1-*glnP*::*ermB* and UAMS-1-*arcD*::*ermB* during the transition from the exponential to post-exponential growth. The data are presented as the percentage difference in PIA accumulation relative to UAMS-1 at 2 h. The results are the mean and SEM of 3 independent cultures. *C*, Representative PIA immunoblot for strains UAMS-1, UAMS-1-*glnP*::*ermB*, and UAMS-1-*glnP*::*ermB* complemented with plasmid pYM6. For all results a $P < 0.01$ vs. UAMS-1 parental strain is indicated by ** and a $P < 0.05$ is indicated by *.

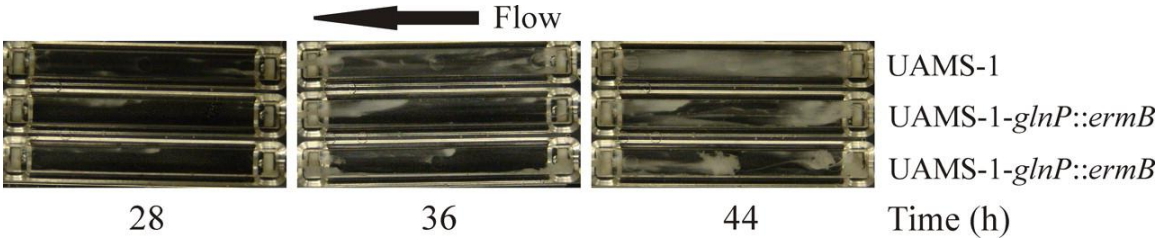

Fig. 3. Growth of *S. aureus* strains UAMS-1and UAMS-1-*glnP*::*ermB* in a 3-chamber flow cell. Bacterial strains were grown at 37° C with a continuous flow (0.5 ml per minute per chamber) of TSB containing 0.5% glucose and 3% NaCl. The results are representative of three independent experiments.

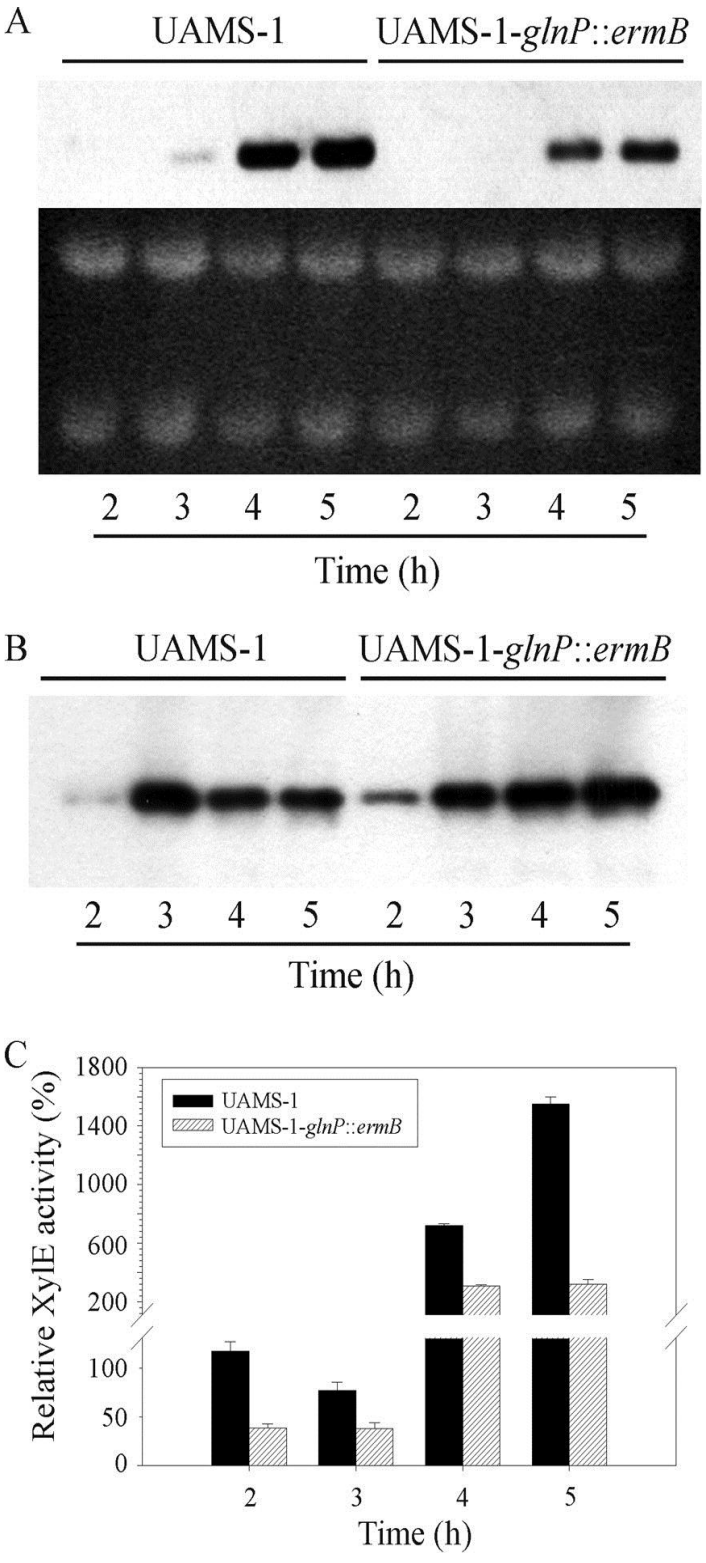
A
UAMS-1
UAMS-1-glnP::ermB
2 3 4 5 2 3 4 5
Time (h)
B
UAMS-1
UAMS-1-glnP::ermB
2 3 4 5 2 3 4 5
Time (h)
C
Relative XylE activity (%)
1800
1400
1000
600
200
100
50
0
UAMS-1
UAMS-1-glnP::ermB
2 3 4 5
Time (h)

Fig. 4. Inactivation of *glnP* alters the temporal pattern of virulence factor synthesis. *A*, RNAIII northern blot showing a decreased level of RNAIII in strain UAMS-1-*glnP*::*ermB* relative to strain UAMS-1. The bottom panel is a photograph of an ethidium bromide stained agarose gel demonstrating equivalent loading of total RNA. *B*, Protein A western blot. The blots in panels *A* and *B* are representative of more than 3 independent experiments. *C*. *sspA* promoter driven catechol 2,3-dioxygenase activity in strains UAMS-1 and UAMS-1-glnP::ermB; for all results the $P < 0.01$ vs. UAMS-1 parental strain. Data are expressed relative to the activity present in strain UAMS-1 at 2 h post-inoculation and represent the mean and SEM of 3 independent experiments.

CHAPTER IV

RPIR HOMOLOGUES LINK *STAPHYLOCOCCUS AUREUS* VIRULENCE FACTOR SYNTHESIS TO THE HEXOSE MONOPHOSPHATE SHUNT

Abstract

Staphylococcus aureus is an important human and animal pathogen that can synthesize a wide range of virulence factors. These virulence factors are regulated by a complex regulatory network that includes the *agr* quorum sensing system and the SarA family of regulators. In addition, the tricarboxylic acid (TCA) cycle has a major function in regulating the synthesis of many staphylococcal virulence factors. Some virulence factors, such as the capsular polysaccharide, are regulated by TCA cycle activity through the supply of biosynthetic intermediates; however, other virulence factors are regulated indirectly by TCA cycle activity. Indirect regulation is likely to occur when a metabolite-responsive regulator (*e.g.*, CcpA) responds to changes in TCA cycle associated biosynthetic intermediates, the redox status, and/or ATP. Previously, we observed that TCA cycle inactivation leads to an increased intracellular concentration of ribose, leading us to hypothesize that a ribose responsive regulator may mediate some of the TCA cycle-dependent regulatory effects. Using a bioinformatic approach to find putative ribose-responsive regulators, three RpiR-family regulators were identified in the *S. aureus* Mu50 genome. RpiR was first identified in *Escherichia coli* as a regulator of ribose-5-phosphate isomerase, an enzyme in the hexose monophosphate shunt. RpiR family proteins have N-terminal domains containing a helix-turn-helix DNA binding motif and a C-terminal sugar isomerase binding domain. To determine if the *S. aureus* RpiR regulators create a bridge between the hexose monophosphate shunt and virulence factor synthesis, the three *rpiR* homologues (orfs SAV0317, SAV0193 and SAV2315) were inactivated, and the effects on the hexose monophosphate shunt activity and virulence factor synthesis were

assessed. Deletion of *sav0193* or *sav2315* decreased transcription of *rpiA* (ribose-5-phosphate isomerase) and *zwf* (glucose-6-phosphate dehydrogenase), indicating the proteins encoded by these genes regulate hexose monophosphate shunt transcription. In addition, inactivation of *sav0193* or *sav2315* dramatically increased the transcription or stability of RNAIII, the effector molecule of the *agr* quorum sensing system. Consistent with this increase in RNAIII levels, deletion of *sav2315* decreased protein A biosynthesis and increased the accumulation of capsular polysaccharides. These data confirm a close linkage of central metabolism and virulence factor synthesis in *S. aureus*.

Introduction

Staphylococcus aureus is an important human and animal pathogen that is capable of causing a wide variety of diseases (Lowy 1998). The pathogenicity of *S. aureus* depends on its ability to synthesize virulence factors that facilitate colonization, immune evasion, and nutrient acquisition. Virulence factor synthesis is controlled by a complex network of regulatory proteins including the *agr* quorum sensing system and the SarA family of transcriptional regulators (please see Chapter 1). In addition, the tricarboxylic acid (TCA) cycle is important for regulation of staphylococcal virulence factor synthesis (Vuong, Kidder et al. 2005; Sadykov, Olson et al. 2008; Somerville and Proctor 2009; Zhu, Xiong et al. 2009; Sadykov, Mattes et al. 2010). The two most common types of regulation are genetic regulation and metabolic regulation; hence, TCA cycle-dependent regulation most likely occurs via one or both of these mechanisms. Genetic regulation occurs through the repression or induction of enzyme synthesis, while metabolic regulation controls enzyme activity through the availability of substrates and co-factors (*e.g.*, feedback inhibition). An example of metabolic regulation is synthesis of capsular polysaccharide, which is regulated by TCA cycle activity through the supply of oxaloacetate for gluconeogenesis (Sadykov, Mattes et al. 2010). Other virulence factors such as polysaccharide intercellular adhesin (PIA) are genetically regulated by TCA cycle activity through transcriptional repression of the operon that codes for the enzymes of PIA biosynthesis (*i.e., icaADBC*) (Vuong, Kidder et al. 2005; Sadykov, Olson et al. 2008). TCA cycle-dependent genetic regulation likely depends on response regulators that can sense metabolic changes associated with TCA cycle activity fluctuations (Somerville and

Proctor 2009).

To identify TCA cycle-responsive regulators, we characterized the metabolomic changes associated with TCA cycle stress (Sadykov *et al.*, submitted for publication). By comparing the intracellular concentrations of metabolites between *S. epidermidis* strain 1457 and its isogenic aconitase mutant, we found that TCA cycle inactivation leads to an increased intracellular concentration of ribose. This implies that if there is a regulator that can respond to the concentration of ribose, then the activity of that regulator will likely be altered. The prototype ribose-responsive regulator, RpiR, was first identified in *Escherichia coli* as a regulator of ribose-5-phosphate isomerase B (*rpiB*), which catalyzes the reversible isomerization of ribulose-5-phosphate and ribose-5-phosphate (Sorensen and Hove-Jensen 1996). Members of RpiR family often act as transcriptional regulators of sugar catabolism and RpiR homologues have been identified as repressors and activators in both Gram-negative and Gram-positive bacteria, including *E. coli*, *Pseudomonas putida*, and *Bacillus subtilis* (Sorensen and Hove-Jensen 1996; Yamamoto, Serizawa et al. 2001; Daddaoua, Krell et al. 2009). To identify putative ribose response regulators, the *S. aureus* Mu50 genome (Kuroda, Ohta et al. 2001) was searched for RpiR family members. Three open reading frames were identified as having significant amino acid homology to RpiR (between 21-23% identity and 45-46% positive); namely, SAV0317, SAV0193 and SAV2315. Similar to other members of the RpiR family of proteins (Bateman 1999), the *S. aureus* homologues are predicted to have an N-terminal helix-turn-helix DNA binding motif and a C-terminal sugar isomerase binding (SIS) domain.

Based on NMR metabolomic data demonstrating that TCA cycle stress alters the

intracellular concentration of ribose, we hypothesized that one or more RpiR homologues may link the hexose monophosphate shunt to virulence regulation in *S. aureus*. To test this hypothesis, three single mutants, three double mutants, and a triple mutant of the RpiR homologues SAV0317, SAV0193 and SAV2315 were made in *S. aureus* strain UAMS-1 and the effects on hexose monophosphate shunt activity, RNAIII transcription, capsular polysaccharide biosynthesis, PIA accumulation, and the ability to form a biofilm were assessed.

Materials and Methods

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in 2x YT broth (Sambrook and Russell 2001) or on a 2x YT agar, and *S. aureus* strains were grown in tryptic soy broth (TSB) (BD Biosciences) or on TSB-containing 1.5% agar. Unless otherwise stated, all bacterial cultures were inoculated 1:200 from an overnight culture (normalized for growth) into TSB, incubated at 37ºC, and aerated at 225 rpm with a flask-to-medium ratio of 10:1. Antibiotics were purchased from Fisher Scientific or Sigma Chemical and, when used, were used at the following concentration: *E. coli* ampicillin at 100 µg/ml; *S. aureus,* erythromycin at 8 or 10 µg/ml, chloramphenicol at 10-15 µg/ml, and tetracycline at 10 µg/ml.

Construction of *S. aureus rpiR* mutants. To inactivate *rpiRA* (orf SAV0317), a 2.559 kb fragment was PCR amplified using primers SAV0316-BamHI and SAV0318-SacI (Table 2) and cloned into the SmaI site of pBluescript II K/S(+) (Stratagene) to generate the plasmid pYF-7. The *ermB* cassette of pEC4 was amplified using primers pEC4ErmBNdeIF and pEC4ErmBNdeIR (Table 2) and ligated into the NdeI site within *rpiRA* of pYF-7 to yield the plasmid pYF-8. The *rpiRA*::*ermB* fragment of pYF-8 was cloned into the BamHI and SacI sites of pTS1 to create the plasmid pYF-9. The temperature-sensitive plasmid pYF-9 was isolated from *S. aureus* strain RN4220 and introduced into strain UAMS-1 by electroporation. Transformed bacteria were used to construct the *rpiRA* mutant using the temperature shift method of Foster (Foster 1998).

To inactivate *rpiRB* (orf SAV0193), the gene splicing by overlap extension (Horton, Ho et al. 1993) technique was used to replace a 741 bp internal region of *rpiRB* with the *cat* gene from plasmid pTS-1. For PCR, genomic DNA from *S. aureus* strain UAMS-1 was used as a template for the amplification of regions flanking *rpiRB*. PCR primers BamHI-SAV0192-f and cat-SAV0193-r (Table 2) were used for amplification of a 1.5 kb region upstream of *rpiRB* and a 1.5 kb region of the *rpiRB* downstream region was amplified using cat-SAV0193-f and SacI-SAV0195-r primers (Table 2). The *cat* gene was amplified from pTS-1 using primers SAV0193-cat-f and SAV0193-cat-r (Table 2). The resulting 3.9 kb PCR product consisted of an internal 816-bp *cat* gene with DNA flanking the *rpiRB* gene. The 3.9 kb PCR product contained BamHI and SacI sites that were used for ligation into pTS1-d digested with SacI and BamHI to generate pYM-4. Plasmid pYM-4 was used to construct an *rpiRB* mutant (UAMS-1-*rpiRB*::*cat*) using the referenced temperature shift method (Foster 1998).

Gene splicing by overlap extension was used to replace a 614 bp internal region of *rpiRC* (orf SAV2315) with the *tetM* gene from plasmid pJF-12 (Table 1). A 1.5 kb region upstream of *rpiRC* was amplified using primers BamHI-SAV2312-f and tetM-SAV2315-r (Table 2) and primers tetM-SAV2315-f and KpnI-SAV2316-r (Table 2) were used for amplification of a 1.6-kb downstream region. *tetM* was amplified from pJF-12 using primers SAV2315-tetM-f and SAV2315-tetM-r (Table 2). A 5.4 kb PCR product consisting of the 2.3 kb *tetM* gene and DNA flanking the *rpiRC* gene with BamHI and KpnI sites was inserted into pTS1-d digested with BamHI and KpnI to generate pYM-5. Plasmid pYM-5 was used to construct a strain UAMS-1 *rpiRC* mutant (UAMS-1-*rpiRC*::*tetM*) using temperature shifts. To minimize the possibility that any phenotype(s)

were the result of random mutations occurring during temperature shifts, all resulting mutations were back-crossed into wild-type strain UAMS-1 using transducing phage Φ85 (Foster 1998). All mutants were verified by PCR and Southern blot analysis (Fig. 1). Construction of the *rpiR* double mutants and the triple mutant were made using transducing phage Φ85.

Construction of *rpiR* complementing plasmids. Plasmids pCL15 and pCL15-*ermB* (Table 1), containing a P_{spac} promoter, were used to construct the *rpiRA*, *rpiRB* and *rpiRC* complementation plasmids pYF-10, pYF-11 and pYF-12. The promoterless genes from *S. aureus* strain UAMS-1 were PCR amplified using the primers listed in Table 2 and ligated into plasmids pCL15 or pCL15-*ermB*. Plasmids were isolated from *S. aureus* strain RN4220 and electroporated into *S. aureus* strain UAMS-1-*rpiRA*, -*rpiRB* and -*rpiRC* mutants.

Northern blot analysis. To determine if inactivation of *rpiR* affected transcription of hexose monophosphate shunt genes, northern blot analysis was performed on ribose 5-phosphate isomerase A (*rpiA*) and glucose-6-dehydrogenase (*zwf*, orf SAV1505). RNAIII transcript levels were also evaluated to determine the effect of *rpiR* inactivation on the *agr* system. Northern blots were performed as described (Sambrook and Russell 2001), except that total RNA was isolated using the FastRNA Pro Blue kit (Qbiogene) and purified using an RNeasy kit (Qiagen). Probes for Northern blotting were generated by PCR amplification of unique internal regions of RNAIII, *rpiA*, and *zwf* (Table 2) and labeled using the North2South random prime labeling kit (Pierce). Detection was

performed using the chemiluminescent nucleic acid detection module (Pierce).

Glucose-6-dehydrogenase activity assay. To determine if *rpiR* inactivation affects hexose monophosphate shunt activity, glucose-6-dehydrogenase (G6PD) activity was measured as described (Bergmeyer, Bergmeyer et al. 1983). Protein concentrations were determined using a modified Lowry assay (Pierce Chemical).

Western blot analysis. To determine if inactivation of *rpiR* affected protein A biosynthesis, protein A was collected as described (Vytvytska, Nagy et al. 2002) and western blot analysis was performed (Towbin, Staehelin et al. 1979).

Hemolytic assay. The mRNA for δ-toxin is contained within RNAIII (Janzon and Arvidson 1990). To determine if inactivation of any *rpiR* homologue altered δ-toxin accumulation, a semi-quantitative microtiter plate assay was used as described (Fitzgerald, Hartigan et al. 2000). Briefly, horse red blood cells (RBCs; Colorado Serum Company) were washed three times in phosphate-buffered saline (PBS; pH 7.2) and suspended at 2% (v/v) in PBS. Bacteria were grown in TSB for 15 hours, centrifuged at for 16100 x g for 5 minutes, supernatants were collected, and 2-fold serial dilutions were made in PBS. Hemolytic assays were started by mixing 100 μL of freshly prepared 2% horse RBCs with 100 μL serial of the appropriate culture supernatant 2-fold serial dilutions. The microtiter plates were incubated at 37°C for 30 min followed by 12 h at 4°C. After incubation, the supernatant fluids were collected and hemoglobin release was

measured at 595 nm. Each experiment was repeated three times and the mean and SEM were calculated.

Polystyrene primary attachment assay. The primary attachment assay was performed as described by Lim et al. (Lim, Jana et al. 2004). Briefly, bacterial cultures (2 h post-inoculation) were diluted into TSB to yield approximately 300 CFU. Bacteria were poured onto polystyrene Petri dishes (Fisher Scientific) and incubated at 37ºC for 30 min. Following incubation, the Petri dishes were rinsed three times with sterile PBS (pH 7.5) and covered with 15 ml of TSB containing 0.8% agar maintained at 48ºC. The percentage of bacteria attached to the polystyrene was defined as the number of CFU remaining in petri dishes after washing compared to the number of CFU in unwashed TSB plates. The experiment was repeated three times and the mean and SEM were calculated.

Capsule immunoblot assay. To determine if *rpiR* inactivation altered capsule biosynthesis, capsule accumulation was quantified using immunoblots as described (Luong, Newell et al. 2003), except that immunoblots were developed using a chemiluminescent horseradish peroxidase (HRP) substrate (Millipore). For the capsule blots, bacteria (1.25 OD_{660} units) were harvested after overnight growth in tryptic soy broth at 37°C, with a flask-to-medium ratio of 20:1, and aerated at 225 rpm.

PIA immunoblot assay. PIA accumulation was determined after 2, 4 and 6 h of growth as described (Zhu, Weiss et al. 2007). The data are presented as the percent difference in PIA accumulation relative that by strain UAMS-1 at 2 h.

Flow cell biofilm formation. *S. aureus* strains were grown in flow cell chambers (Stovall Life Science) as described (Zhu, Weiss et al. 2007). To assess bacterial growth, at 12 h post-inoculation and every 4 h thereafter, effluent samples were collected, the pH was measured, and the chamber was photographed.

Proteomic Analyses. Bacterial cells (2 h and 6 h post-inoculation) were harvested by centrifugation and suspended in 1.0 ml of lysis buffer containing 50 mM ammonium bicarbonate and 1.5 mM phenylmethylsulfonyl fluoride (PMSF). The samples were homogenized for 40 seconds at speed 6.0 m/s in a FastPrep instrument (MP Biomedical). The lysate was centrifuged for 5 min at 20,800 x g at 4ºC. The protein extract was suspended, denatured, alkylated, and digested by Trypsin (Roche) at 37ºC. The tryptic peptides were then desalted using PepClean C-18 spin columns according to manufacturer's instructions (Thermo scientific).

Fully automated off-line two dimensional chromatographic experiments were performed with a ultimate 3000 Dionex MDLC system (Dionex Corporation) integrated with a nanospray source and LCQ Fleet Ion Trap mass spectrometer (Thermofinnigan). The first dimension LC separation (Strong Cation-exchange (SCX) chromatography) with fraction collection was performed followed by the second dimension LC separation (reverse phase chromatography) and detection by tandem mass spectrometry was carried out. The first dimensional separation was performed on a 15 cm x 300 µm I.D. SCX column (Polysulfoethyl, 1mm I.D x 15 cm, 5 µm, 300A Dionex). 50 µl of samples were loaded onto first dimension SCX column and eluted using a salt gradient (0-600 mM) of

45 minutes. The second dimension separation was included on-line sample pre-concentration and desalting using a monolithic C 18 trap column (Pep Map, 300 um I.D, 5 µm, 100A, 1mm monolithic C18 column). The loading of the sample on the monolithic trap column was conducted using the micro pump at a flow rate of 300 nl/min. The desalted peptides was then eluted and separated on a C 18 Pep Map, 75 um I.D X 15 cm, 3 µm, 100A column applying an acetonitrile gradient (ACN plus 0.1% formic acid, 90 minute gradient including 25 minutes reequlibriation at a flow rate of 300 nl/min) and introduced into the mass spectrometer using the nano spray source. The LCQ Fleet mass spectrometer was operated with the following parameters: nano spray voltage 2.0 kV, heated capillary temperature 200°C, full scan m/z range 400-2000. The LCQ was operated in data dependent mode with 4 ms/ms spectra for every full scan, 5 micros can averaged for full scans and ms/ms scans, 3 m/z isolation width for ms/ms isolations and 35% collision energy for collision induced dissociation. Dynamic exclusion was enabled with exclusion duration of 1 min.

The MS/MS spectra were searched against *S. aureus* MRSA252 databases using MASCOT (Version 2.2 Matrix Science). Database search criteria were as follows: enzyme: Trypsin, Missed Cleavages: 2; mass: monoisotropic; fixed modification: carbamidomethyl (C); peptide tolerance: 2Da; MS/MS fragment ion tolerance: 1Da. Probability assessment of peptide assignments and protein identifications were accomplished by Scaffold (version Scaffold 3.0 Proteome Software Inc.). Only peptides with ≥95% probability were considered. Criteria for protein identification included detection of at least 2 unique identified peptides and a protein probability score of ≥95%.

Hydrogen peroxide susceptibility assay. To determine if the *rpiR* inactivation affects hydrogen peroxide susceptibility, *S. aureus* strains UAMS-1, UAMS-1-*rpiRC* and UAMS-1-*rpiRABC* were grown in TSB for 15 h and diluted to an optical density at 600nm (OD_{600}) of 0.05 into sterile medium containing increasing concentrations of hydrogen peroxide (Fisher Scientific). . Cultures were grown at 37°C with shaking (225 rpm) for 4 h. Bacterial densities were determined by measuring the OD_{600}.

Results

Characterization of *rpiR* mutants. Bioinformatic analysis of the Mu50 genome (Kuroda, Ohta et al. 2001) revealed the presence of three RpiR homologues; specifically, SAV0317, SAV0193 and SAV2315. For simplicity, these homologues were designated RpiRA (SAV0317), RpiRB (SAV0193), and RpiRC (SAV2315). To assess the effects of inactivating the *rpiR* homologue genes on growth, growth studies were performed and the optical densities and pH of the culture media were measured (Fig. 2A). Inactivation of any single *rpiR* homologue in UAMS-1 did not alter the growth rate, growth yield, or the pH profile of the culture medium (Fig. 2A). Similarly, the double mutants and the triple mutant had the growth rates and growth yields equivalent to the wild-type strain UAMS-1 (Fig.2B). Interestingly, the pH profile of the culture medium for the triple mutant had an increased rate of alkalization relative to the wild-type strain, suggesting it had an increased rate of acetic acid utilization (Fig.2B). These results demonstrate the growth of the *rpiR* mutants is equivalent to that of the isogenic wild-type strain.

***RpiR* homologues regulate hexose monophosphate shunt activity.** As stated, RpiR was first identified in *E. coli* as a repressor of the hexose monophosphate shunt gene *rpiB* (Sorensen and Hove-Jensen 1996). Similarly, the *Pseudomonas putida* RpiR homologue, HexR, regulates *zwf* which encodes G6PD, the rate-controlling enzyme of the hexose monophosphate shunt (Daddaoua, Krell et al. 2009). These data led us to hypothesize that one, or more, of the RpiR homologues in *S. aureus* would regulate transcription of hexose monophosphate shunt genes. To test this hypothesis, transcription of the hexose

monophosphate shunt genes *rpiA* (ribose-5-phosphate isomerase A) and *zwf* (*sav1505*; codes for G6PD) in the *rpiR* mutant strains was assessed by northern blot (Fig. 3A and 3C). In addition, the enzymatic activity of G6PD was measured in the UAMS-1 *rpiR* mutant strains and strain UAMS-1. As expected, inactivation of *rpiRB* and *rpiRC* decreased transcription of both *rpiA* and *zwf* relative to the parental strain UAMS-1; however, *rpiRA* inactivation had only a minor effect on *rpiA* and *zwf* mRNA levels (Fig. 3A). Complementation of the UAMS-1 *rpiRB* and *rpiRC* mutants, increased levels of *rpiA* mRNA (Fig. 3C), confirming the transcriptional changes are due to the inactivation of the correct *rpiR* genes. Surprisingly, inactivation of *rpiRA* in either an *rpiRB* or *rpiRC* mutant strain increased the transcription or stability of *rpiA* and *zwf* mRNA to wild-type levels (Fig. 3A), suggesting an antagonistic effect between RpiRA and both RpiRB and RpiRC. Because *zwf* mRNA migrates on an agarose gel near ribosomal RNA and to confirm the northern blot data, the activity of G6PD was assessed in the wild-type and *rpiR* mutant strains (Fig. 3B). Consistent with the northern blot data, mutation of *rpiRB* or *rpiRC* leads to decreased exponential growth phase (2 h) G6PD enzymatic activity (Fig. 3B) relative to the wild-type strain UAMS-1. Also consistent with the northern blot data is the antagonist effect of RpiRA on G6PD activity in both *rpiRB* and *rpiRC* mutant backgrounds. In contrast, only *rpiRB* inactivation significantly decreased G6PD enzymatic activity during the post-exponential growth phase (8 h) relative to the wild-type strain (Fig 3B). Overall, these data demonstrated that *S. aureus* RpiR homologues RpiRB and RpiRC have a positive regulatory function in the hexose monophosphate shunt regulation and that RpiRA is antagonistic to this regulatory function.

Inactivation of *rpiRC* delays biofilm development and decreases the synthesis of cell wall-associated virulence determinants. NMR metabolomic data indicated the intracellular concentration of ribose varied in response to stressors that induce biofilm formation and PIA accumulation in *S. epidermidis* (Sadykov *et al.*, unpublished data). Because the RpiR homologues have been reported to respond to hexose monophosphate shunt intermediates in other bacteria (Daddaoua, Krell et al. 2009), we wanted assess the effects of *rpiR* inactivation on PIA accumulation and biofilm formation (Fig. 4). Inactivation of any *rpiR* homologue resulted in minimal or no change in the accumulation of PIA (Fig. 4A and 4B). In *S. aureus*, biofilms can form in the absence of PIA biosynthesis (Boles, Thoendel et al. 2010); therefore, the lack of any significant effect of *rpiR* inactivation on PIA accumulation did not preclude the possibility that one or more of the RpiR homologues would affect biofilm formation. Inactivation of *rpiRC* delayed biofilm maturation; however, the gross morphology of the biofilms formed by the wild-type and *rpiRC* mutant strains was similar after 24 h of growth (Fig. 4C). The delay in biofilm maturation and the absence of any attenuation of PIA accumulation was consistent with a PIA-independent biofilm being formed. PIA-independent biofilms frequently involve protein adhesins such as protein A (Merino, Toledo-Arana et al. 2009). To determine if inactivation of *rpiRC* decreased adhesin synthesis, polystyrene attachment assays were performed on the wild-type and *rpiR* mutant strains (Fig. 4D). Consistent with delay in biofilm formation, strains containing a mutation in *rpiRC* had a significantly decreased ($p < 0.01$) ability to attach to polystyrene relative to the parental strain (Fig. 4D). Taken together, these data suggest the synthesis of cell wall-associated adhesins was decreased by *rpiRC* inactivation.

The association of protein A with PIA-independent biofilm formation and the decreased ability of *rpiR* mutant strains to adhere to polystyrene, suggest that cell-associated adhesin synthesis is impaired by inactivation of one or more RpiR homologues. Protein A is primarily synthesized during the exponential growth phase and it is considered representative of cell wall-associated protein synthesis. To determine if *rpiR* inactivation altered the exponential growth phase expression of protein A and potentially other cell wall-associated proteins, the exponential growth phase accumulation of protein A was assessed by western blotting. Mutations in either *rpiRA* or *rpiRB* did not affect the accumulation of protein A; however, inactivation of *rpiRC* completely inhibited the exponential growth phase accumulation of protein A (Fig. 4E). Interestingly, *rpiRA* inactivation did not antagonize the expression of protein A in the *rpiRC* mutant background, suggesting the antagonistic effects of RpiRA are confined to regulation of the hexose monophosphate shunt. In total, these data suggest that RpiRC acts as a regulatory bridge between the hexose monophosphate shunt and virulence factor synthesis in *S. aureus*.

RpiRC represses RNAIII transcription or message stability. RNAIII is the effector RNA of the *agr* quorum sensing system and a negative regulator of protein A (*spa*) (Novick and Geisinger 2008). Mutation of *rpiRC* eliminated the exponential growth phase accumulation of protein A (Fig. 4E); raising the possibility that RNAIII transcription or stability was increased. To determine if *rpiR* inactivation affected RNAIII levels, northern blot analysis of RNAIII was performed on all *rpiR* mutant strains throughout a 12 hour growth cycle (Fig. 5A and Fig. 5B). As expected, *rpiRC* inactivation

increased the RNAIII transcript level relative to the parental strain during the exponential growth phase (2 hour) (Fig. 5A). Complementation of the *rpiRC* mutation with pYF12 decreased the level of RNAIII relative to the *rpiRC* mutant strain, confirming the increased RNAIII level was due to *rpiRC* inactivation (Fig. 5C). Consistent with the protein A western blot and the attachment assays (Fig. 4E and 4D), we did not observe an antagonistic effect of *rpiRA* inactivation on the exponential growth phase (2 h) transcription or stability of RNAIII in either an *rpiRB* or *rpiRC* mutant background (Fig. 5A). Although RNAIII levels were largely independent of RpiRA or RpiRB in the exponential growth phase, *rpiRB* inactivation increased the post-exponential growth phase RNAIII transcript levels (Fig 5B). RNAIII is both a riboregulator and the coding sequence for δ-toxin (Janzon and Arvidson 1990); therefore, if RNAIII levels are increased, then it is likely that δ-toxin synthesis is increased. Using a hemolytic titer assay, the increased RNAIII levels correlated with an increase in hemolysis activity due to δ-toxin (Fig. 6A). In total, these data indicate that RpiRC represses RNAIII transcription during the exponential growth phase, while RpiRB represses RNAIII transcription during the post-exponential growth phase.

Inactivation of *rpiRC* dramatically increases capsule accumulation. RNAIII is a positive regulator of capsule gene (*cap*) transcription (Dassy, Hogan et al. 1993; Pohlmann-Dietze, Ulrich et al. 2000; Luong, Sau et al. 2002); thus, an increase in RNAIII levels should correlate with an increase in capsule biosynthesis. To determine if *rpiR* inactivation effects capsule biosynthesis, capsule accumulation was assessed using a

capsule immunoblot. Consistent with the increased RNAIII levels, inactivation of all three *rpiR* genes increased capsule accumulation (Fig. 6B); however, it was most apparent in strains having a mutation in *rpiRC*. These data strongly suggest that the RpiR-dependent de-repression of RNAIII facilitates virulence determinant expression and that the RpiR proteins act as a bridge between the hexose monophosphate shunt and virulence factor synthesis.

Inactivation of *rpiRC* alters the proteome. To identify changes in cytosolic protein content in strain UAMS-1 and the *rpiRC* mutant strains, cell-free lysates were prepared from strains UAMS-1, UAMS-1-*rpiRC*, and UAMS-1-*rpiRABC* grown to the exponential and post-exponential growth phases and analyzed using LC-MS (Appendix A and B). Although *rpiRC* inactivation resulted in numerous proteomic changes, we were specifically interested in changes to hexose monophosphate shunt enzymes and proteins that might clarify the increased RNAIII transcript levels. Proteomic analysis identified the hexose monophosphate shunt enzymes transaldolase and ribose-phosphate pyrophosphokinase as being present at lower concentrations in strains UAMS-1-*rpiRC* and UAMS-1-*rpiRABC* than in strain UAMS-1, consistent with RpiRC regulating the hexose monophosphate shunt. In addition, proteomic analysis suggested there was an increase in proteins associated with σ^B; specifically, inactivation of *rpiRC* increased the concentrations of the alkaline shock protein A (Asp23) and RsbU (gi|49242422) (Appendix A and B). Because transcription of *asp23* is exclusively controlled by σ^B, Asp23 is used as an indicator of σ^B activity (Kullik, Giachino et al. 1998; Miyazaki, Chen

et al. 1999). RsbU is a phosphatase that de-phosphorylates (activates) the anti-anti-sigma factor RsbV, which then binds the anti-sigma factor RsbW in a competitive manner to increase the concentration of free σ^B (Giachino, Engelmann et al. 2001). In addition to regulating transcription of *asp23*, σ^B regulates transcription of *sarA* from the *sar* P3 promoter (Miyazaki, Chen et al. 1999). SarA is an activator of *agrACDB* and RNAIII (Cheung, Bayer et al. 1997) and *rpiRC* inactivation increased RNAIII mRNA levels relative to the wild-type strain (Fig. 5), suggesting that *rpiRC* inactivation might increase the availability of SarA. Consistent with this suggestion, *rpiRC* inactivation increased the cytosolic concentration of SarA during both the exponential and post-exponential growth phases (Appendix A and B). Although not conclusive, these data strongly suggest the increased RNAIII levels in *rpiRC* mutants are due to an increased availability of σ^B, which increases *sarA* transcription and translation, increasing *agr* and RNAIII transcription.

Inactivation of *rpiRC* decreases peroxide susceptibility. In some strains of *S. aureus*, σ^B has been implicated in the susceptibility to peroxide (Kullik, Giachino et al. 1998; Giachino, Engelmann et al. 2001). This observation and the fact that strain UAMS-1-*rpirC* had increased ferritin and catalase levels relative to strain UAMS-1, led us to assess the susceptibility of strains UAMS-1, UAMS-1-*rpiRC*, and UAMS-1-*rpiRABC* to peroxide stress (Fig. 7). As expected, inactivation of *rpiRC* significantly decreased the susceptibility of strain UAMS-1-*rpiRC* and UAMS-1-*rpiRABC* to hydrogen peroxide relative to strain UAMS-1 (Fig. 7).Taken together, these data demonstrate that the *S.*

aureus RpiR family of proteins creates a bridge between the hexose monophosphate shunt and virulence determinant regulation.

Discussion

Three central metabolic pathways (*i.e.*, glycolysis, the hexose monophosphate shunt, and the TCA cycle) provide the thirteen biosynthetic intermediates needed to synthesize all macromolecules produced in bacteria. By default, virulence determinants are synthesized from the thirteen biosynthetic intermediates of central metabolism; hence, virulence determinant synthesis is dependent on the endogenous or exogenous availability of these intermediates or by-products of these intermediates. Because of the importance of these intermediates, bacteria have evolved metabolite responsive regulators (*e.g.*, CcpA, CodY) that "sense" the availability of these intermediates or compounds derived from them (Somerville and Proctor 2009). These metabolite-responsive regulators not only function to maintain metabolic homeostasis but many also regulate virulence determinant synthesis (Somerville and Proctor 2009). Although metabolite responsive regulators have been identified in *S. aureus* that respond to changes in glycolytic and TCA cycle intermediates or derivatives, none have been identified that respond to changes in hexose monophosphate shunt intermediates. To that end, three RpiR family members, RpiRA, RpiRB and RpiRC were identified and inactivated in *S. aureus* strain UAMS-1 and the phenotypic changes associated with each RpiR homologue were characterized.

Hexose monophosphate shunt regulation. RpiRB and RpiRC positively regulate the exponential growth phase transcription of the hexose monophosphate shunt genes *rpiA* and *zwf* (Fig 3A). In addition, RpiRC positively affects expression of transaldolase and

ribose-phosphate pyrophosphokinase (Appendix A and B). Although RpiRB and RpiRC are paralogs, there appears to be minimal overlap in function of the two regulatory proteins as inactivation of either *rpiRB* or *rpiRC* decreases transcription of *rpiA* and *zwf* to the same extent (Fig. 3A). In other words, RpiRB does not compensate for the loss of RpiRC and RpiRC does not compensate for the loss of RpiRB. Interestingly, RpiRA has only a slight effect on *rpiA* and *zwf* transcription; however, it does antagonize the regulatory effects of both RpiRB and RpiRC (Fig. 3A). In double mutants, inactivation of *rpiRA* restores transcription of *rpiA* and to a lesser extent *zwf* to near wild-type levels. Interestingly, this antagonism only involves RpiRB- and RpiRC-dependent regulation of *rpiA* and *zwf* but not the RpiRC-dependent regulation of RNAIII (Fig. 5A and 5B). Taken together, these data confirm the *S. aureus* RpiR homologues regulate the hexose monophosphate shunt.

RNAIII regulation. Synthesis of RNAIII is under the control of the *agr* cell-density sensing system (Novick, Ross et al. 1993); hence, transcription of RNAIII usually begins late in the exponential phase of growth (4 h) (Fig. 5A). The growth rates and growth yields of the UAMS-1 *rpirA*, *rpiRB*, and *rpiRC* mutant strains are equivalent to the parental strain (Fig. 2A); thus, it was surprising to find that transcription of RNAIII was de-repressed during the early exponential growth phase (2 h) in strain UAMS-1-*rpiRC* (Fig. 5A) relative to strain UAMS-1. This RpiRC-dependent de-repression persists into the post-exponential (4-6 h) but declines thereafter (Fig. 5A). The more likely explanations for the RpiRC-dependent de-repression of RNAIII transcription are either an increase in expression of the *agr* cell-density sensing system or an *agr*-independent

increase in RNAIII transcription. Proteomic analysis of the cytosolic fractions of strains UAMS-1, UAMS-1-*rpiRC*, and UAMS-1-*rpiRABC* (Appendix A and B) demonstrated that inactivation of *rpiRC* increased the intracellular concentration of SarA during the exponential (2 h) and post-exponential (6 h) growth phases relative to the parental strain UAMS-1. The increased level of SarA is likely mediated by an increase in free σ^B due to over-expression of RsbU phosphatase activity (Appendix A and B). Consistent with an increase in free σ^B, proteomic analysis revealed that *rpiRC* inactivation resulted in an increase in the accumulation of the σ^B-regulated alkaline shock protein A. These data suggest the increased level of RNAIII in strains lacking RpiRC is due to an increase in SarA-mediated transcription of RNAIII. Further, these data confirm a direct linkage between central metabolism (*i.e.*, the hexose monophosphate shunt) and three major virulence regulators (SarA, σ^B, and RNAIII) in *S. aureus*. Finally, these data demonstrate that metabolite-responsive regulators can override the normal temporal pattern of virulence determinant synthesis by altering the cell-density sensing system.

Conclusions. Dr. Richard Novick postulated in a "black-box" model (Novick 2003) that an "energy signal" derived from intermediary metabolism would, in an unknown (*i.e.*, "black-box") fashion, regulate the transcription of the *agr* cell-density sensing system. Since the introduction of the "black-box" model, several regulators (*e.g.*, CcpA and CodY) have been identified that link metabolism to the regulation of virulence determinants (reviewed in (Somerville and Proctor 2009)). In the present study, it was observed that RNAIII synthesis is co-regulated with central metabolism, specifically the hexose

monophosphate shunt, through the action of three RpiR family regulators. Although the "black-box" model is largely accurate, based on data presented here and from other studies (Seidl, Stucki et al. 2006; Majerczyk, Sadykov et al. 2008; Majerczyk, Dunman et al. 2010), the "energy signal" responsible for regulating the transcription of *agr* is more than likely a "carbon signal".

In *Pseudomonas putida*, the DNA binding activity of the RpiR homologue, HexR, is modulated by the Entner-Doudoroff pathway intermediate 2-keto-3-deoxy-6-phosphogluconate (Daddaoua, Krell et al. 2009). All three *S. aureus* RpiR homologues have sugar isomerase binding domains, suggesting their regulatory activity may be controlled by intermediates of the hexose monophosphate shunt. Collaborative studies are underway to identify the metabolites to which the RpiR homologues bind; hopefully, this information will fill in one of the "black-boxes" in *S. aureus* virulence factor regulation.

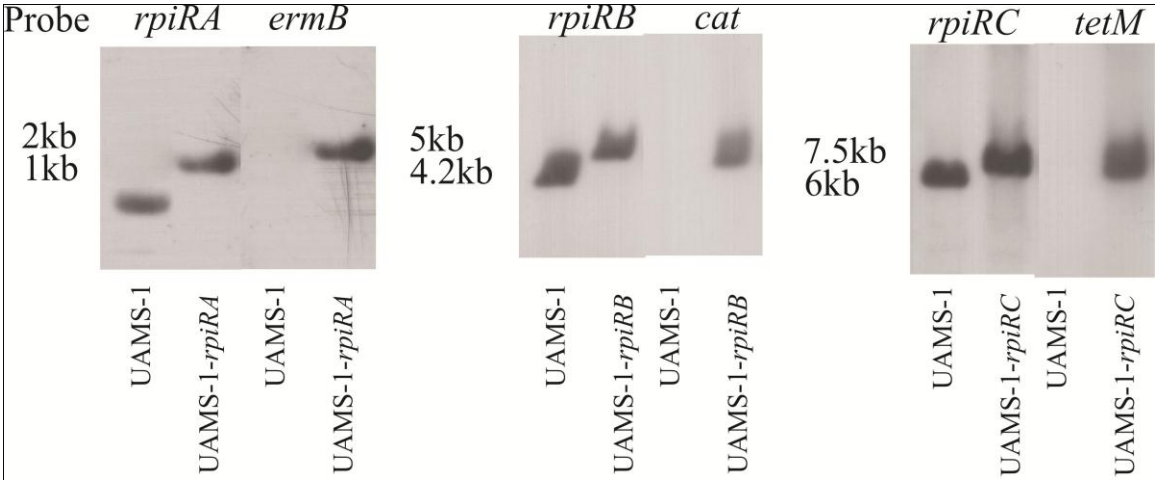

Fig.1. Southern blots demonstrating the inactivation of *rpiRA*, *rpiRB* and *rpiRC* in strain UAMS-1 by the insertion of antibiotic cassettes *ermB*, *cat*, and *tetM* respectively.

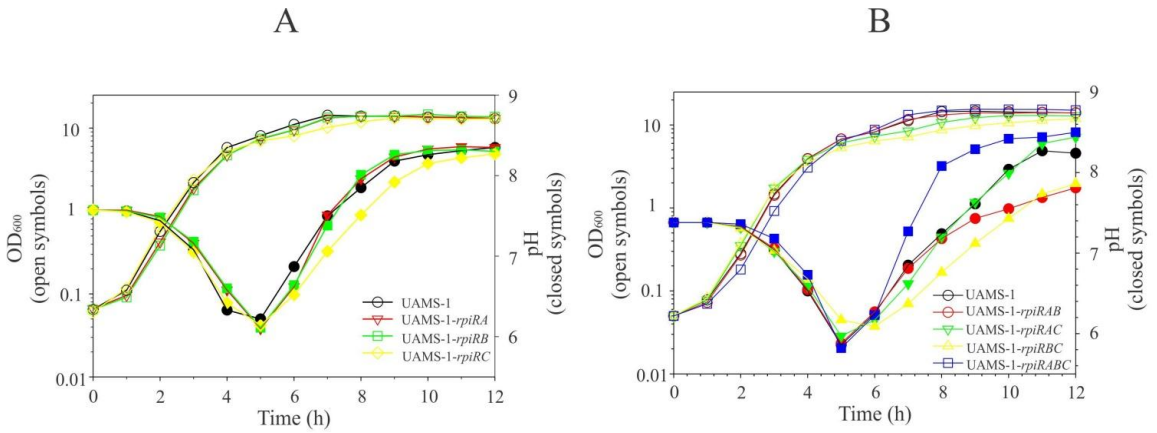

Fig.2. Growth curves (A) and culture medium pH profiles (B) for strains UAMS-1, UAMS-1-*rpiRA*, UAMS-1-*rpiRB*, UAMS-1-*rpiRC* mutants under aerobic growth conditions.

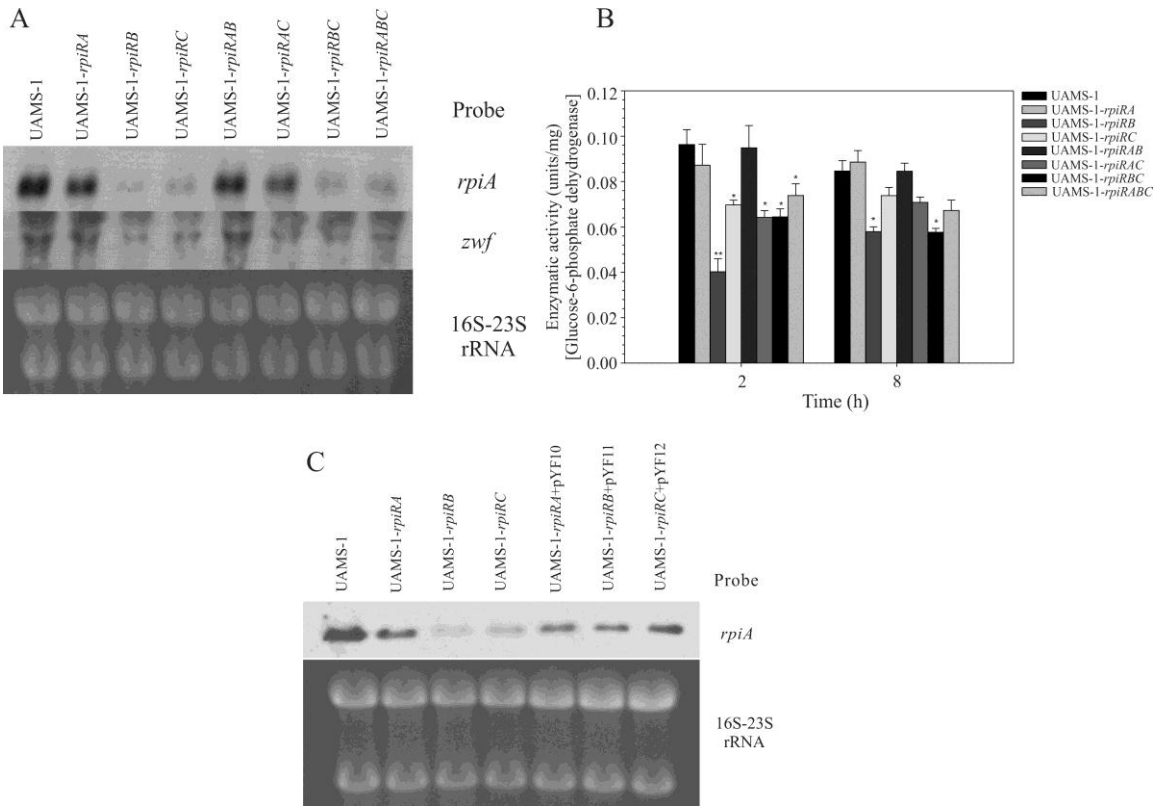

Fig. 3. Inactivation of *rpiR* homologues alters hexose monophosphate shunt activity. (A) Northern blot demonstrating that inactivation of *rpiRB* or *rpiRC* decreases transcription of ribose phosphate isomerase A (*rpiA*) and G6PD (*zwf*). Ethidium bromide staining of 23S and 16S rRNA is shown to demonstrate equivalent loading of total RNA. (B) Glucose-6-phosphate dehydrogenase activity of strain UAMS-1 and *rpiR* mutants. Data are presented as the means and SEM of two independent experiments each determined in triplicate. **, $P<0.01$; *, $P<0.05$. (C) Northern blot demonstrating that complementation of *rpiRB* and *rpiRC* increase transcription of *rpiA*.

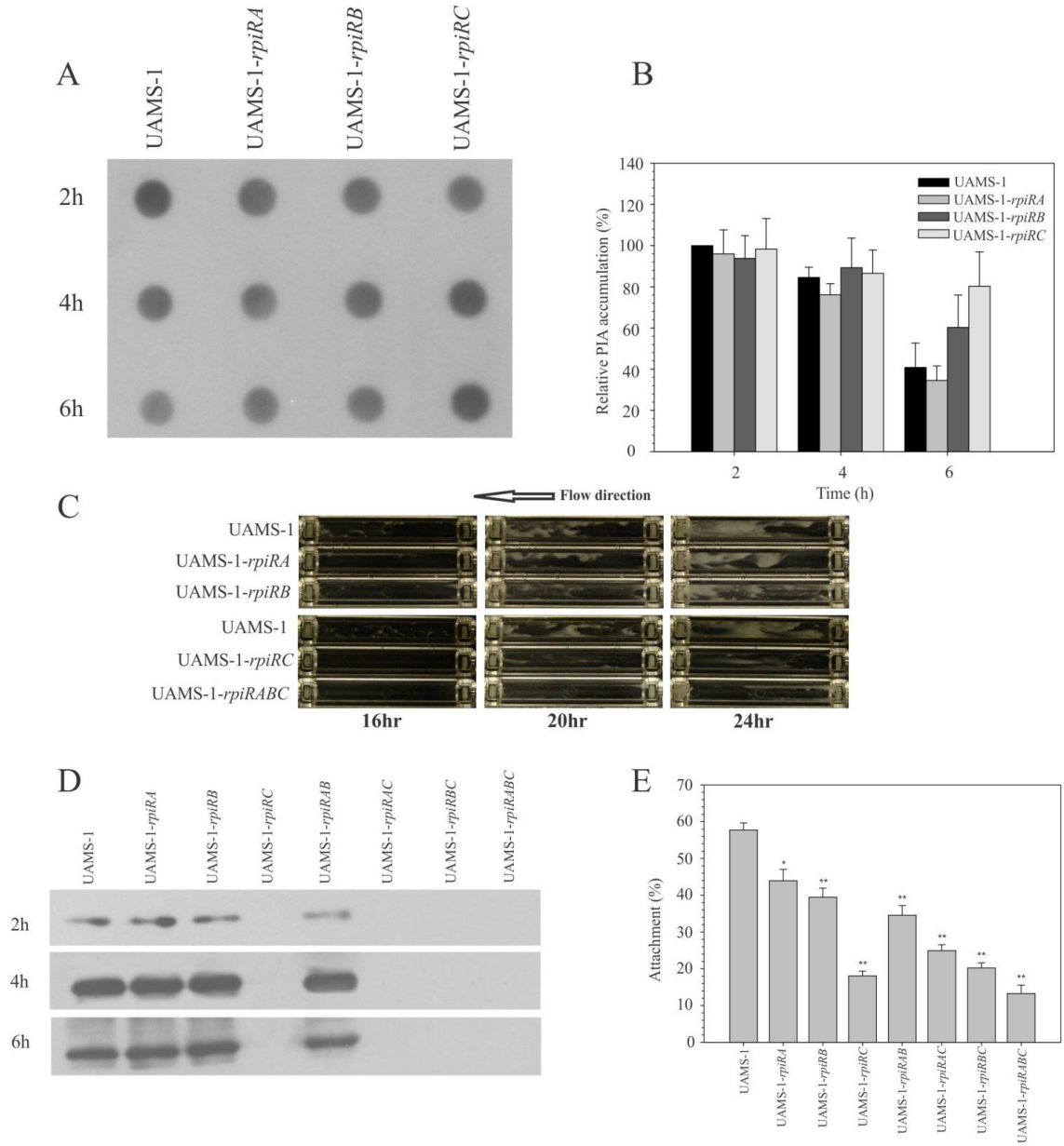
A
UAMS-1
UAMS-1-rpiRA
UAMS-1-rpiRB
UAMS-1-rpiRC
2h
4h
6h
B
Relative PIA accumulation (%)
Time (h)
UAMS-1
UAMS-1-rpiRA
UAMS-1-rpiRB
UAMS-1-rpiRC
C
Flow direction
UAMS-1
UAMS-1-rpiRA
UAMS-1-rpiRB
UAMS-1
UAMS-1-rpiRC
UAMS-1-rpiRABC
16hr
20hr
24hr
D
UAMS-1
UAMS-1-rpiRA
UAMS-1-rpiRB
UAMS-1-rpiRC
UAMS-1-rpiRAB
UAMS-1-rpiRAC
UAMS-1-rpiRBC
UAMS-1-rpiRABC
2h
4h
6h
E
Attachment (%)
UAMS-1
UAMS-1-rpiRA
UAMS-1-rpiRB
UAMS-1-rpiRC
UAMS-1-rpiRAB
UAMS-1-rpiRAC
UAMS-1-rpiRBC
UAMS-1-rpiRABC

Fig.4. Inactivation of rpiR homologues alters the expression of virulence factors and delays biofilm formation. (A) Representative PIA immunoblot for strains UAMS-1, UAMS-1-*rpiRA*, UAMS-1-*rpiRB*, UAMS-1-*rpiRC* mutant. (B) Relative PIA accumulation by strains UAMS-1, UAMS-1-*rpiRA*, UAMS-1-*rpiRB*, UAMS-1-*rpiRC* mutant during the transition from exponential to post-exponential growth. The data are presented as the differences in PIA accumulation relative to PIA accumulation by UAMS-1 at 2h. The results are the means and SEM of three independent cultures. (C) Growth of *S. aureus* strain UAMS-1, UAMS-1-*rpiRA*, UAMS-1-*rpiRB*, UAMS-1-*rpiRC* and UAMS-1-*rpiRABC* in a three-chamber flow cell. Bacterial strains were grown at 37°C with a continuous flow (0.5ml per minute per chamber) of TSB containing 0.5% glucose and 3% NaCl. The results are representative of three independent experiments. (D) Adhesion of *S. aureus* cells to polystyrene. **, $P<0.01$; *, $P<0.05$. (E) Protein A Western blot. The blot is representative of three independent experiments.

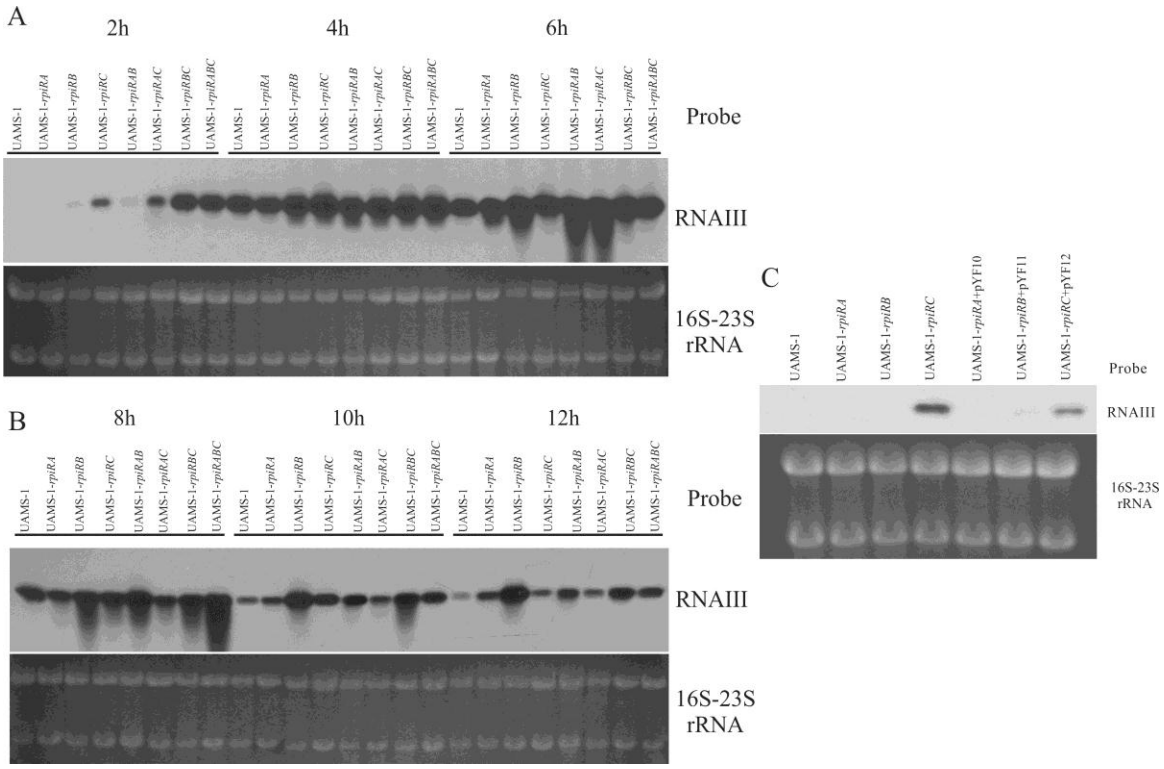

FIG. 5. RNAIII Northern blots. (A) Inactivation of *rpiRC* increases transcription or stability of RNAIII relative to that in strain UAMS-1 at early exponential phase (2h and 4h). Double mutant of *rpiRB* and *rpiRC* have synergistic effect. (B) Deletion of *rpiRB* increases transcription or stability of RNAIII at post-exponential phase. The bottom panel is a photograph of an ethidium bromide-stained agarose gel demonstrating equivalent loading of total RNA. (C) Complementation of *rpiRC* results in decreased transcription of RNAIII.

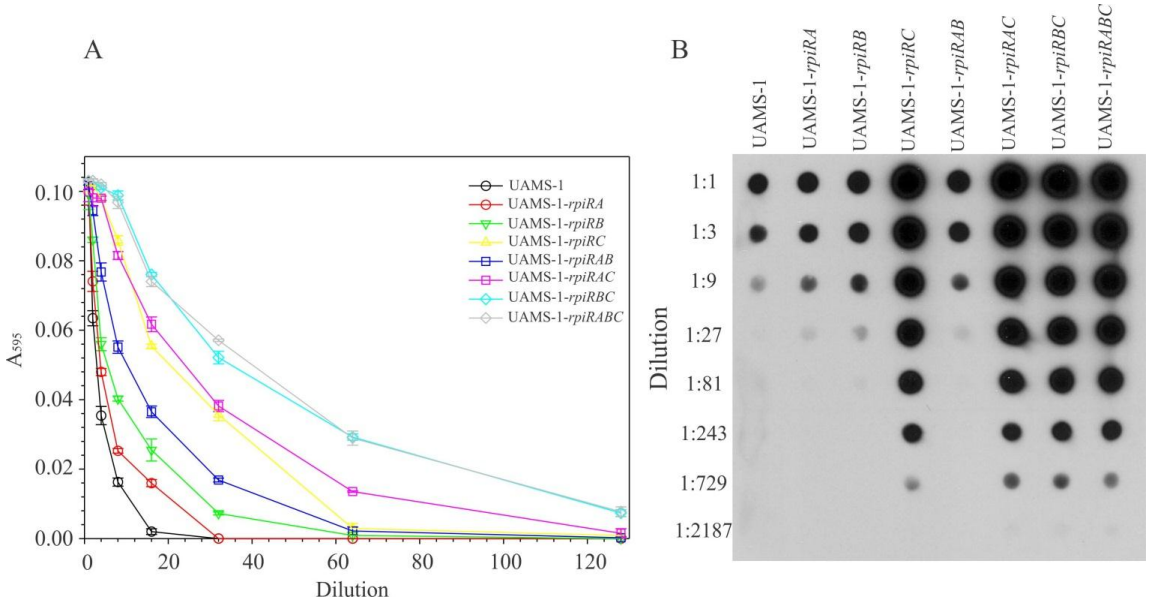

FIG.6 Inactivation of *rpiR* homologues alters hemolytic activity and capsule biosynthesis. (A) Hemolytic activity of strain UAMS-1 and *rpiR* mutants. All above data represent the means and standard errors of the means of three independent experiments. (B) Capsule polysaccharide Immunoblot. The blot is representative of three independent experiments.

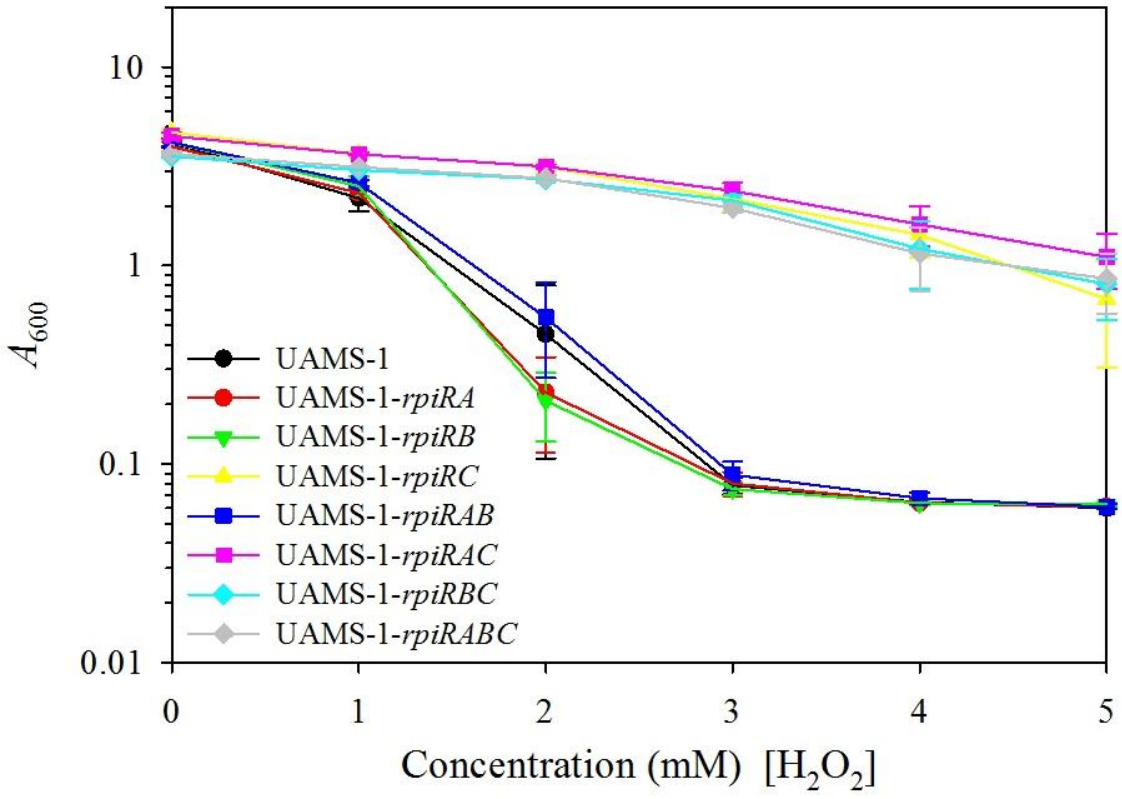

FIG.7 Susceptibility of *S. aureus* strains to hydrogen peroxide. Data are presented as the means and standard errors of the means (SEM) of three independent experiments. **, $P<0.01$; *, $P<0.05$ (versus the UAMS-1 parental strain).

Table 1. Strains and plasmids used in this study.

Plasmid or strain	Relevant genotype and/or characteristic(s)	Source or Reference
pBluescript II KS(+)	E. coli phagemid cloning vector	Stratagene
pTS1	*S. aureus* - *E. coli* temperature-sensitive shuttle vector; $Amp^r Cam^r$	(Greene, McDevitt et al. 1995)
pTS1-d	Derivative of pTS1 with depletion of plasmid-encoded 3' region of *ermC*	(Sadykov, Olson et al. 2008)
pEC4	pBluescript II KS(+) with *ermB* inserted into ClaI site	(Bruckner 1997)
pJF12	Plasmid pCR2.1 containing *tetM*; $Amp^r Min^r$	J. Finan and G. Archer
pYF-7	pBluescript II KS(+) containing a portion of SAV0317	This study
pYF-8	pYF-7 containing the *ermB* cassette of pEC4 inserted into the NdeI site of SAV0317	This study
pYF-9	SAV0317::*ermB* PCR fragment of pYF-8 inserted into the BamHI and SacI sites of pTS-1	This study
pYM-4	Derivative of pTS-1 with SAV0193::*cat* fragment	This study
pYM-5	Derivative of pTS-1 with SAV2315::*tetM* fragment	This study
pCL15	Expression vector; derivative of pSI-1; Cam^r	Chia Lee
pCL15-*ermB*	Replacement of *cat* with *ermB* in expression plasmid pCL15; Erm^r	This study
pYF-10	pCL15 with SAV0317 gene under the control of the P_{spac} promoter; Cam^r	This study
pYF-11	pCL15-*ermB* with SAV0193 gene under the control of the P_{spac} promoter; Erm^r	This study
pYF-12	pCL15 with SAV2315 gene under the control of the P_{spac} promoter; Cam^r	This study
RN4220	Restriction-negative *S. aureus*	(Novick 1991)
DH5α	*E. coli* cloning host	Invitrogen
UAMS-1	*S. aureus* clinical isolate	Mark Smeltzer strain collection
UAMS-1-*rpiRA*	SAV0317 insertion mutant of UAMS-1; Erm^r	This study
UAMS-1-*rpiRB*	SAV0193 deletion mutant of UAMS-1; Cam^r	This study
UAMS-1-*rpiRC*	SAV2315 deletion mutant of UAMS-1; Min^r	This study
UAMS-1-*rpiRAB*	SAV0317, SAV0193 double mutant of UAMS-1; Erm^r Cam^r	This study
UAMS-1-*rpiRAC*	SAV0317, SAV2315 double mutant of UAMS-1; Erm^r Min^r	This study
UAMS-1-*rpiRBC*	SAV0193, SAV2315 double mutant of UAMS-1; Cam^r Min^r	This study
UAMS-1-*rpiRABC*	SAV0317, SAV0193, SAV2315 triple mutant of UAMS-1; Erm^r Cam^r Min^r	This study

[a] Amp^r, ampicillin resistant; Cam^r, chloramphenicol resistant; Erm^r, erythromycin resistant; Min^r, minocycline resistant.

Table 2. Primers used in this study.

Primer target	Primer designation	Nucleotide sequence (5′ - 3′)	PCR product use
SAV0317	SAV0316-BamHI	GCTGGATCCCGACTGAACAATGAACGCCTAAGTC	PCR cloning
SAV0317	SAV0318-SacI	CCTGAGCTCATCAACGCCGGACAACAAAAGTG	PCR cloning
ermB	pEC4ErmBNdeI-f	GCGCATATGCGTTAGATTAATTCCTACCAGTGAC	PCR cloning
ermB	pEC4ErmBNdeI-r	GCGCATATGCTCATAGAATTATTTCCTCCCG	PCR cloning
SAV0193	BamHI-SAV0192-f	CCAGGATCCAGAACGAATTATTGCTGCAGTAGG	PCR cloning
SAV0193	cat-SAV0193-r	CCACTTTATCCAATTTTCGTTTGTTGTTCACCGTCATATCAATGATTTTATGTGG	PCR cloning
cat	SAV0193-cat-f	CCACATAAAATCATTGATATGACGGTGAACAACAAACGAAAATTGGATAAAGTGGG	PCR cloning
cat	SAV0193-cat-r	GCAAGATGCTTCCGGTAATTATCAAGCGACTGTAAAAAGTACAGTCGGC	PCR cloning
SAV0193	cat-SAV0193-f	GCCGACTGTACTTTTTACAGTCGCTTGATAATTACCGGAAGCATCTTGC	PCR cloning
SAV0193	SacI-SAV0195-r	GCAGAGCTCGTTGAATAAGTGCTTCTACCGCATAC	PCR cloning
SAV2315	BamHI-SAV2312-f	CCAGGATCCGATTCCTAAACTATGGAGTCGATGGG	PCR cloning
SAV2315	tetM-SAV2315-r	CGTAAGAGCATATTTGTAAAGGAATCTCCGAGACCTCATTTTAATCACCTTTTGAGG	PCR cloning
tetM	SAV2315-tetM-f	CCTCAAAAGGTGATTAAAATGAGGTCTCGGAGATTCCTTTACAAATATGCTCTTACG	PCR cloning
tetM	SAV2315-tetM-r	GAAGTTGTTGCTCCCATATGCATCCGATCTCCTCCTTTCCACTTTAATTC	PCR cloning
SAV2315	tetM-SAV2315-f	GAATTAAAGTGGAAAGGAGGAGATCGGATGCATATGGGAGCCTTC	PCR cloning
SAV2315	KpnI-SAV2316-r	GAAGGTACCAATGGATTGTAGTTGGTATGAGTGAG	PCR cloning
RNAIII	SARNAIII-f	GAAGGAGTGATTTCAATGGCACAAG	Northern blot
RNAIII	SARNAIII-r	GGCTCACGACCATACTTATTATTAAGGG	Northern blot
rpiA	rpiaf	GTGACATGACGCTGGGAATTGG	Northern blot
rpiA	rpiar	GTATCCTGTCTCAAACACACCTGTCAG	Northern blot
SAV1505	SAV1505f	GCACCACAATTCTTTGGCGTTATTTC	Northern blot
SAV1505	SAV1505r	AGTACGAATATAGAATGGTACACCAGCC	Northern blot
SAV0317	BamHI-SD-rpiR-f	CAAGGATCCATTAAGATGAAGGGGTGACACAATG	Complementation
SAV0317	SacI-rpiR-r	CAAGAGCTCAATCACGATGATTGTCTACAGTTGC	Complementation
SAV0193	BamHI-SAV0193-f	CTAGGATCCATGACAAATATTTTATATCGCATTGATAAACAGTTGAG	Complementation
SAV0193	SAV0193-r	CAAACAACTGAATCACATCAAAAACTTCAATTG	Complementation
SAV2315	BamHI-SAV2315-f	CTAGGATCCATGTCAAACGTACTAACAGAAATAGATAGTCAATATCC	Complementation
SAV2315	SAV2315-r	GCGTATGTTATACAAGATAAAAGACATGTAAGCTTTG	Complementation

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Appendix A: 2h post-inoculation proteomic analyses

	Identified Proteins	Accession Number	UAMS-1 (Spectral count)	UAMS-1 *-rpiRC* (Spectral count)	UAMS-1 *-rpiRC* (ratio)	UAMS-1 *-rpiRABC* (Spectral count)	UAMS-1 *-rpiRABC* (ratio)
1	translation elongation factor Tu	gi|49240907	153	502	3.3	581	3.8
2	dihydrolipoamide succinyltransferase E2 component of 2-oxoglutarate dehydrogenase complex	gi|49241731	648	218	0.3	202	0.3
3	very large surface anchored protein	gi|49241754	37	11	0.3	10	0.3
4	glyceraldehyde 3-phosphate dehydrogenase 1	gi|49241159	4	126	28.7	130	29.8
5	putative arginyl-tRNA synthetase	gi|49240966	31	12	0.4	13	0.4
6	alkaline shock protein 23	gi|49242531	26	55	2.1	108	4.1
7	50S ribosomal protein L7/L12	gi|49240899	28	84	3.0	77	2.7
8	chaperone protein	gi|49241957	9	57	6.5	55	6.3
9	putative bifunctional purine biosynthesis protein	gi|49241366	1	1	1.0	1	1.4
10	putative membrane protein	gi|49242107	77	19	0.2	17	0.2
11	putative PTS multidomain regulator	gi|49240693	7	3	0.4	1	0.2
12	putative lipoprotein	gi|49240817	9	10	1.1	8	0.9
13	putative surface anchored protein	gi|49242132	4	4	1.0	1	0.2
14	putative helicase	gi|49242827	1	5	5.2	1	1.0
15	nitrate reductase alpha chain	gi|49242742	15	4	0.3	4	0.3
16	putative exodeoxyribonuclease VII large subunit	gi|49241902	1	1	1.0	1	1.4
17	conserved hypothetical protein	gi|49242216	24	36	1.5	52	2.2
18	putative enolase	gi|49241163	11	40	3.6	41	3.7
19	glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	gi|49242505	1	2	1.7	4	3.6
20	translation elongation factor G	gi|49240906	2	46	21.0	40	18.1
21	elongation factor Ts	gi|49241549	1	44	44.2	37	36.7
22	hypothetical phage protein	gi|49241813	7	3	0.4	1	0.2
23	DNA-directed RNA polymerase beta chain protein	gi|49240901	2	15	6.7	9	4.3

24	putative membrane protein	gi|49241927	2	19	8.7	12	5.6
25	putative exonuclease	gi|49241667	9	1	0.1	2	0.2
26	conserved hypothetical protein	gi|49241201	15	41	2.7	32	2.1
27	30S ribosomal protein S2	gi|49241548	2	32	14.6	38	17.4
28	putative 3-dehydroquinate synthase	gi|49241783	39	14	0.4	8	0.2
29	50S ribosomal protein L21	gi|49242020	4	28	6.3	34	7.7
30	pyruvate kinase	gi|49242068	1	24	24.2	30	29.5
31	hypothetical phage protein	gi|49242324	1	3	3.5	4	4.3
32	glutamyl-tRNA amidotransferase subunit B	gi|49242269	1	3	3.5	1	1.4
33	triosephosphate isomerase	gi|49241161	13	28	2.1	33	2.5
34	conserved hypothetical protein	gi|49241727	7	2	0.3	1	0.2
35	DNA-binding protein HU	gi|49241789	7	29	4.3	32	4.8
36	thioredoxin	gi|49241437	13	23	1.7	36	2.7
37	putative lipoprotein	gi|49241909	2	2	0.8	2	1.0
38	glutamate synthase, large subunit	gi|49240825	2	1	0.5	1	0.7
39	topoisomerase IV subunit A	gi|49241677	1	2	1.7	1	1.4
40	collagen adhesin precursor	gi|49243016	4	1	0.2	1	0.2
41	putative stress response-related Clp ATPase	gi|49240883	2	3	1.2	1	0.7
42	50S ribosomal protein L11	gi|49240896	7	24	3.7	19	2.9
43	putative peptidase	gi|49242127	4	1	0.2	1	0.3
44	putative membrane protein	gi|49240437	7	2	0.3	3	0.4
45	putative glycosyl transferase	gi|49240623	1	1	1.0	1	1.4
46	putative rod shape-determining protein	gi|49242022	2	3	1.6	5	2.3
47	putative pyruvate dehydrogenase E1 component, beta subunit	gi|49241386	2	20	9.1	22	9.9
48	putative membrane protein	gi|49240649	4	2	0.4	1	0.2
49	putative GMP synthase	gi|49240771	1	2	1.7	3	2.9
50	preprotein translocase SecA subunit	gi|49241139	1	3	3.5	5	5.0
51	alkyl hydroperoxide reductase subunit C	gi|49240760	1	16	16.5	23	23.1

52	isoleucyl-tRNA synthetase	gi|49241485	9	4	0.5	5	0.6
53	dihydrolipoamide dehydrogenase	gi|49241388	1	17	17.3	19	18.7
54	conserved hypothetical protein	gi|49241469	2	1	0.5	1	0.7
55	putative membrane protein	gi|49242969	4	4	1.0	3	0.7
56	trigger factor (prolyl isomerase)	gi|49242047	2	22	9.9	16	7.2
57	putative UDP-N-acetylglucosamine 1-carboxyvinyltransferase	gi|49242477	9	3	0.3	4	0.4
58	putative thiamine biosynthesis protein	gi|49242085	1	2	1.7	1	1.0
59	DNA-directed RNA polymerase beta' chain protein	gi|49240902	4	3	0.6	2	0.5
60	maltose operon transcriptional repressor	gi|49241887	1	5	5.2	9	8.6
61	50S ribosomal protein L29	gi|49242587	1	23	23.4	19	18.7
62	60 kDa chaperonin	gi|49242384	1	8	7.8	9	9.4
63	immunodominant antigen A	gi|49242897	1	16	15.6	17	17.3
64	putative 50S ribosomal protein L25	gi|49240857	1	19	19.1	16	15.8
65	bifunctional autolysin precursor	gi|49241346	4	4	1.0	5	1.2
66	acetate kinase	gi|49242081	1	4	4.3	2	2.2
67	putative ATP-dependent DNA helicase	gi|49241106	2	3	1.2	1	0.7
68	putative exported protein	gi|49242128	31	12	0.4	6	0.2
69	30S ribosomal protein S13	gi|49242571	2	9	4.0	9	4.3
70	cold shock protein	gi|49243029	24	12	0.5	11	0.4
71	50S ribosomal protein L14	gi|49242585	1	16	15.6	9	9.4
72	aspartyl-tRNA synthetase	gi|49242004	4	1	0.2	1	0.2
73	putative aldehyde-alcohol dehydrogenase	gi|49240520	7	1	0.2	1	0.2
74	50S ribosomal protein L32	gi|49241419	2	21	9.5	16	7.2
75	putative chromosome partition protein	gi|49241526	4	1	0.2	1	0.3
76	zinc metalloproteinase aureolysin precursor	gi|49242963	4	1	0.2	4	1.0
77	putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gi|49241162	1	9	8.7	4	3.6
78	putative lipoprotein	gi|49240798	4	2	0.4	6	1.5

79	putative cell division protein	gi|49240867	1	1	1.0	1	1.4
80	ABC transporter ATP-binding protein	gi|49242565	1	1	1.0	3	2.9
81	putative alanyl-tRNA synthetase	gi|49241992	7	8	1.2	2	0.3
82	DeoR ramily regulatory protein	gi|49241083	1	1	1.0	5	5.0
83	putative inosine-5'-monophosphate dehydrogenase	gi|49240770	1	9	8.7	12	12.2
84	30S ribosomal protein S6	gi|49240724	1	14	13.9	19	18.7
85	putative ATPase subunit of an ATP-dependent protease	gi|49241265	2	3	1.6	9	3.9
86	ATP synthase alpha chain	gi|49242458	1	9	8.7	9	9.4
87	conserved hypothetical protein	gi|49241995	1	2	1.7	1	1.0
88	50S ribosomal protein L16	gi|49242588	1	10	9.5	10	10.1
89	putative exported protein	gi|49240647	1	1	1.0	1	1.4
90	putative pyruvate dehydrogenase E1 component, alpha subunit	gi|49241385	1	11	11.3	17	16.6
91	sensor kinase protein	gi|49242433	7	3	0.5	1	0.2
92	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-dia minopimelate ligase	gi|49241311	2	1	0.5	2	1.0
93	50S ribosomal protein L10	gi|49240898	4	15	3.4	13	3.0
94	adenylate kinase	gi|49242574	1	14	13.9	7	7.2
95	fructose-bisphosphate aldolase class I	gi|49242931	1	16	15.6	12	12.2
96	penicillin-binding protein PBP2B	gi|49241930	1	3	3.5	1	1.0
97	NAD-dependent malic enzyme	gi|49242072	1	1	1.0	1	1.4
98	putative transketolase	gi|49241663	1	9	8.7	9	9.4
99	ATP synthase beta chain	gi|49242456	2	12	5.5	9	4.3
100	conserved hypothetical protein	gi|49240841	1	2	1.7	3	2.9
101	6-phosphogluconate dehydrogenase, decarboxylating	gi|49241890	2	3	1.6	3	1.3
102	hypothetical protein	gi|49242182	1	1	1.0	2	2.2
103	putative lipoprotein	gi|49240757	1	2	1.7	7	7.2
104	50S ribosomal protein L1	gi|49240897	1	6	6.1	9	8.6
105	conserved hypothetical protein	gi|49241296	7	2	0.3	1	0.2

106	putative tagatose-bisphosphate aldolase	gi|49242478	1	14	13.9	14	14.4
107	30S ribosomal protein S5	gi|49242578	1	8	7.8	4	4.3
108	hypothetical protein	gi|49240656	1	3	3.5	5	5.0
109	putative membrane protein	gi|49240652	9	3	0.4	3	0.3
110	putative cell division protein	gi|49241477	1	7	6.9	4	4.3
111	putative extracellular sugar-binding lipoprotein	gi|49240576	1	2	1.7	3	2.9
112	50S ribosomal protein L15	gi|49242576	1	8	7.8	14	13.7
113	50S ribosomal protein L27	gi|49242018	1	7	6.9	6	6.5
114	putative glycosyl transferase	gi|49240922	24	3	0.1	1	0.1
115	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	gi|49241387	1	12	12.1	12	12.2
116	30S ribosomal protein S3	gi|49242589	1	10	9.5	6	6.5
117	penicillin-binding protein 2	gi|49241768	1	2	1.7	1	1.0
118	aconitate hydratase	gi|49241672	7	2	0.3	4	0.5
119	ribose-phosphate pyrophosphokinase	gi|49240856	1	2	1.7	1	1.0
120	putative AMP-binding enzyme	gi|49242934	4	2	0.4	1	0.2
121	50S ribosomal protein L2	gi|49242592	2	6	2.8	6	3.0
122	putative type I restriction modification DNA specificity protein	gi|49242185	4	2	0.4	1	0.2
123	30S ribosomal protein S4	gi|49242089	4	7	1.6	8	1.8
124	bone sialoprotein-binding protein	gi|49240921	2	1	0.5	1	0.7
125	factor essential for expression of methicillin resistance	gi|49241696	1	2	1.7	1	1.0
126	conserved hypothetical protein	gi|49241381	1	3	3.5	3	2.9
127	MHC class II analog	gi|49242309	2	1	0.5	3	1.3
128	putative lipoprotein	gi|49241200	4	3	0.6	3	0.7
129	acyl carrier protein	gi|49241524	9	12	1.4	11	1.2
130	phosphoenolpyruvate-protein phosphotransferase	gi|49241376	2	10	4.3	6	3.0
131	putative N utilization substance protein A	gi|49241558	1	3	3.5	4	4.3
132	50S ribosomal protein L18	gi|49242579	1	7	6.9	10	10.1

133	30S ribosomal protein S9	gi|49242560	1	8	7.8	6	6.5
134	putative DNA-binding protein	gi|49240765	1	6	6.1	6	6.5
135	hypothetical phage protein	gi|49241822	18	1	0.1	2	0.1
136	cell division protein FtsZ	gi|49241478	1	9	8.7	10	10.1
137	putative phosphofructokinase	gi|49241084	1	2	1.7	1	1.0
138	glutamine synthetase	gi|49241601	1	10	9.5	7	7.2
139	GrpE protein (Hsp-70 cofactor)	gi|49241958	9	8	0.9	7	0.8
140	primosomal protein n'	gi|49241504	1	3	2.6	2	2.2
141	conserved hypothetical protein	gi|49240924	4	5	1.2	4	0.8
142	alanine racemase	gi|49242425	4	1	0.2	1	0.2
143	putative thiol peroxidase	gi|49242083	1	3	2.6	4	4.3
144	putative regulatory protein	gi|49240484	1	2	1.7	1	1.0
145	putative exported protein	gi|49242108	1	11	11.3	12	11.5
146	putative exported protein	gi|49240646	1	3	2.6	2	2.2
147	Spo0B-associated GTP-binding protein	gi|49242017	1	3	2.6	4	3.6
148	sodium/hydrogen exchanger family protein	gi|49240986	4	1	0.2	1	0.2
149	putative lipoprotein	gi|49242273	1	1	1.0	1	1.4
150	30S ribosomal protein S8	gi|49242581	2	11	5.1	9	3.9
151	ribosome recycling factor	gi|49241551	4	5	1.2	4	1.0
152	putative protein-export membrane protein	gi|49242010	1	1	1.0	2	2.2
153	ATP-dependent Clp protease ATP-binding subunit ClpX	gi|49242046	2	5	2.4	4	2.0
154	putative 30S ribosomal protein S1	gi|49241792	1	11	11.3	6	5.8
155	putative lipoprotein	gi|49240750	1	8	7.8	6	6.5
156	50S ribosomal protein L6	gi|49242580	1	7	6.9	8	7.9
157	adenine phosphoribosyltransferase	gi|49242008	4	1	0.2	1	0.2
158	30S ribosomal protein S7	gi|49240905	2	10	4.7	5	2.3
159	conserved hypothetical protein	gi|49241773	4	1	0.2	1	0.2
160	putative monooxygenase	gi|49242650	1	3	2.6	1	1.0
161	conserved hypothetical protein	gi|49241567	4	3	0.6	1	0.2

162	putative riboflavin biosynthesis protein	gi|49241564	7	6	0.9	4	0.7
163	hypothetical protein	gi|49240472	11	3	0.2	1	0.1
164	3-oxoacyl-[acyl-carrier-protein] synthase II	gi|49241274	1	2	1.7	4	3.6
165	50S ribosomal protein L13	gi|49242561	2	5	2.4	6	3.0
166	putative malate:quinone oxidoreductase 2	gi|49242932	1	5	5.2	7	7.2
167	DNA-directed RNA polymerase alpha chain	gi|49242569	4	5	1.2	4	1.0
168	ABC transporter ATP_binding protein	gi|49242710	1	3	2.6	2	2.2
169	putative phosphoglucosamine mutase	gi|49242512	2	1	0.5	3	1.3
170	putative membrane protein	gi|49241264	1	3	2.6	1	1.0
171	MarR family regulatory protein	gi|49241071	1	8	7.8	5	5.0
172	serine hydroxymethyltransferase	gi|49242466	2	3	1.2	2	1.0
173	putative fatty oxidation complex protein	gi|49240594	1	1	1.0	1	1.4
174	putative GMP reductase	gi|49241658	11	1	0.1	1	0.1
175	glutamyl-tRNA amidotransferase subunit A	gi|49242270	1	3	3.5	5	5.0
176	putative excinuclease ABC subunit C	gi|49241438	7	1	0.2	1	0.2
177	fumarate hydratase, class-II	gi|49242222	1	1	1.0	1	1.4
178	50S ribosomal protein L4	gi|49242594	4	7	1.6	6	1.3
179	50S ribosomal protein L19	gi|49241533	1	3	2.6	4	3.6
180	phosphoglycerate kinase	gi|49241160	1	5	5.2	7	7.2
181	DEAD/DEAH box helicase family protein	gi|49241797	1	2	1.7	1	1.0
182	putative pyridoxine biosynthesis protein	gi|49240877	1	4	4.3	3	2.9
183	conserved hypothetical protein	gi|49242516	1	2	1.7	1	1.0
184	BipA family GTPase	gi|49241401	1	1	1.0	1	1.4
185	putative thioredoxin	gi|49241192	1	5	5.2	6	5.8
186	superoxide dismutase	gi|49241931	1	7	6.9	6	5.8
187	conserved hypothetical protein	gi|49241518	1	2	1.7	2	2.2
188	deoxyribose-phosphate aldolase	gi|49240510	1	4	4.3	4	3.6
189	formate acetyltransferase	gi|49240587	1	1	1.0	1	1.4
190	putative membrane protein	gi|49240396	1	1	1.0	1	1.4

191	putative pyridine nucleotide-disulphide oxidoreductase	gi|49241230	1	3	3.5	3	2.9
192	conserved hypothetical protein	gi|49241608	1	2	1.7	1	1.0
193	cold shock protein	gi|49241721	2	12	5.5	4	2.0
194	putative DNA repair protein	gi|49241898	2	2	0.8	1	0.5
195	putative oligopeptidase	gi|49241292	2	3	1.2	1	0.7
196	poly A polymerase family protein	gi|49241775	1	1	1.0	1	1.4
197	6,7-dimethyl-8-ribityllumazine synthase	gi|49242141	1	3	2.6	2	2.2
198	sensor kinase protein	gi|49241733	2	1	0.5	1	0.7
199	50S ribosomal protein L5	gi|49242583	1	4	4.3	5	5.0
200	putative membrane protein	gi|49242225	1	1	1.0	3	2.9
201	response regulator protein	gi|49241734	1	1	1.0	1	1.4
202	hypothetical protein	gi|49242280	1	3	2.6	1	1.0
203	30S ribosomal protein S21	gi|49241952	1	6	6.1	4	4.3
204	hypothetical protein	gi|49242953	1	1	1.0	3	2.9
205	putative glycyl-tRNA synthetase	gi|49241942	1	4	4.3	3	2.9
206	UDP-GlcNAc 2-epimerase	gi|49242464	2	2	0.8	1	0.5
207	putative 3-oxoacyl-[acyl-carrier-protein] synthase III	gi|49241273	1	1	1.0	2	2.2
208	30S ribosomal protein S11	gi|49242570	1	5	5.2	6	5.8
209	conserved hypothetical protein	gi|49241502	1	3	3.5	5	5.0
210	50S ribosomal protein L3	gi|49242595	1	1	1.0	3	2.9
211	putative peptidase	gi|49241689	1	1	1.0	1	1.4
212	putative phosphate acetyltransferase	gi|49240947	1	1	1.0	2	2.2
213	putative 50S ribosomal protein L31	gi|49242473	2	4	2.0	4	1.6
214	putative gluconokinase	gi|49242836	15	1	0.1	1	0.1
215	conserved hypothetical protein	gi|49242497	1	1	1.0	4	3.6
216	putative UDP-N-acetylglucosamine 1-carboxyvinyltransferase	gi|49242453	2	2	0.8	1	0.5
217	ribonucleoside-diphosphate reductase beta chain	gi|49241118	1	5	5.2	4	3.6
218	50S ribosomal protein L36	gi|49242572	1	3	3.5	3	2.9

219	staphylococcal accessory regulator A	gi|49240975	1	2	1.7	1	1.4
220	hypothetical protein	gi|49241371	1	1	1.0	3	2.9
221	ribonucleoside-diphosphate reductase alpha chain	gi|49241117	1	3	2.6	1	1.0
222	putative DNA-binding protein	gi|49240719	1	2	1.7	1	1.4
223	conserved hypothetical protein	gi|49241968	1	3	3.5	4	3.6
224	coenzyme A disulfide reductase	gi|49241260	4	1	0.2	1	0.2
225	HPr kinase/phosphatase	gi|49241146	1	2	1.7	1	1.4
226	plasmid recombination enzyme	gi|49240411	1	1	1.0	1	1.4
227	putative exported protein	gi|49242825	1	1	1.0	1	1.4
228	glutamyl-tRNA amidotransferase subunit C	gi|49242271	2	3	1.2	2	1.0
229	penicillin-binding protein 1	gi|49241473	1	1	1.0	1	1.4
230	putative mannitol-1-phosphate 5-dehydrogenase	gi|49242510	1	3	2.6	1	1.0
231	50S ribosomal protein L17	gi|49242568	1	3	2.6	4	3.6
232	conserved hypothetical protein	gi|49241989	9	3	0.4	2	0.2
233	putative membrane protein	gi|49242841	2	2	0.8	1	0.5
234	hypothetical protein	gi|49240758	7	1	0.2	1	0.2
235	penicillin-binding protein 4	gi|49240999	1	2	1.7	1	1.0
236	10 kDa chaperonin	gi|49242385	1	3	2.6	3	2.9
237	fumarylacetoacetate (FAA) hydrolase family protein	gi|49241257	1	2	1.7	3	2.9
238	conserved hypothetical protein	gi|49241510	1	2	1.7	1	1.0
239	conserved hypothetical protein	gi|49240648	1	3	3.5	1	1.4
240	conserved hypothetical protein	gi|49241102	1	2	1.7	1	1.0
241	putative deoxyadenosine kinase protein	gi|49240916	1	2	1.7	1	1.0
242	putative glucosyl transferase	gi|49241089	1	2	1.7	1	1.0
243	glycine cleavage system H protein	gi|49241194	1	5	5.2	1	1.4
244	putative membrane protein	gi|49240944	1	1	1.0	1	1.4
245	putative glutamyl-tRNA synthetase	gi|49240886	1	1	1.0	1	1.4
246	30S ribosomal protein S16	gi|49241530	1	4	4.3	1	1.4
247	putative universal stress protein	gi|49242080	1	1	1.0	1	1.4

248	putative CTP synthase	gi|49242480	1	1	1.0	2	2.2
249	conserved hypothetical protein	gi|49242812	1	1	1.0	1	1.4
250	conserved hypothetical protein	gi|49242817	1	1	1.0	2	2.2
251	putative membrane protein	gi|49242683	1	1	1.0	2	2.2
252	putative exodeoxyribonuclease VII small subunit	gi|49241901	1	2	1.7	4	3.6
253	30S ribosomal protein S10	gi|49242596	1	3	2.6	1	1.0
254	DNA-directed RNA polymerase delta subunit	gi|49242481	1	3	2.6	3	2.9
255	putative DNA-binding protein	gi|49242218	1	3	3.5	2	2.2
256	translation initiation factor IF-1	gi|49242573	1	1	1.0	1	1.4
257	30S ribosomal protein S19	gi|49242591	1	3	2.6	2	2.2
258	conserved hypothetical protein	gi|49241484	1	1	1.0	3	2.9
259	membrane anchored protein	gi|49242387	1	3	2.6	1	1.0
260	ABC transporter ATP-binding protein	gi|49241000	4	1	0.2	1	0.2
261	DNA polymerase III, beta chain	gi|49240384	1	2	1.7	1	1.0
262	conserved hypothetical protein	gi|49241308	1	1	1.0	2	2.2
263	putative lipoprotein	gi|49241062	7	1	0.2	1	0.2
264	putative ATP-dependent Clp protease proteolytic subunit	gi|49241154	1	1	1.0	1	1.4
265	hypothetical phage protein	gi|49242329	1	2	1.7	1	1.0
266	conserved hypothetical protein	gi|49241999	15	34	2.2	25	1.6
267	hypothetical protein	gi|49241998	1	1	1.0	1	1.4
268	adenylosuccinate lyase	gi|49242278	1	1	1.0	1	1.4
269	putative transcription regulator	gi|49242660	4	1	0.2	1	0.2

Appendix B: 6h post-inoculation proteomic analyses

	Identified Proteins	Accession Number	UAMS-1 (Spectral count)	UAMS-1 -*rpiRC* (Spectral count)	UAMS-1 -*rpiRC* (ratio)	UAMS-1 -*rpiRABC* (Spectral count)	UAMS-1 -*rpiRABC* (ratio)
1	dihydrolipoamide succinyltransferase E2 component of 2-oxoglutarate dehydrogenase complex	gi|49241731	733	955	1.3	1412	1.9
2	putative ATP synthase delta chain	gi|49242459	66	121	1.8	175	2.7
3	translation elongation factor Tu	gi|49240907	877	758	0.9	1003	1.1
4	DNA-binding protein HU	gi|49241789	665	577	0.9	160	0.2
5	very large surface anchored protein	gi|49241754	24	28	1.2	38	1.6
6	putative arginyl-tRNA synthetase	gi|49240966	46	73	1.6	99	2.2
7	putative membrane protein	gi|49242107	44	114	2.6	134	3.1
8	alkaline shock protein 23	gi|49242531	184	347	1.9	91	0.5
9	conserved hypothetical protein	gi|49241727	4	9	2.3	6	1.5
10	MHC class II analog	gi|49242309	41	18	0.4	16	0.4
11	putative membrane protein	gi|49240652	31	29	0.9	33	1.1
12	putative enolase	gi|49241163	133	83	0.6	112	0.8
13	conserved hypothetical protein	gi|49241995	12	10	0.8	5	0.4
14	glyceraldehyde 3-phosphate dehydrogenase 1	gi|49241159	133	121	0.9	63	0.5
15	hypothetical protein	gi|49240742	2	2	1.0	3	1.5
16	factor essential for expression of methicillin resistance	gi|49241696	9	17	1.9	28	3.1
17	ferritin	gi|49242263	60	94	1.6	127	2.1
18	hypothetical phage protein	gi|49241813	21	2	0.1	3	0.1
19	conserved hypothetical protein	gi|49242216	42	90	2.2	132	3.2
20	DeoR ramily regulatory protein	gi|49241083	4	9	2.3	8	2.0
21	putative regulatory protein	gi|49241547	2	2	1.0	8	4.0
22	putative pyruvate carboxylase	gi|49241406	2	12	6.0	10	5.0
23	putative lipoprotein	gi|49240757	59	21	0.4	22	0.4

24	conserved hypothetical protein	gi|49241469	11	1	0.1	2	0.2
25	fibronectin-binding protein precursor	gi|49242833	2	9	4.5	10	5.0
26	putative pyruvate dehydrogenase E1 component, beta subunit	gi|49241386	123	54	0.4	37	0.3
27	hypothetical phage protein	gi|49242324	8	23	2.9	10	1.3
28	dihydrolipoamide dehydrogenase	gi|49241388	93	55	0.6	59	0.6
29	putative copper importing ATPase A	gi|49242887	1	3	3.0	1	1.0
30	pyruvate kinase	gi|49242068	99	55	0.6	50	0.5
31	thioredoxin	gi|49241437	84	48	0.6	89	1.1
32	putative 30S ribosomal protein S1	gi|49241792	74	50	0.7	39	0.5
33	conserved hypothetical protein	gi|49240648	69	72	1.0	52	0.8
34	putative peptidase	gi|49240574	1	3	3.0	1	1.0
35	DNA polymerase I	gi|49242061	3	5	1.7	5	1.7
36	DNA-directed RNA polymerase beta' chain protein	gi|49240902	11	14	1.3	11	1.0
37	translation elongation factor G	gi|49240906	46	42	0.9	37	0.8
38	putative ATPase subunit of an ATP-dependent protease	gi|49241265	21	6	0.3	9	0.4
39	putative bifunctional purine biosynthesis protein [includes: phosphoribosylaminoimidazolecarboxamide formyltransferase and IMP cyclohydrolase]	gi|49241366	31	19	0.6	29	0.9
40	putative bifunctional protein	gi|49242654	1	8	8.0	12	12.0
41	putative surface anchored protein	gi|49242132	1	5	5.0	15	15.1
42	alkyl hydroperoxide reductase subunit C	gi|49240760	78	48	0.6	29	0.4
43	hypothetical protein	gi|49240472	1	1	1.0	2	2.0
44	putative rod shape-determining protein	gi|49242022	1	4	4.0	1	1.0
45	aconitate hydratase	gi|49241672	31	49	1.6	31	1.0
46	preprotein translocase SecA subunit-like protein	gi|49242975	1	2	2.0	5	5.0
47	RpiR family transcriptional regulator	gi|49240564	1	2	2.0	3	3.0
48	putative deoxyribose-phosphate aldolase	gi|49242490	127	6	0.0	2	0.0
49	hypothetical protein	gi|49242953	17	4	0.2	1	0.1
50	putative universal stress protein	gi|49242080	113	22	0.2	4	0.0

51	putative CoA synthetase protein	gi|49241537	27	37	1.4	52	1.9
52	putative membrane protein	gi|49242326	1	2	2.0	5	5.0
53	putative inosine-5'-monophosphate dehydrogenase	gi|49240770	41	35	0.9	25	0.6
54	bifunctional autolysin precursor	gi|49241346	20	15	0.8	17	0.9
55	fructose-bisphosphate aldolase class I	gi|49242931	62	24	0.4	23	0.4
56	arsenical pump membrane protein 2	gi|49242147	1	6	6.0	6	6.0
57	translation initiation factor IF-2	gi|49241561	13	15	1.2	3	0.2
58	preprotein translocase SecA subunit	gi|49241139	8	7	0.9	14	1.8
59	putative helicase	gi|49242827	3	2	0.7	2	0.7
60	putative malate:quinone oxidoreductase 2	gi|49242932	22	39	1.8	25	1.1
61	aldehyde dehydrogenase family protein	gi|49242475	5	1	0.2	3	0.6
62	putative exonuclease	gi|49241667	2	2	1.0	3	1.5
63	phosphoenolpyruvate carboxykinase	gi|49242162	20	20	1.0	27	1.4
64	ATP synthase beta chain	gi|49242456	33	18	0.5	7	0.2
65	putative AMP-binding enzyme	gi|49242934	4	9	2.3	10	2.5
66	formate--tetrahydrofolate ligase	gi|49242101	28	23	0.8	11	0.4
67	cell division protein FtsZ	gi|49241478	37	32	0.9	26	0.7
68	putative stress response-related Clp ATPase	gi|49240883	7	4	0.6	1	0.1
69	glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	gi|49242505	8	1	0.1	3	0.4
70	chaperone protein	gi|49241957	32	14	0.4	18	0.6
71	hypothetical protein	gi|49241015	3	3	1.0	3	1.0
72	glutamate synthase, large subunit	gi|49240825	1	2	2.0	1	1.0
73	potassium-transporting ATPase B chain	gi|49240444	2	6	3.0	10	5.0
74	maltose operon transcriptional repressor	gi|49241887	7	26	3.7	5	0.7
75	DNA-directed RNA polymerase beta chain protein	gi|49240901	11	34	3.1	20	1.8
76	ABC transporter ATP-binding protein	gi|49243023	1	7	7.0	10	10.0
77	trigger factor (prolyl isomerase)	gi|49242047	16	30	1.9	19	1.2
78	putative chromosome partition protein	gi|49241526	2	3	1.5	1	0.5

79	UTP--glucose-1-phosphate uridylyltransferase	gi|49242832	3	3	1.0	1	0.3
80	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	gi|49241387	49	15	0.3	12	0.2
81	conserved hypothetical protein	gi|49241293	18	1	0.1	1	0.1
82	teicoplanin resistance associated membrane protein	gi|49242700	8	5	0.6	5	0.6
83	putative amidophosphoribosyltransferase precursor	gi|49241363	6	4	0.7	8	1.3
84	putative lipoprotein	gi|49240750	25	31	1.2	23	0.9
85	elongation factor Ts	gi|49241549	28	31	1.1	25	0.9
86	polyribonucleotide nucleotidyltransferase	gi|49241566	7	5	0.7	5	0.7
87	putative glycyl-tRNA synthetase	gi|49241942	13	32	2.5	28	2.2
88	DNA gyrase subunit A	gi|49240388	7	7	1.0	6	0.9
89	putative type I restriction enzyme	gi|49240566	1	2	2.0	2	2.0
90	putative restriction and modification system specificity protein	gi|49240790	6	5	0.8	2	0.3
91	putative transketolase	gi|49241663	29	20	0.7	25	0.9
92	triosephosphate isomerase	gi|49241161	36	27	0.8	24	0.7
93	putative pyruvate dehydrogenase E1 component, alpha subunit	gi|49241385	29	20	0.7	17	0.6
94	catalase	gi|49241655	19	33	1.7	27	1.4
95	collagen adhesin precursor	gi|49243016	3	1	0.3	2	0.7
96	putative membrane protein	gi|49242225	6	1	0.2	4	0.7
97	conserved hypothetical protein	gi|49241256	2	2	1.0	2	1.0
98	putative GMP reductase	gi|49241658	1	17	17.0	1	1.0
99	60 kDa chaperonin	gi|49242384	19	15	0.8	16	0.8
100	penicillin-binding protein 2 prime	gi|49240420	4	1	0.3	1	0.3
101	putative thiolase	gi|49240593	1	3	3.0	4	4.0
102	putative RNA binding protein	gi|49242418	2	4	2.0	4	2.0
103	DNA topoisomerase I	gi|49241542	1	7	7.0	2	2.0
104	excinuclease ABC subunit A	gi|49241145	1	6	6.0	21	21.1
105	putative phosphoglycerate mutase	gi|49242761	9	3	0.3	10	1.1

106	30S ribosomal protein S13	gi|49242571	22	17	0.8	14	0.6
107	putative lipoprotein	gi|49242273	1	5	5.0	2	2.0
108	acetate kinase	gi|49242081	37	7	0.2	8	0.2
109	AcrB/AcrD/AcrF family protein	gi|49242605	3	1	0.3	1	0.3
110	conserved hypothetical protein	gi|49241308	21	24	1.1	25	1.2
111	preprotein translocase SecY subunit	gi|49242575	1	4	4.0	1	1.0
112	putative membrane protein	gi|49242969	1	6	6.0	3	3.0
113	putative septation ring formation regulator	gi|49242087	1	10	10.0	8	8.0
114	bone sialoprotein-binding protein	gi|49240921	1	3	3.0	4	4.0
115	phosphoenolpyruvate-protein phosphotransferase	gi|49241376	21	19	0.9	14	0.7
116	alkaline phosphatase synthesis transcriptional regulatory protein	gi|49242064	2	4	2.0	1	0.5
117	glutamyl-tRNA amidotransferase subunit A	gi|49242270	9	5	0.6	8	0.9
118	DNA mismatch repair protein MutL	gi|49241588	1	2	2.0	3	3.0
119	putative thioredoxin reductase	gi|49241150	30	1	0.0	2	0.1
120	adenylate kinase	gi|49242574	17	14	0.8	25	1.5
121	putative succinate dehydrogenase iron-sulfur protein	gi|49241441	6	26	4.3	27	4.5
122	putative phosphofructokinase	gi|49241084	5	2	0.4	6	1.2
123	conserved hypothetical protein	gi|49242982	3	1	0.3	1	0.3
124	cell surface elastin binding protein	gi|49241796	19	4	0.2	7	0.4
125	50S ribosomal protein L2	gi|49242592	10	10	1.0	14	1.4
126	ABC transporter ATP-binding protein	gi|49241105	2	2	1.0	1	0.5
127	threonyl-tRNA synthetase	gi|49242054	3	19	6.4	10	3.4
128	putative exodeoxyribonuclease VII large subunit	gi|49241902	1	1	1.0	3	3.0
129	putative exported protein	gi|49242128	5	10	2.0	28	5.6
130	putative exported protein	gi|49241950	12	10	0.8	13	1.1
131	glutamyl-tRNA amidotransferase subunit B	gi|49242269	5	7	1.4	5	1.0
132	putative thiol peroxidase	gi|49242083	12	6	0.5	13	1.1
133	sensor kinase protein	gi|49242433	1	13	13.0	8	8.0

134	ATP-dependent DNA helicase	gi|49242275	5	2	0.4	5	1.0
135	putative surface anchored protein	gi|49240920	2	2	1.0	3	1.5
136	conserved hypothetical protein	gi|49241883	1	1	1.0	2	2.0
137	putative ATP-dependent protease ATP-binding subunit	gi|49241546	5	12	2.4	3	0.6
138	putative phosphoribosylformylglycinamidine synthase II	gi|49241362	9	15	1.7	7	0.8
139	topoisomerase IV subunit A	gi|49241677	4	1	0.3	2	0.5
140	putative 5'-nucleotidase	gi|49240405	2	6	3.0	2	1.0
141	aerobic glycerol-3-phosphate dehydrogenase	gi|49241593	5	3	0.6	1	0.2
142	6-phosphogluconate dehydrogenase, decarboxylating	gi|49241890	10	30	3.0	13	1.3
143	sensor kinase protein	gi|49241733	1	1	1.0	2	2.0
144	NAD-dependent malic enzyme	gi|49242072	5	3	0.6	7	1.4
145	conserved hypothetical protein	gi|49241255	1	2	2.0	1	1.0
146	putative membrane protein	gi|49240649	12	2	0.2	1	0.1
147	putative exported protein	gi|49242193	2	2	1.0	1	0.5
148	putative alanyl-tRNA synthetase	gi|49241992	6	6	1.0	3	0.5
149	putative GMP synthase	gi|49240771	1	17	17.0	21	21.1
150	putative membrane protein	gi|49240720	2	4	2.0	4	2.0
151	isoleucyl-tRNA synthetase	gi|49241485	1	5	5.0	26	26.1
152	putative sodium:dicarboxylate symporter protein	gi|49240762	3	12	4.0	11	3.7
153	putative gluconate permease	gi|49242835	1	3	3.0	6	6.0
154	putative regulatory protein	gi|49242954	1	3	3.0	1	1.0
155	formate acetyltransferase	gi|49240587	17	1	0.1	1	0.1
156	putative transaldolase	gi|49242155	14	7	0.5	6	0.4
157	acetyl-coenzyme A synthetase	gi|49242102	8	15	1.9	6	0.8
158	putative membrane protein	gi|49240653	1	2	2.0	3	3.0
159	multicopper oxidase protein	gi|49241054	1	2	2.0	1	1.0
160	putative peptidyl-prolyl cis-isomerase	gi|49242212	2	1	0.5	1	0.5
161	fumarate hydratase, class-II	gi|49242222	11	7	0.6	9	0.8
162	ABC transporter ATP-binding protein	gi|49241711	1	7	7.0	1	1.0

163	seryl-tRNA synthetase	gi|49240391	10	19	1.9	10	1.0
164	primosomal protein n'	gi|49241504	9	2	0.2	6	0.7
165	glutamine synthetase	gi|49241601	9	17	1.9	17	1.9
166	putative phosphoribosylaminoimidazole carboxylase ATPase subunit	gi|49241358	2	5	2.5	4	2.0
167	30S ribosomal protein S6	gi|49240724	4	12	3.0	20	5.0
168	FtsK/SpoIIIE family protein	gi|49242110	5	1	0.2	1	0.2
169	hypothetical protein	gi|49240656	1	5	5.0	7	7.0
170	isocitrate dehydrogenase	gi|49242065	11	19	1.7	18	1.6
171	putative DNA repair protein	gi|49241898	2	1	0.5	1	0.5
172	putative non-ribosomal peptide synthetase	gi|49240550	1	3	3.0	9	9.0
173	putative glycosyl transferase	gi|49240623	2	1	0.5	1	0.5
174	50S ribosomal protein L13	gi|49242561	1	28	28.0	13	13.0
175	putative hexulose-6-phosphate synthase	gi|49240928	2	2	1.0	5	2.5
176	lipoprotein	gi|49240487	11	2	0.2	6	0.5
177	putative tRNA pseudouridine synthase B	gi|49241563	4	2	0.5	5	1.3
178	conserved hypothetical protein	gi|49240716	2	1	0.5	11	5.5
179	putative NAD synthetase	gi|49242283	9	8	0.9	5	0.6
180	resolvase	gi|49242119	4	5	1.3	3	0.8
181	alkyl hydroperoxide reductase subunit F	gi|49240759	9	5	0.6	2	0.2
182	2-oxoglutarate dehydrogenase E1 component	gi|49241732	8	3	0.4	1	0.1
183	putative pyrimidine-nucleoside phosphorylase	gi|49242489	10	4	0.4	3	0.3
184	putative lipoprotein	gi|49241909	1	3	3.0	5	5.0
185	phosphoglycerate kinase	gi|49241160	14	4	0.3	6	0.4
186	putative exported protein	gi|49242970	2	1	0.5	1	0.5
187	peptide chain release factor 1	gi|49242471	1	3	3.0	1	1.0
188	putative UDP-N-acetylglucosamine 1-carboxyvinyltransferase	gi|49242477	4	8	2.0	7	1.8
189	putative cell division protein	gi|49240867	3	7	2.3	2	0.7

190	putative anti repressor	gi|49242367	1	2	2.0	1	1.0
191	putative phosphoglucosamine mutase	gi|49242512	14	11	0.8	8	0.6
192	putative UDP-N-acetylglucosamine pyrophosphorylase	gi|49240855	1	2	2.0	1	1.0
193	acyl carrier protein	gi|49241524	13	24	1.9	12	0.9
194	putative exported protein	gi|49242160	7	3	0.4	1	0.1
195	penicillin-binding protein PBP2B	gi|49241930	4	4	1.0	1	0.3
196	urocanate hydratase	gi|49242674	12	16	1.3	15	1.3
197	putative succinate dehydrogenase flavoprotein subunit	gi|49241440	1	13	13.0	12	12.0
198	putative ketoacyl-CoA thiolase	gi|49240934	2	4	2.0	1	0.5
199	putative cell division protein	gi|49241477	12	1	0.1	6	0.5
200	putative glucose-6-phosphate isomerase	gi|49241251	6	8	1.3	1	0.2
201	putative lipoprotein	gi|49241093	1	8	8.0	14	14.1
202	serine hydroxymethyltransferase	gi|49242466	9	20	2.2	10	1.1
203	ornithine aminotransferase	gi|49241246	3	13	4.3	17	5.7
204	conserved hypothetical protein	gi|49241153	3	3	1.0	1	0.3
205	putative peptidase	gi|49241986	1	1	1.0	2	2.0
206	FolD bifunctional protein [includes: methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase]	gi|49241356	10	7	0.7	20	2.0
207	DNA-directed RNA polymerase alpha chain	gi|49242569	6	21	3.5	15	2.5
208	nitrate reductase alpha chain	gi|49242742	1	1	1.0	5	5.0
209	putative glycine cleavage system P-protein	gi|49241914	8	1	0.1	1	0.1
210	D-3-phosphoglycerate dehydrogenase	gi|49242093	2	2	1.0	1	0.5
211	adenylosuccinate lyase	gi|49242278	13	11	0.8	9	0.7
212	30S ribosomal protein S4	gi|49242089	5	13	2.6	19	3.8
213	putative tagatose-bisphosphate aldolase	gi|49242478	16	19	1.2	13	0.8
214	putative phosphate acetyltransferase	gi|49240947	6	6	1.0	7	1.2
215	putative lipoprotein	gi|49240798	1	2	2.0	1	1.0
216	acetyltransferase (GNAT) family protein	gi|49241468	5	18	3.6	3	0.6

217	50S ribosomal protein L16	gi|49242588	2	18	9.1	21	10.6
218	putative pyruvate flavodoxin/ferredoxin oxidoreductase	gi|49241581	3	5	1.7	1	0.3
219	putative CTP synthase	gi|49242480	2	1	0.5	3	1.5
220	50S ribosomal protein L1	gi|49240897	9	8	0.9	13	1.5
221	putative membrane protein	gi|49240704	2	15	7.5	14	7.1
222	putative membrane protein	gi|49240796	6	10	1.7	13	2.2
223	putative dUTP pyrophosphatase	gi|49242347	2	2	1.0	3	1.5
224	putative DNA topoisomerase	gi|49242599	2	1	0.5	3	1.5
225	putative oligopeptidase	gi|49241292	1	8	8.0	5	5.0
226	putative homoserine dehydrogenase	gi|49241649	1	2	2.0	2	2.0
227	hypothetical protein	gi|49241267	1	1	1.0	4	4.0
228	putative lipoic acid synthetase	gi|49241214	12	6	0.5	4	0.3
229	3-oxoacyl-[acyl-carrier-protein] synthase II	gi|49241274	11	4	0.4	1	0.1
230	putative succinyl-CoA ligase	gi|49241538	13	20	1.5	12	0.9
231	putative molybdate-binding lipoprotein precursor	gi|49242623	7	3	0.4	5	0.7
232	recombinase A	gi|49241577	3	1	0.3	2	0.7
233	RGD-containing lipoprotein	gi|49240571	1	8	8.0	9	9.0
234	conserved hypothetical protein	gi|49240841	2	1	0.5	1	0.5
235	putative ribonuclease R	gi|49241167	1	3	3.0	1	1.0
236	ABC transporter ATP-binding protein	gi|49241203	1	13	13.0	17	17.1
237	conserved hypothetical protein	gi|49241567	6	11	1.8	6	1.0
238	L-lactate dehydrogenase 2	gi|49242927	13	9	0.7	3	0.2
239	fumarylacetoacetate (FAA) hydrolase family protein	gi|49241257	9	8	0.9	4	0.4
240	DNA polymerase III PolC-type	gi|49241556	1	2	2.0	1	1.0
241	putative monooxygenase	gi|49242650	1	1	1.0	2	2.0
242	arginine deiminase	gi|49242961	13	1	0.1	1	0.1
243	putative O-acetylserine (thiol)-lyase	gi|49240869	11	9	0.8	6	0.5
244	putative cell division protein	gi|49241476	2	2	1.0	1	0.5
245	putative thiamine pyrophosphate enzyme	gi|49240559	1	3	3.0	3	3.0

246	aspartyl-tRNA synthetase	gi|49242004	8	3	0.4	3	0.4
247	conserved hypothetical protein	gi|49241500	1	3	3.0	1	1.0
248	putative NAD-specific glutamate dehydrogenase	gi|49241247	8	11	1.4	7	0.9
249	DNA ligase	gi|49242274	2	3	1.5	9	4.5
250	immunoglobulin G binding protein A precursor	gi|49240483	9	1	0.1	1	0.1
251	putative exported protein	gi|49240646	3	2	0.7	1	0.3
252	histidyl-tRNA synthetase	gi|49242005	2	2	1.0	1	0.5
253	putative peptidase	gi|49242127	8	15	1.9	18	2.3
254	putative aminotransferase	gi|49240913	3	20	6.7	13	4.4
255	putative asparaginyl-tRNA synthetase	gi|49241772	1	12	12.0	21	21.1
256	putative membrane protein	gi|49242896	3	1	0.3	1	0.3
257	cytosol aminopeptidase family protein	gi|49241231	3	2	0.7	1	0.3
258	conserved hypothetical protein	gi|49241773	2	1	0.5	3	1.5
259	manganese-dependent inorganic pyrophosphatase	gi|49242290	3	8	2.7	4	1.3
260	putative ATP-dependent protease ATP-binding subunit ClpL	gi|49242878	2	3	1.5	1	0.5
261	conserved hypothetical protein	gi|49241518	4	16	4.0	14	3.5
262	putative L-serine dehydratase, alpha chain	gi|49242860	5	6	1.2	4	0.8
263	50S ribosomal protein L5	gi|49242583	1	13	13.0	14	14.1
264	DNA gyrase subunit B	gi|49240387	1	6	6.0	7	7.0
265	nucleoside permease	gi|49240879	1	3	3.0	4	4.0
266	putative imidazolonepropionase	gi|49242673	4	3	0.8	3	0.8
267	putative extracellular glutamine-binding protein	gi|49242229	3	2	0.7	1	0.3
268	putative sigma factor sigB regulation protein	gi|49242422	1	9	9.0	5	5.0
269	dihydropteroate synthase	gi|49240870	1	1	1.0	2	2.0
270	putative helicase	gi|49242435	2	1	0.5	2	1.0
271	putative pyridine nucleotide-disulphide oxidoreductase	gi|49241230	10	19	1.9	3	0.3
272	50S ribosomal protein L6	gi|49242580	7	17	2.4	10	1.4
273	chromosome replication initiation/membrane attachment	gi|49242056	3	1	0.3	2	0.7

	protein						
274	S-adenosylmethionine synthetase	gi|49242161	3	2	0.7	3	1.0
275	putative mannitol-1-phosphate 5-dehydrogenase	gi|49242510	5	1	0.2	1	0.2
276	peptide chain release factor 2	gi|49241140	2	1	0.5	4	2.0
277	porphobilinogen deaminase	gi|49242042	3	1	0.3	1	0.3
278	conserved hypothetical protein	gi|49240912	4	1	0.3	1	0.3
279	aminotransferase class-V protein	gi|49242086	1	2	2.0	1	1.0
280	putative purine nucleoside phosphorylase	gi|49242491	3	11	3.7	7	2.3
281	putative ornithine carbamoyltransferase	gi|49242960	22	1	0.0	1	0.0
282	conserved hypothetical protein	gi|49241999	32	1	0.0	1	0.0
283	putative N utilization substance protein A	gi|49241558	3	6	2.0	1	0.3
284	putative choline dehydrogenase	gi|49242937	1	2	2.0	1	1.0
285	putative methionyl-tRNA synthetase	gi|49240846	1	4	4.0	6	6.0
286	ABC transporter ATP_binding protein	gi|49242710	1	2	2.0	10	10.0
287	putative transcriptional regulator	gi|49242846	5	12	2.4	10	2.0
288	DNA polymerase III, beta chain	gi|49240384	10	9	0.9	1	0.1
289	putative exported protein	gi|49241174	2	1	0.5	1	0.5
290	putative pur operon repressor	gi|49240852	3	1	0.3	1	0.3
291	putative adenylosuccinate synthetase	gi|49240399	5	19	3.8	1	0.2
292	50S ribosomal protein L4	gi|49242594	5	18	3.6	12	2.4
293	putative excinuclease ABC subunit C	gi|49241438	1	1	1.0	2	2.0
294	30S ribosomal protein S8	gi|49242581	3	7	2.3	11	3.7
295	putative single-stranded-DNA-specific exonuclease	gi|49242009	2	1	0.5	1	0.5
296	conserved hypothetical protein	gi|49242516	1	4	4.0	1	1.0
297	conserved hypothetical protein	gi|49241968	29	7	0.2	1	0.0
298	putative DNA-binding protein	gi|49240765	5	3	0.6	4	0.8
299	arginase family protein	gi|49242677	2	2	1.0	1	0.5
300	conserved hypothetical protein	gi|49242284	2	1	0.5	1	0.5
301	ABC transporter ATP-binding protein	gi|49242504	3	1	0.3	1	0.3

302	putative exported protein	gi|49240668	16	3	0.2	1	0.1
303	putative lipoprotein	gi|49241200	7	1	0.1	4	0.6
304	ATP synthase alpha chain	gi|49242458	6	7	1.2	13	2.2
305	putative phosphoribosylamine--glycine ligase	gi|49241367	5	9	1.8	6	1.2
306	putative transport protein	gi|49242882	2	4	2.0	1	0.5
307	putative phosphoribosylaminoimidazole-succinocarboxamide synthase	gi|49241359	4	10	2.5	7	1.8
308	citrate synthase II	gi|49242066	7	10	1.4	5	0.7
309	30S ribosomal protein S9	gi|49242560	13	13	1.0	9	0.7
310	putative S30EA family ribosomal protein	gi|49241138	17	8	0.5	6	0.4
311	lipoprotein	gi|49241122	10	3	0.3	8	0.8
312	serine protease	gi|49242188	1	3	3.0	1	1.0
313	aldo/keto reductase family protein	gi|49242159	3	18	6.0	11	3.7
314	putative selenocysteine lyase	gi|49241205	3	1	0.3	1	0.3
315	sodium:neurotransmitter symporter family protein	gi|49240812	5	12	2.4	6	1.2
316	putative exported protein	gi|49241326	2	1	0.5	1	0.5
317	3-hydroxy-3-methylglutaryl coenzyme A synthase	gi|49242876	3	8	2.7	15	5.0
318	penicillin-binding protein 2	gi|49241768	2	2	1.0	1	0.5
319	aldehyde dehydrogenase family protein	gi|49242884	4	1	0.3	1	0.3
320	ATP-dependent Clp protease ATP-binding subunit ClpX	gi|49242046	5	2	0.4	1	0.2
321	putative exported protein	gi|49242108	8	13	1.6	12	1.5
322	50S ribosomal protein L29	gi|49242587	9	8	0.9	9	1.0
323	putative exported protein	gi|49241180	1	4	4.0	1	1.0
324	putative protease	gi|49241315	1	4	4.0	1	1.0
325	putative cardiolipin synthetase	gi|49242441	1	2	2.0	1	1.0
326	putative DNA polymerase	gi|49241848	1	1	1.0	3	3.0
327	putative adenosylmethionine-8-amino-7-oxononanoate aminotransferase	gi|49242771	2	1	0.5	1	0.5
328	hypothetical protein	gi|49241949	5	2	0.4	3	0.6

329	putative 2-amino-3-ketobutyrate coenzyme A ligase	gi|49240909	1	6	6.0	1	1.0
330	putative fatty acid synthesis protein	gi|49241521	2	1	0.5	1	0.5
331	glyceraldehyde 3-phosphate dehydrogenase 2	gi|49242058	5	3	0.6	5	1.0
332	carbamate kinase	gi|49242958	7	2	0.3	1	0.1
333	fibrinogen and keratin-10 binding surface anchored protein	gi|49242956	2	1	0.5	1	0.5
334	50S ribosomal protein L32	gi|49241419	4	10	2.5	7	1.8
335	site-specific recombinase	gi|49240435	1	1	1.0	3	3.0
336	6-phosphofructokinase	gi|49242069	2	6	3.0	1	0.5
337	putative GTP-binding protein	gi|49241961	2	1	0.5	4	2.0
338	Spo0B-associated GTP-binding protein	gi|49242017	2	1	0.5	1	0.5
339	putative exported protein	gi|49240516	1	1	1.0	3	3.0
340	putative potassium-transporting ATPase B chain	gi|49242431	1	1	1.0	2	2.0
341	RNA polymerase sigma factor	gi|49241939	1	2	2.0	2	2.0
342	ribose-phosphate pyrophosphokinase	gi|49240856	5	2	0.4	3	0.6
343	acetyl-CoA acetyltransferase	gi|49240713	7	1	0.1	1	0.1
344	alcohol dehydrogenase	gi|49240964	7	1	0.1	1	0.1
345	amidase	gi|49241803	1	2	2.0	2	2.0
346	putative lipoprotein	gi|49241384	1	2	2.0	2	2.0
347	30S ribosomal protein S3	gi|49242589	4	3	0.8	8	2.0
348	putative chaperonin	gi|49240868	1	2	2.0	1	1.0
349	putative type I restriction modification DNA specificity protein	gi|49242185	7	6	0.9	2	0.3
350	hypothetical protein	gi|49241063	5	5	1.0	1	0.2
351	immunodominant antigen A	gi|49242897	14	6	0.4	4	0.3
352	putative membrane protein	gi|49240417	2	7	3.5	4	2.0
353	lipase precursor	gi|49240681	1	2	2.0	1	1.0
354	peptide chain release factor 3	gi|49241313	1	1	1.0	2	2.0
355	alanine racemase	gi|49242425	1	2	2.0	2	2.0

356	putative glucose-6-phosphate 1-dehydrogenase	gi|49241884	1	8	8.0	9	9.0
357	putative protease	gi|49242097	4	1	0.3	1	0.3
358	putative lipoprotein	gi|49241802	8	1	0.1	1	0.1
359	extracellular solute-binding lipoprotein	gi|49242759	1	9	9.0	2	2.0
360	conserved hypothetical protein	gi|49241381	1	2	2.0	2	2.0
361	50S ribosomal protein L7/L12	gi|49240899	10	8	0.8	2	0.2
362	beta-lactamase precursor	gi|49242122	1	4	4.0	1	1.0
363	putative betaine aldehyde dehydrogenase	gi|49242938	7	2	0.3	4	0.6
364	sodium/hydrogen exchanger family protein	gi|49240986	2	1	0.5	1	0.5
365	transcription antitermination protein	gi|49240895	7	5	0.7	1	0.1
366	putative peptidase	gi|49241689	8	1	0.1	1	0.1
367	putative sulfatase	gi|49241104	1	1	1.0	3	3.0
368	putative peptidase	gi|49241908	1	7	7.0	4	4.0
369	ABC transporter extracellular binding protein	gi|49240988	3	8	2.7	2	0.7
370	lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex	gi|49241894	7	4	0.6	1	0.1
371	conserved hypothetical protein	gi|49241360	7	8	1.1	10	1.4
372	putative membrane protein	gi|49241264	1	3	3.0	1	1.0
373	putative aldolase	gi|49240494	11	4	0.4	3	0.3
374	putative membrane protein	gi|49241534	1	1	1.0	2	2.0
375	glycerol kinase	gi|49241592	1	8	8.0	4	4.0
376	50S ribosomal protein L21	gi|49242020	16	3	0.2	5	0.3
377	staphylococcal accessory regulator A	gi|49240975	1	3	3.0	5	5.0
378	putative D-isomer specific 2-hydroxyacid dehydrogenase	gi|49242649	1	4	4.0	1	1.0
379	putative UTP--glucose-1-phosphate uridylyltransferase	gi|49242520	3	1	0.3	1	0.3
380	transcription elongation factor	gi|49241984	3	6	2.0	3	1.0
381	integrase	gi|49242376	1	7	7.0	5	5.0
382	prephenate dehydrogenase	gi|49241688	1	1	1.0	2	2.0
383	superoxide dismutase	gi|49241931	12	6	0.5	9	0.8

384	putative single-strand DNA-binding protein	gi|49240725	1	3	3.0	1	1.0
385	beta-lactamase regulatory protein	gi|49242121	1	1	1.0	2	2.0
386	putative thioredoxin	gi|49241192	3	8	2.7	9	3.0
387	putative dioxygenase	gi|49242852	1	2	2.0	1	1.0
388	putative UDP-N-acetylglucosamine 1-carboxyvinyltransferase	gi|49242453	2	3	1.5	1	0.5
389	uracil phosphoribosyltransferase	gi|49242465	7	3	0.4	6	0.9
390	50S ribosomal protein L17	gi|49242568	1	7	7.0	6	6.0
391	putative carbamoyl-phosphate synthase, pyrimidine-specific, large chain	gi|49241495	2	2	1.0	1	0.5
392	3-oxoacyl-[acyl-carrier protein] reductase	gi|49241523	3	1	0.3	2	0.7
393	mevalonate kinase	gi|49240949	1	2	2.0	1	1.0
394	ABC transporter ATP-binding protein	gi|49242773	1	1	1.0	2	2.0
395	hypothetical protein	gi|49240767	7	7	1.0	3	0.4
396	putative peptidase	gi|49241891	6	3	0.5	1	0.2
397	50S ribosomal protein L15	gi|49242576	5	8	1.6	3	0.6
398	putative extracellular sugar-binding lipoprotein	gi|49240576	4	1	0.3	1	0.3
399	putative peptidoglycan pentaglycine interpeptide biosynthesis protein	gi|49242606	3	1	0.3	1	0.3
400	putative lipoprotein	gi|49240795	2	2	1.0	1	0.5
401	30S ribosomal protein S12	gi|49240904	2	4	2.0	4	2.0
402	putative glycoprotease	gi|49242404	1	1	1.0	2	2.0
403	D-alanine--D-alanine ligase	gi|49242437	2	12	6.0	1	0.5
404	antibacterial protein	gi|49241466	10	1	0.1	14	1.4
405	putative aldehyde dehydrogenase	gi|49240539	5	3	0.6	4	0.8
406	putative lipoprotein	gi|49240799	3	8	2.7	6	2.0
407	L-lactate dehydrogenase 1	gi|49240604	3	1	0.3	1	0.3
408	conserved hypothetical protein	gi|49242849	2	1	0.5	1	0.5
409	conserved hypothetical protein	gi|49241484	6	6	1.0	4	0.7

410	putative membrane protein	gi|49240396	1	1	1.0	2	2.0
411	6,7-dimethyl-8-ribityllumazine synthase	gi|49242141	3	6	2.0	7	2.3
412	hypothetical protein	gi|49242788	1	2	2.0	2	2.0
413	conserved hypothetical protein	gi|49241443	3	5	1.7	1	0.3
414	conserved hypothetical protein	gi|49241207	1	8	8.0	6	6.0
415	conserved hypothetical protein	gi|49241875	2	1	0.5	2	1.0
416	signal recognition particle protein	gi|49241529	1	1	1.0	3	3.0
417	putative oligopeptide transport ATP-binding protein	gi|49241282	3	1	0.3	1	0.3
418	probable cadmium-transporting ATPase	gi|49241056	3	1	0.3	1	0.3
419	putative membrane protein	gi|49242947	1	2	2.0	3	3.0
420	diaminopimelate decarboxylase	gi|49241719	6	1	0.2	1	0.2
421	HPr kinase/phosphatase	gi|49241146	1	3	3.0	1	1.0
422	putative zinc-binding dehydrogenase	gi|49242536	2	2	1.0	1	0.5
423	putative 6-carboxyhexanoate--CoA ligase	gi|49242768	1	3	3.0	1	1.0
424	conserved hypothetical protein	gi|49242653	1	2	2.0	2	2.0
425	membrane anchored protein	gi|49242387	1	5	5.0	8	8.0
426	putative phosphoribosylformylglycinamidine cyclo-ligase	gi|49241364	7	3	0.4	1	0.1
427	ABC transporter ATP-binding protein	gi|49242684	4	1	0.3	1	0.3
428	putative naphthoate synthase	gi|49241340	2	4	2.0	6	3.0
429	hypothetical phage protein	gi|49242357	1	1	1.0	3	3.0
430	ribonucleoside-diphosphate reductase beta chain	gi|49241118	3	7	2.3	6	2.0
431	50S ribosomal protein L10	gi|49240898	6	4	0.7	6	1.0
432	putative cyclophilin type peptidyl-prolyl cis-trans isomerase	gi|49241243	1	7	7.0	5	5.0
433	autolysis and methicillin resistant-related protein	gi|49241350	1	3	3.0	2	2.0
434	putative exodeoxyribonuclease VII small subunit	gi|49241901	16	3	0.2	1	0.1
435	PTS system, glucose-specific IIA component	gi|49241742	7	3	0.4	2	0.3
436	putative 3-oxoacyl-[acyl-carrier-protein] synthase III	gi|49241273	1	3	3.0	1	1.0
437	conserved hypothetical protein	gi|49241517	1	5	5.0	2	2.0

438	putative 50S ribosomal protein L25	gi|49240857	8	3	0.4	8	1.0
439	chorismate synthase	gi|49241784	2	1	0.5	1	0.5
440	transposase A 1	gi|49240431 (+1)	1	11	11.0	5	5.0
441	cation efflux family protein	gi|49242762	1	1	1.0	5	5.0
442	ribonucleoside-diphosphate reductase alpha chain	gi|49241117	1	1	1.0	2	2.0
443	putative molybdenum cofactor biosynthesis protein	gi|49242617	2	1	0.5	1	0.5
444	signal peptidase Ib	gi|49241254	3	1	0.3	1	0.3
445	hypothetical protein	**gi|49240622**	**1**	**6**	**6.0**	**1**	**1.0**
446	glycine cleavage system H protein	gi|49241194	17	1	0.1	1	0.1
447	acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	gi|49242071	2	3	1.5	1	0.5
448	putative 6-phospho-3-hexuloisomerase	gi|49240929	1	3	3.0	1	1.0
449	glycolytic operon regulator	gi|49241158	1	2	2.0	1	1.0
450	arginase	gi|49242515	4	3	0.8	1	0.3
451	aldo/keto reductase family protein	gi|49241088	1	6	6.0	6	6.0
452	GrpE protein (Hsp-70 cofactor)	gi|49241958	6	3	0.5	1	0.2
453	ImpB/MucB/SamB family protein	gi|49241687	1	1	1.0	3	3.0
454	haloacid dehalogenase-like hydrolase	gi|49241218	3	5	1.7	1	0.3
455	conserved hypothetical protein	gi|49241102	1	1	1.0	4	4.0
456	conserved hypothetical protein	gi|49241152	1	2	2.0	2	2.0
457	putative acyltransferase	gi|49242096	3	1	0.3	1	0.3
458	capsular polysaccharide synthesis enzyme	gi|49240528	1	2	2.0	2	2.0
459	hypothetical phage protein	gi|49241851	1	1	1.0	4	4.0
460	putative quinol oxidase polypeptide II precursor	gi|49241354	5	2	0.4	5	1.0
461	putative glutamyl-tRNA synthetase	gi|49240886	4	3	0.8	2	0.5
462	putative ATP-dependent Clp protease proteolytic subunit	gi|49241154	2	4	2.0	4	2.0
463	putative phosphoribosylformylglycinamidine synthase I	gi|49241361	5	3	0.6	2	0.4
464	D-specific D-2-hydroxyacid dehydrogenase	gi|49242856	5	1	0.2	1	0.2

465	putative polypeptide deformylase 2	gi|49241383	2	1	0.5	1	0.5
466	putative exported protein	gi|49242176	1	1	1.0	3	3.0
467	putative cobalt transport protein	gi|49242563	2	1	0.5	1	0.5
468	putative nucleoside diphosphate kinase	gi|49241785	1	3	3.0	1	1.0
469	hypothetical phage protein	gi|49241861	2	1	0.5	2	1.0
470	putative membrane protein	gi|49242533	4	7	1.8	8	2.0
471	urease alpha subunit	gi|49242634	1	1	1.0	2	2.0
472	30S ribosomal protein S2	gi|49241548	6	1	0.2	4	0.7
473	hypothetical protein	gi|49242048	1	1	1.0	5	5.0
474	hypothetical protein	gi|49240758	1	4	4.0	4	4.0
475	putative peptidase	gi|49241704	3	3	1.0	8	2.7
476	putative 50S ribosomal protein L31	gi|49242473	4	3	0.8	5	1.3
477	translation initiation factor IF-3	gi|49242052	1	3	3.0	1	1.0
478	DNA-directed RNA polymerase delta subunit	gi|49242481	4	3	0.8	3	0.8
479	putative phosphoribosylaminoimidazole carboxylase catalytic subunit	gi|49241357	4	5	1.3	1	0.3
480	putative membrane protein	gi|49242500	1	7	7.0	4	4.0
481	sucrose-specific PTS tranporter protein	gi|49240563	1	4	4.0	1	1.0
482	conserved hypothetical protein	gi|49241025	1	2	2.0	1	1.0
483	hypothetical protein	gi|49242527	2	1	0.5	1	0.5
484	conserved hypothetical protein	gi|49240865	1	1	1.0	2	2.0
485	catabolite control protein A	gi|49242105	2	1	0.5	1	0.5
486	hypothetical protein	gi|49240748	2	1	0.5	1	0.5
487	prolyl-tRNA synthetase	gi|49241555	2	1	0.5	1	0.5
488	alanine dehydrogenase 1	gi|49242079	4	1	0.3	2	0.5
489	30S ribosomal protein S10	gi|49242596	1	4	4.0	1	1.0
490	response regulator protein	gi|49241734	2	3	1.5	1	0.5
491	ABC transporter ATP-binding protein	gi|49241000	1	2	2.0	1	1.0
492	conserved hypothetical protein	gi|49242074	1	2	2.0	3	3.0

493	putative membrane protein	gi|49241171	1	3	3.0	2	2.0
494	hypothetical protein	gi|49242299	1	9	9.0	3	3.0
495	conserved hypothetical protein	gi|49242388	3	6	2.0	1	0.3
496	conserved hypothetical protein	gi|49241151	2	1	0.5	1	0.5
497	putative dihydroorotate dehydrogenase	gi|49242916	2	1	0.5	1	0.5
498	putative pyridoxine biosynthesis protein	gi|49240877	2	1	0.5	1	0.5
499	putative hydrolase	gi|49242908	3	2	0.7	1	0.3
500	putative thioredoxin	gi|49242113	4	1	0.3	1	0.3
501	undecaprenyl pyrophosphate synthetase	gi|49241552	1	4	4.0	1	1.0
502	AhpC/TSA family protein	gi|49242233	3	6	2.0	1	0.3
503	conserved hypothetical protein	gi|49242981	11	1	0.1	1	0.1
504	putative peptidase	gi|49242379	1	2	2.0	1	1.0
505	hypothetical phage protein	gi|49241867	2	3	1.5	1	0.5
506	signal transduction protein	gi|49242205	1	1	1.0	5	5.0
507	glycerophosphoryl diester phosphodiesterase	gi|49240418	1	1	1.0	6	6.0
508	putative acyl-CoA dehydrogenase	gi|49240595	2	2	1.0	1	0.5
509	30S ribosomal protein S7	gi|49240905	1	3	3.0	1	1.0
510	putative non-heme iron-containing ferritin	gi|49242492	1	7	7.0	1	1.0
511	conserved hypothetical protein	gi|49242133	5	1	0.2	1	0.2
512	conserved hypothetical protein	gi|49242497	4	3	0.8	1	0.3
513	hypothetical protein	gi|49241579	1	1	1.0	8	8.0
514	30S ribosomal protein S16	gi|49241530	2	4	2.0	1	0.5
515	FdhD/NarQ family protein	gi|49242624	2	1	0.5	1	0.5
516	glutamine transport ATP-binding protein	gi|49242228	1	2	2.0	1	1.0
517	putative membrane protein	gi|49242523	2	1	0.5	1	0.5
518	D-alanyl carrier protein	gi|49241223	1	1	1.0	9	9.0
519	30S ribosomal protein S11	gi|49242570	3	2	0.7	1	0.3
520	30S ribosomal protein S19	gi|49242591	1	1	1.0	3	3.0
521	response regulator protein	gi|49241871	2	1	0.5	1	0.5

522	hypothetical protein	gi|49241998	1	1	1.0	3	3.0
523	50S ribosomal protein L23	gi|49242593	1	2	2.0	1	1.0
524	conserved hypothetical protein	gi|49242813	2	1	0.5	1	0.5
525	putative cytochrome ubiquinol oxidase	gi|49241378	2	1	0.5	1	0.5
526	putative membrane protein	gi|49242779	2	1	0.5	1	0.5
527	putative 4-oxalocrotonate tautomerase	gi|49241686	4	2	0.5	1	0.3
528	stage V sporulation protein G	gi|49240854	3	1	0.3	1	0.3
529	flavohemoprotein	gi|49240603	2	1	0.5	1	0.5
530	hypothetical phage protein	gi|49241810	1	2	2.0	1	1.0
531	putative methyltransferase	gi|49241955	1	1	1.0	3	3.0
532	hypothetical protein	gi|49242669	1	2	2.0	1	1.0
533	conserved hypothetical protein	gi|49241583	2	1	0.5	1	0.5
534	conserved hypothetical protein	gi|49241297	1	1	1.0	2	2.0
535	putative small heat shock protein	gi|49242733	1	3	3.0	1	1.0
536	putative methyltransferase	gi|49242124	2	1	0.5	1	0.5