4-7-2017

Non-classical regulators in Staphylococcus aureus

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Non-classical regulators in *Staphylococcus aureus*

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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College of Arts and Sciences
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Date of Approval:
March 8, 2017

Keywords: small RNA polymerase subunits, RpoE, δ subunit, RpoZ, ω subunit, small regulatory RNA, sRNA

ACKNOWLEDGEMENTS

The road to my Ph.D. was at no point a solitary one. At any given time of my academic journey, I have been surrounded by mentors and friends who have supported me in a myriad of ways and without whom I may never have achieved this goal.

First, I would like to thank Dr. Lindsey Shaw, my mentor and PI. He brought me into his lab, gave me freedom to develop my own ideas, and handed me the intellectual tools necessary for success in the scientific world. He has not only offered professional but also personal advice, and in this light, the German expression “Doktorvater” is highly appropriate and reflects the paternal guidance he has shown me. Furthermore, I would like to thank the members of my committee, Dr. Kathleen Scott, Dr. James Riordan and Dr. Burt Anderson, all of whom have challenged and pushed me wherever necessary and whose support has helped me to become a better scientist.

I would also like to thank one of my mentors who did not formally appear on my committee nor any of my paperwork. Dr. Ronan Carroll, as a postdoc in Dr. Shaw’s lab and later an independent researcher after his departure, has always supported me both as a colleague and a friend. I am grateful for his patience and for each beer shared over (mostly) scientific conversations.

Although a lab community is a transient thing, with new students constantly coming and going, I was fortunate to cross paths and develop friendships with many outstanding individuals. Past and present Shaw lab members have helped foster my joy for science

as well as preserve my sanity during late hours and weekends in the lab. I am thankful to consider each of them a close friend and our time thus far as only the first step in future endeavors together.

Not all of the help I received was professional, but more often than not, emotional in nature. This special kind of support came from my family. It wasn't always easy for me to pursue a degree almost 5,000 miles from home, but it must have been even harder to let a son, brother or grandson venture so far away. Despite this, I never once heard a complaint or felt pressure to live closer, which was not due to a lack of wish on their behalf, but instead testimony to their wholehearted support. Though each of my family members has added their piece to the puzzle of who I am today, I want to thank in particular my mom and dad. Both have given me more than anyone could expect from another person and now that I have seen more of the world, I can fully appreciate how uniquely loving my past was and how lucky I am to have been raised by such outstanding parents.

Lastly, and most importantly, I want to thank my wonderful wife, Amy. Of all the sections and paragraphs written in this dissertation, this might be the hardest. Not because of the lack of things to say, of memorable anecdotes, adventures or challenges that we have overcome together, but because of the shortage of sufficient language that would allow to express how thankful I am for everything that she has done for me. When Amy stepped into my life, everything became a little brighter and clearer. She made all of the seemingly impossible challenges appear miniscule, and her support allowed me to work a little bit harder in order to achieve not my, but our goals. Without her, many of the pages in this dissertation and indeed my life would have remained empty. ILD

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ABSTRACT

Staphylococcus aureus is a highly problematic human pathogen due to its ability to cause devastating infections, paired with a capacity to withstand the action of a large fraction of available antibiotics. Both pathogenicity and antibiotic resistance are encoded by numerous genomic elements, though the expression of these factors is energetically costly and not always beneficial for cellular survival. Therefore, *S. aureus* has developed sophisticated regulatory networks to integrate a multitude of signals, enabling it to navigate the delicate balance between its pathogenic lifestyle and baseline needs for cellular energy homeostasis. It is thus imperative to study *S. aureus* behavior and its underlying regulatory circuits not as isolated entities, but rather holistically as part of an optimized, highly interconnected system. To do so, we must seek to achieve a comprehensive understanding of all encoded regulators, that is, not only historically well defined elements like transcription factors, two-component systems and σ factors, but also the lesser studied 'non-classical' regulators like small regulatory RNAs and regulatory subunits of RNA-dependent RNA polymerase (RNAP). To this end, we describe here the identification of numerous, previously unidentified sRNAs and their incorporation into a new standardized cataloging and annotation system. The identification and annotation procedures are based on a number of RNAseq experiments performed in three different *S. aureus* backgrounds (MRSA252, NCTC 8325, and USA300). We then apply RNAseq to evaluate the expression patterns of

these elements when grown in human serum, thus probing for possible connections between sRNAs and *S. aureus* pathogenicity. In addition, we characterize the role of two small RNAP subunits, δ and ω, for *S. aureus* RNAP function. δ is of particular interest, as it is unique to Gram-positive bacteria; deletion of the subunit results in a loss of transcriptional stringency and decreased expression of numerous virulence determinants. These alterations are accompanied by impaired survival of the δ mutant in whole human blood, increased phagocytosis by human leukocytes, and decreased survival in a murine model septicemia when compared to its parental strain. In contrast, there is no indication of direct and gene-specific transcriptional functions for ω. Rather, we describe a role for ω in the structural integrity of the RNAP complex, where its loss leads to a structural disturbance in the RNAP complex that causes altered affinities for (alternative) σ factors and the δ subunit. Overall, the findings presented here contribute to a better understanding of the intricate regulatory systems that guide the lifestyle of an organism that presents an immense burden to patients and our health care system alike.

CHAPTER 1: INTRODUCTION

A CASE FOR THE COMPLEXITY OF THE *STAPHYLOCOCCUS AUREUS* LIFESTYLE AND THE NEED FOR CONSTANT ADAPTATION

"Life is not always a matter of holding good cards, but sometimes, playing a poor hand well."
- **Jack London**

Staphylococcus aureus is a Gram-positive, facultative anaerobe. Phylogenetically, this coccal bacterium belongs to the phylum Firmicutes, whose members are characterized by a low G-C content. This bacterium can be found as a commensal asymptomatically colonizing the human body, but can alternatively present itself as a formidable pathogen. It is thus characterized as an opportunistic pathogen. Since its first isolation in 1880 [1], the bacterium has garnered widespread attention driven by several key events, the most notable of which being the development of resistance to a number of widely used antibiotics [2-5] and large-scale outbreaks in nosocomial settings, as well as in healthy communities in Northern America, Europe, and the Asia-Pacific region. A recent study reported ~500,000 *S. aureus*-related hospitalizations in the USA in 2005, associated with annual costs of an estimated $830 million to $9.6 billion [6], highlighting the immense socio-economic burden this organism presents.

From the hospital to the community: The adaptation of *S. aureus* to the healthy population

Historically, *S. aureus* infections have occurred in high rates within the nosocomial setting. These trends have escalated due to the appearance of numerous resistance mechanisms against most available antibiotics [7, 8]. These “waves of resistance’ [8] have been the result of the acquisition of genetic elements that render the bacterium, which is ‘naturally susceptible to virtually every antibiotic that has ever been developed’ [8], resistant to precisely these antimicrobial agents. Soon after the introduction of the very first antibiotic, penicillin, in 1941 the first resistant strains were reported in 1942 [2] and soon spread through the hospital system. As of 1959, it was possible to combat these strains for a limited time through the use of methicillin. Nevertheless, the appearance of methicillin-resistant *S. aureus* (MRSA) [3], which acquired the *mecA* locus in the early 1960’s, caused a second large wave of infections (reviewed by [9]). These infections were largely nosocomial, so the causative strains were referred to as hospital acquired-MRSA (HA-MRSA). Subsequently, a third large wave occurred in the late 70’s and mid 80’s [8] and led to high usage of vancomycin, a last resort antibiotic against MRSA infection. The bacterium quickly adapted, and intermediate (vancomycin intermediate *S. aureus*, VISA) [4] and full vancomycin resistance (vancomycin resistance *S. aureus*, VRSA) [5] appeared. This development seriously endangered the treatment of complicated infections, but the concerning strains were still largely refined to hospitals. However, novel strains appeared concurrently and were able to infect the otherwise healthy population (community acquired MRSA, CA-MRSA) in Western Australia [10] as well as in the US [11]. Research into the unique features and

differences between HA-MRSA and CA-MRSA is an active field of research and has been reviewed in detail on several occasions [12-15]. In order to delineate between *S. aureus* subgroups in context of the health care setting, the Center for Disease Control and Prevention (CDC) has defined a case as CA-MRSA-related when MRSA isolates from outpatients were obtained within 48 h after admission to the hospital and underlying risk factors like a history of hospitalizations, surgery, dialysis, or residence in a long-term care facility within one year prior to hospitalization are absent. Disqualifying criteria for CA-MRSA also include the presence of an indwelling catheter or percutaneous device and previous isolation of MRSA from the patient [16].

From an epidemiological standpoint, CA-MRSA strains in the US belong to the USA300 and USA400 pulsed-field gel electrophoresis (PFGE) pulsotypes, while HA-MRSA are commonly USA100 and USA200 [17]. As mentioned before, outbreaks with CA-MRSA strains usually affect parts of the population that did not have any previous hospitalization or underlying risk factors. Nevertheless, certain groups with elevated risks for infection are present in the population, and David and Daum [12] have produced a comprehensive overview of outbreaks in a variety of community settings. Briefly, parts of the population that are proposed to be at particular risk for CA-MRSA infections are, amongst others, children of different ages, athletes of various sports (usually those involving skin to skin contact), underserved urban communities, indigenous populations, inmates in correctional facilities, military members, and individuals that are in close contact with animals (e.g. livestock handlers, pet owners, veterinarians) [12]. The newly acquired ability to infect young and otherwise healthy

individuals has attracted a lot of attention from the scientific community, and we have consequently developed a clearer understanding of the genomic changes that allow transition from the hospital to the community. Several hallmarks of CA-MRSA strains, when compared to their nosocomial counterparts, were identified [14]:

i) A major finding was that there are obvious differences in the genomic content concerning the presence of distinct mobile genetic elements in HA- and CA-MRSA strains. As mentioned above, MRSA strains carry the *mecA* gene, which encodes for penicillin binding protein 2a, conferring resistance to methicillin [18]. Together with two regulators of *mecA*, *mecI* and *mecR*, this locus is encoded on the staphylococcal cassette chromosome *mec* (SCC*mec*) [19]. At least 11 different SCC*mec* types with varying structural organization and content have been found ([20]; an updated list is available under http://www.sccmec.org/). While HA-MRSA strains carry the larger SCC*mec* types I-III, the smaller types IV or V are found in CA-MRSA. Though the size of the latter two is connected to a lower fitness cost [21] and a higher mobility of the elements themselves [22], types I-III carry additional antibiotic resistance genes [23], explaining the multi-drug resistance (MDR) phenotypes of HA-MRSA. In contrast, CA-MRSA strains are, with exceptions [24], sensitive to non-β lactam antibiotics. Combined, these findings concerning the distribution of SCC*mec* elements aided our understanding of the differences between HA- and CA-MRSA strains, ultimately leading Lee and colleagues [21] to propose that the presence of type IV and V elements in CA-MRSA is driven by 'the selection for factors that contribute to ecological fitness [and] may outweigh the need for multiple resistance determinants'.

ii) As CA-MRSA strains are able to infect otherwise healthy individuals, it was long suspected that community and hospital strains differ in the expression of virulence determinants (*S. aureus* encodes for a large number of proteins that allow for colonization and survival in or on the host, termed virulence factors, and collectively referred to as the 'virulome' [25]). Such a notion has now been confirmed for several proteins that directly contribute to *S. aureus* pathogenicity. These factors include the Panton-Valentine Leukocidin (PVL), several Phenol Soluble Modulins (PSMs), and α-toxin, all of which are cytolytic (though acting against different cell types). The two-component leukocidin PVL was first described and connected to infection in 1894 [26] and 1932 [27], respectively. The factor was the first to be thoroughly researched for a connection to the appearance of CA-MRSA strains, as it was observed early that the encoding genes (*lukS-PV* and *lukF-PV*) are found in nearly all CA-MRSA isolates [28-31] and are often absent in HA-MRSA strains [28]. Although this β-pore-forming toxin is well known to lyse human neutrophils [32] and is therefore considered a *bona fide* virulence factor, there is debate as to how much the factor actually contributes to the CA-MRSA specific pathophysiology (discussed by [12, 14, 33]). This doubt is derived mainly from the finding, amongst others, that PVL action is model-dependent [32] (e.g. mouse [34] vs. rabbit [35]) and therefore studies have produced partially contradicting results.

Two additional (groups of) toxins that have been connected to the appearance of CA-MRSA strains are PSMs [36] and the α-toxin [37], both of which are effective cytolysins (reviewed by [38] and [39, 40], respectively). The seven PSMs (PSMα1-PSMα4,

PSMβ1, PSMβ2 and δ-toxin) encoded by *S. aureus* are small amphipathic α-helical peptides that have been linked to *S. aureus* pathogenicity [36, 41], largely due to their ability to lyse neutrophils [36], erythrocytes [42] and osteoblasts [43]. Alpha-toxin, also known as α-hemolysin, due to its ability to lyse erythrocytes (amongst a variety of other cell types [40]), has also been shown to contribute to the ability of *S. aureus* to cause disease [41, 44]. In contrast to PVL, there is no obvious difference in the presence of α-toxin and PSM genes between HA- and CA-MRSA isolates. Nevertheless, differences in their corresponding expression patterns (and subsequently protein abundance) when comparing HA- and CA-MRSA have been described [36, 45]. Since both PSM [46] and α-toxin [47] loci are controlled by the major regulatory system Agr (discussed in more detail below), the discovery of increased Agr activity in CA-MRSA [48] ultimately explained the higher abundance of such toxins in these strains.

iii) Another factor that was connected to the success of *S. aureus* as a pathogen within the healthy community was the acquisition of an additional mobile genetic element, ACME (arginine catabolic mobile element). This element, acquired originally from *Staphylococcus epidermidis* [49], is omnipresent in the USA300 pulsotype and has been linked to its ability to cause disease in a rabbit model [49]. At least two avenues for how the ACME-encoded genes aid colonization and infection by *S. aureus* have been reported. First, the gene cluster encodes for an arginine deiminase system (Arc), which enables *S. aureus* to persist in acidic environments [50], such as that found on human skin [51]. A second gene, *speG*, encodes for a spermine/spermidine-acetyltransferase, which confers resistance to polyamines (e.g. spermine and spermidine) [52].

Polyamines are naturally produced by the human body as important factors in wound healing [50, 53]. Interestingly, it was previously thought that all forms of life were able to synthesize and therefore resist possible inhibitory effects of polyamines. Contrary to this assumption, it was recently shown that *S. aureus* inherently is incapable of producing polyamines and is highly sensitive to their presence [52]. By the acquisition of ACME, and in particular *speG*, CA-MRSA strains are able to overcome these effects. Thus, the presence of ACME allows *S. aureus* to effectively colonize and infect the skin, which explains the high incident rate of skin infections with CA-MRSA strains (as discussed in the following section). These findings extend our epidemiological understanding of *S. aureus* in other contexts, too: since ACME was described to be solely present in the USA300 lineage [54], it has been suggested that the element aided in the replacement of other CA-MRSA lineages, e.g. USA400 [50].

Overall, the differences between HA- and CA-MRSA are multifactorial, but can be summarized as a fine balance between virulence and fitness. While certain virulence genes are upregulated (PSMs and α-toxin) or omnipresent (PVL) in CA-MRSA strains, these lineages sacrificed larger SCC*mec* elements that carry higher number of resistance genes for smaller and more mobile elements with a smaller fitness burden on the cell. Simultaneously, the acquisition of novel elements, such as ACME, presents a fitness advantage on the skin, therefore making parts of the human body more accessible to CA-MRSA strains. These alterations have been the result of both horizontal gene transfer (e.g. in case of PVL or ACME) as well as modifications of existing genomic content (e.g. increased activity of Agr and a concomitant increase in

expression of virulence determinants). Ultimately, the findings summarized in this section highlight the extraordinary ability of this pathogen to adjust to novel conditions and challenges, e.g. the increased usage of antibiotics, and explain our difficulties in overcoming the negative impact of HA- as well as CA-MRSA strains; despite all of the advancements made in our health care system.

Not a one-trick pony: The broad spectrum of diseases caused by *S. aureus*

The increasing attention cast towards *S. aureus* can be explained by its versatility as a pathogen, allowing the bacterium to infect almost every site within the human body (comprehensively reviewed by [55]). Amongst the different disease manifestations, perhaps the most concerning is the occurrence of invasive infections, and the development of acute *Staphylococcus aureus* bacteremia (SAB) [56-60]. In this regard, several studies found *S. aureus* to be the leading cause of bacteremia in Europe [61] and the Americas [62, 63]. Although advances in health care and in particular the introduction of antibiotics (i.e. penicillin [64]), have rapidly decreased mortality rates from untreated *S. aureus* infections (75%-82%) [65, 66], mortality within 30 days of the onset of infection at the end of the 20th and beginning of the 21st century is still ~20% [67-69]. These numbers represent the immense progress modern medicine has made to date, but also highlight existing limitations for treatment of SAB and the threat that an impending postantibiotic era due to increasing (multi-) drug resistance amongst bacterial pathogens presents [70]. In addition to general and systemic implications of SAB, infection of the endocardium (invasive endocarditis, IE) is especially problematic and commonly seen during invasive infection with *S. aureus*. At this point, *S. aureus* is

still a leading cause of IE and was found to be the most prevalent pathogen when investigating ~1800 cases of IE in 16 countries [71]. Even more concerning, the number of patients in the US affected by IE as well as overall mortality rates have been increasing during the first decade of the 21st century [72]. Beyond these directly life-threatening invasive infections, *S. aureus* is the leading cause of osteoarticular infections, including osteomyelitis, septic arthritis, and prosthetic joint infection [55]. In particular, the latter presents a large burden in a steadily aging population, where arthroplasty is and will continue to be a common procedure [73]; with ~2% of patients affected by *S. aureus* [74, 75].

Although invasive infections present a major concern, not all diseases caused by *S. aureus* require deep penetration of the bacterium into the human body: the clinical picture of *S. aureus* also commonly comprises skin and soft tissue infections (SSTIs), including cutaneous abscesses as well as purulent and non-purulent cellulitis, impetigo, and necrotizing fasciitis [12]. Although these infections have been prevalent and historically connected to *S. aureus*, the number of such diseases has drastically increased since the appearance of CA-MRSA strains, and is particularly driven by the USA300 lineage [76-78]. Severity of these infections can vary widely, but increasing numbers of hospitalizations have been observed [78], adding to the challenges that *S. aureus* presents for society and the healthcare system. Lastly, along with the disease manifestations above, *S. aureus* is a common cause for a variety of pleuropulmonary-, gastrointestinal-, and urinary tract infections, as well as for bacterial meningitis, which won't be discussed in detail here, but have been reviewed elsewhere [55].

These high numbers of infections within the hospital and in the community ultimately raise the question of the natural reservoir and/or path of infection. In a nosocomial environment, transmissions have been well tracked and it is widely acknowledged that improved hygienic standards can effectively prevent *S. aureus* outbreaks and cross-contamination between patients [79-83]. In contrast, the mode of transmission within the community is more complex, though we now have a better understanding of the survival of the bacterium on different fomites [84], as well as its transmission dynamics (reviewed by [12]). Such findings ultimately let the CDC to release a list of risk factors for MRSA transmission ('5 C's'): i) **C**rowding ii) frequent skin-to-skin **C**ontact iii) **C**ompromised skin iv) **C**ontaminated items and surfaces and v) lack of **C**leanliness (http://www.cdc.gov/niosh/topics/mrsa/). Furthermore, nose picking was related to higher chances of *S. aureus* nasal colonization and therefore should be avoided where possible [85].

Despite these hygiene and safety measures, we now appreciate that large numbers of individuals are asymptomatically colonized with *S. aureus* at a variety of sites of the human body [86]. Most notably, *S. aureus* has a natural reservoir in vestibulum nasi within the squamous epithelium [86]. Additionally, extra-nasal colonization sites include skin, throat, perineum, vagina, and gastrointestinal tract [86-89].

Adjustment to highly variable environments: From nasal colonization to invasive infection

As discussed in the previous sections, *S. aureus* strains have evolved to interact with

healthy and predisposed individuals alike, causing a variety of clinical manifestations. Nevertheless, *S. aureus* can also reside on the human host without causing any symptoms. This asymptomatic colonization with a highly virulent pathogen raises the question: how does the bacterium employ its genetic content to foster a balance that allows for niche survival without triggering a strong immune response, such that it can survive in close proximity to its host? Therefore, this section will consider the adaptation of *S. aureus* to various host environments, with a focus on the nose as the primary reservoir for *S. aureus* colonization.

Colonization with *S. aureus*

Numerous studies have investigated the extent of colonization with *S. aureus* within the healthy population, i.e. asymptomatic colonization. This is of particular interest, as nasal colonization is associated with risk of infection by the pathogen [86, 90, 91]. Historically, three colonization patterns have been identified to describe *S. aureus* carrier subpopulations. These include **i)** persistent carriers who always carry the bacterium in their nose (~20% of the population), **ii)** intermittent carriers who carry the bacterium for short times (~30%), and **iii)** non-carriers who are never colonized (~50%) (reviewed by [86]). These categories, however, have been brought into question recently, and it has been recommended to distinguish only between carriers and 'others' [92], as intermittent and non-carriers show similarly contrasting features from persistent carriers when comparing **i)** immune response to colonization [92], **ii)** bacterial loads during colonization [92], **iii)** survival rates of the bacterium in the nose [92], and **iv)** risk of infection [93]. This latter point is additionally supported by evidence that the

endogenous strain is usually that found during invasive infection [91]. Nevertheless, persistent colonization has been associated with lower mortality rates in the event of an invasive infection [94].

Microbial interaction during nose colonization

Once *S. aureus* is transferred and begins to colonize the nose, the bacterium encounters a number of unfavorable factors, from nutrient limitation, to mechanical forces, to the action of the immune system. Moreover, due to its constant stream of airflow, the nose is an organ that is heavily colonized with a number of other microorganisms [95], and we are only starting to understand the complex interplay of *S. aureus* with other members of the nasal microbiome [96]. Antagonistic interactions have been reported for many of these co-colonizers, including *Lactobacillus* [90, 97], *Corynebacterium* sp. [98, 99], *Streptococcus pneumoniae* [100, 101], *Streptococcus mitis* [90] and *S. epidermidis* [99, 102, 103]. Particular focus has been given to competition with *Staphylococcus ludgenensis*, as this bacterium produces a thiazolidine-containing cyclic peptide antibiotic, Lugdunin, which inhibits *S. aureus* growth and could be used as a novel therapeutic [104]. Although information concerning the interaction of *S. aureus* with the nasal microbiota is steadily growing, it is noteworthy that some studies reported partially conflicting results [105] (here in the case of *S. epidermidis* and *Corynebacterium* sp.), which may be attributable to strain- and species-specific differences and highlight the need to further investigate polybacterial interactions in this niche.

Adaptation to the nasal environment

Adhesion to (biotic or abiotic) surfaces is the first and most important step for colonization, and ultimate success, of a pathogen (with the exception of toxin-mediated diseases). Although the colonization and formation of biofilms on abiotic medical devices, e.g. catheters or artificial heart valves, is an immense problem in the hospital setting [55], this section will primarily focus on the attachment to biotic surfaces. Attachment is typically differentiated into **i)** primary attachment through binding to a (host) surface, and **ii)** the formation of a biofilm, which necessitates proliferation and establishment of contact between dividing bacteria (intercellular adherence) [106]. During initial attachment to surfaces, bacterial proteins interact with host extracellular matrix components via both proteinaceous and non-proteinaceous factors. Components that are attached to the cell wall, usually by action of the protein sortase [107], are termed **m**icrobial **s**urface **c**omponents **r**ecognizing **a**dhesive **m**atrix **m**olecules (MSCRAMMs) [108]. In addition, secreted proteins that are not anchored to the cell wall but still affect adhesion (and in some cases immune response) are referred to as **s**ecretable **e**xpanded **r**epertoire **a**dhesive **m**olecules (SERAMs) [109]. Lastly, non-specific interactions have also been suggested to play a role, e.g. in the form of hydrophobic interactions [110]. Numerous host factors are bound by MSCRAMMs and SERAMs, including fibronectin, fibrinogen, fibrin, collagen, elastin, vitronectin, thrombospondin, bone sialoprotein, elastin, collagen, prothrombin, and von Willebrand factor (reviewed by [111]).

Although there are numerous MSCRAMMs and SERAMs, only a few have been shown to significantly affect colonization of the nose [112, 113]. Two MSCRAMMs, clumping factor B (ClfB) [114-117] and iron-regulated surface determinant A (IsdA) [118-120], as well as wall teichoic acids (WTA) [121, 122], have been identified as the main contributors to nasal attachment. Additionally, the MSCRAMMs *S. aureus* surface protein G (SasG) [123] and serine-aspartic acid repeat proteins C and D (SdrC/D) [124] allow binding to human desquamated nasal epithelial cells, while fibronectin binding proteins (FnBPs) can mediate internalization into a number of (non-phagocytic) cell types [125]. Some other factors (summarized by [126]) have also been linked to nose colonization, although to a lesser extent than those listed above.

Several of these adhesion factors are seemingly redundant as they are able to bind to the same cell types. Burian *et al.* [127] shed light on this apparent redundancy with their investigation into temporal expression patterns over 10 days of nose colonization using a cotton rat model. The study revealed that WTA biosynthesis genes are highly expressed during early stages of attachment, while later phase attachment is driven primarily by high expression of *clfB* and *isdA*. This finding is consistent with a study investigating the effects of loss of adhesion factors on the adhesion process [128], in which, as expected, a WTA-deficient strain had a severely impaired ability to colonize rat noses. Inversely, a sortase mutant deficient in MSCRAMM display on the cell surface (ClfB and IsdA, amongst others) was able to undergo early colonization, but was prematurely cleared some time between 6 and 14 days post infection, indicating a

role in colonization persistence, which is also in line with other group's findings [116, 117].

In addition to investigating the expression patterns of adhesion factors, numerous studies (including those referred to above) have sought to understand how cellular physiology is shaped by exposure to the environmental conditions found in the nose, using *in vitro* (employing artificial nasal medium) [129] and *in vivo* transcriptomics [127, 130-132], as well as proteomic [133] approaches. All of these studies have consistently shown that the environment in the nose not only strongly favors attachment but also induces the expression of immune evasion proteins [130, 131] and simultaneous decreases virulence factor and toxin production [129, 130], pointing towards a commensal state for the bacterium. Correspondingly, known regulators of virulence, including *agr* and *sae, sigB, sarR, graR*, have each been reported by one or more studies to be lowly expressed [127, 129-131]. Interestingly, gene expression patterns can differ from host to host, indicating that a host-specific response is present, further complicating the host-pathogen interaction [130, 131]. This notion is made evident by a recent study using a mouse model, which showed that the transcriptional profiles of invading *S. aureus* are strongly dependent on (the level of) the immune response of the host [132]. Such a notion was furthermore supported by the finding that persistent carriers decontaminated for *S. aureus* tend be re-colonized with their previously endogenous strain when exposed to a mixture of strains, ultimately pointing towards the existence of optimal host-pathogen pairings [92].

All of these adaptation processes are guided by complex regulatory networks that determine the balance between commensalism and invasive disease. In this regard, Edwards *et al.* [112] postulate that an absence of evolutionary pressure for virulence expression in the nose is consistent with low expression of the *agr* system, and the presence of *agr*$^-$ genotypes in nasal and blood isolates [134-136]. Nevertheless, virulence factor expression is determinative for *S. aureus* infection, and cues must therefore be present that switch the balance towards virulence, and thus favor invasive behavior, rather than adhesion and asymptomatic colonization.

After entering the body

Although the human skin is a formidable physical barrier with various additional defense mechanisms [137, 138], *S. aureus* is still able to penetrate and cause invasive infection. For a long time, it was believed that a breach in the skin is required for infection, e.g. due to contact in sports (such as football or wrestling) [139, 140], intravenous drug use [141], or shaving and subsequent skin-to-skin contact during sex [142]. Nevertheless, recent studies investigating the invasion of healthy skin showed that *S. aureus* is able to provoke programmed cell death (pyroptosis) and thereby penetrate through the keratinocyte barrier without necessitating previous structural damage [143, 144].

Once inside the body, information about site-specific *in vivo* interactions with the host is less complete, due to the complexity of the system, and the inaccessibility of sites compared to the nose. We (chapter 4 of this dissertation) and others have attempted to perform transcriptomics either under conditions that are thought to mimic the conditions

found within the host [145] or from specimens collected directly from infection sites [146]. Nevertheless, this picture is far from complete, as a multitude of factors influence the behavior of the pathogen when entering the human body. This section thus provides only a brief overview of this complex field of investigation.

A comprehensive review of the adjustment of *S. aureus* to various sites and conditions was recently published by Balasubramanian and colleagues [147]. The authors describe in great detail the response of *S. aureus* to different environments and conditions within the host as well as the bacterial regulatory response, with a focus on the expression of virulence determinants. In general, four main categories of stimuli are delineated, all of which can differ significantly between different organs and organ systems: **i)** oxygen content, **ii)** nutrient (e.g. carbohydrates or amino acids) availability, **iii)** iron availability, and **iv)** organ-specific immune responses:

i) The human body varies widely in local oxygen levels, ranging from high oxygen levels in the blood to nearly anaerobic conditions in the intestines. Since bacteria as well as recruited immune cells consume oxygen, levels have been shown to further decrease at sites of infection ([148, 149] and reviewed by [150]). In the context of hypoxia (a state of oxygen deprivation), osteomyelitis has been studied intensively. Osseous tissue is considered hypoxic [151], particularly during infection [152]. This environment triggers a complex response in *S. aureus* involving multiple regulatory systems (e.g. SrrAB), leading to an increased cytotoxicity towards both murine and human cell types [152]. NreBC [153, 154] and AirSR/YhcSR [155, 156] regulatory networks, too, have been

connected to adaptation to hypoxic conditions, underscoring the complex response of the bacterium to oxygen levels as an environmental stimulus.

ii) Similar to oxygen sensitivity, cells are able to react to fluctuating nutrient availability, including carbon sources and amino acids, which can vary greatly between distinct sites in the human body. Furthermore, the amount of available compounds depends on the individual and any comorbid conditions, e.g. increased glucose in patients suffering from diabetes mellitus triggers an alternative reaction by the pathogen. In line with these findings, high glucose levels following surgery were connected to increased occurrence of infection (not limited to *S. aureus*) in diabetic patients [157]. These patients have a increased risk for pneumonia caused by *S. aureus* and are often affected by infections of foot ulcers [158], leading to further complications and increased mortality [159]. Similarly, poorer outcomes after infection have been reported during animal studies using a diabetic mouse model compared to healthy counterparts [160].

The effects of high glucose levels on *S. aureus* were investigated to elucidate how comorbid conditions affect the pathogen. A connection between glucose availability and disease manifestation was established when mutants deficient in glucose uptake were shown to display attenuated virulence in a murine SSTI model [161]. Likewise, high glucose levels are connected to elevated expression of virulence factors dependent on the regulator CcpA [162]. CcpA is known to be involved in carbon catabolite repression, a common theme in Gram-positive bacteria [163, 164]. Other regulators that connect the physiological state of the cell with the expression of virulence determinants are the

regulators RpiRc [165, 166] and CodY [167, 168]. While RpiRc repression of virulence is induced during metabolic shifts, CodY repression of virulence genes is induced during shortage of branched chain amino acids. These examples serve to highlight the response of *S. aureus* to changing environments, linking metabolism and virulence by connecting nutritional status to the invasive and pathogenic behavior of *S. aureus*.

iii) Another response by the human host to infection is driven by the iron requirement (as well as other transition metals) for all forms of life. Due to its vital importance, host and invading pathogen constantly compete for this valuable resource. This interplay has been extensively researched and reviewed on several occasions [169-171]. Briefly, the host is able to efficiently bind and store iron, creating an extremely iron-deprived environment with extracellular concentrations of free iron at attomolar (10^{-18} M) levels [172]. These levels are actively decreased further in response to infection, leaving bacteria extremely iron-starved, effectively supporting the activity of the immune system in a process described as 'nutritional immunity' [173]. In response to these low iron levels, *S. aureus* has developed sophisticated mechanisms to free iron from the host, including scavenging iron from host glycoproteins (siderophore-mediated), or internalization of host heme and recovery of bound iron. The important survival function of these mechanisms is demonstrated by studies showing that depletion of involved proteins leaves the bacterium significantly impaired in its ability to cause disease in a mouse model [174-176]. As would be expected for an important and limited resource, several regulatory circuits within the bacterium are in place to ensure sufficient iron pools. These are directed by the main regulator Fur, which represses iron-

transport/import in the presence of iron [177] to prevent toxic effects caused by excess iron accumulation. Thus, this regulator, just like CodY, RpiRc and CcpA, connects nutritional status to virulence by repressing virulence determinants in response to iron starvation [178].

iv) Although our understanding of the human immune system has dramatically improved over the last few decades, only limited information is available about organ-specific (innate) immune responses to *S. aureus*. Nonetheless, this is currently a highly active area of investigation whose work will be indispensable to our understanding of *S. aureus* infection (discussed in detail elsewhere [179, 180]). For now, a model has been posited to guide efforts in this direction with emphasis on the influence of the unique interaction that each organ maintains with the environment (i.e. varying exposure to microorganisms). In this model, each organ has a colonization threshold depending on its proximal environment and function (among other factors) ranging from 'sterile' to heavily colonized organs [180]. Therefore, *S. aureus* likely has to exploit various mechanisms to avoid eradication by the immune system with location-dependent specificity, and a consideration for macro- and micronutrient levels.

Regulatory circuits guiding *S. aureus*

S. aureus encodes an exceptionally large arsenal of virulence determinants that aid in its ability to infect various sites in the human body [181-183]. As the expression of these virulence determinants requires precise spatio-temporal control at each colonization site, complex regulatory networks must be in place to secure a precise orchestration of

these factors [184]. These 'classical' regulatory proteins include 115 transcriptional regulators [185] and 16 two-component systems (TCSs) [185, 186]. Furthermore, *S. aureus* encodes four σ factors, including σ^A [187], the house keeping factor; σ^B [188], the primary alternative σ factor; and two additional alternative factors, σ^H [189] and σ^S [190] with more elusive roles.

Due to their importance for *S. aureus* pathogenicity, regulatory proteins have been identified as possible targets for the development of antimicrobials against *S. aureus* [191-194]. Accordingly, these regulatory networks have long been the focus of *S. aureus* research. Initial investigation into these systems was vastly advanced with the discovery of the Agr (accessory gene regulator) system, still widely regarded as the most important regulatory system of the bacterium ([195-197] and reviewed by [198]). The *agr* locus (*agrBDCA*) was found to encode a quorum sensing system [199] that allows the activation of virulence once the population reaches a critical mass [200]. In this system, *agrD* encodes for a propeptide that is processed and secreted by the membrane protein AgrB in concert with the peptidase SpsB [201]. Processing of the propeptide results in the mature autoinducing peptide (AIP), which, once a threshold extracellular concentration is reached, is bound by the receptor AgrC. This sensory protein coordinates with the response regulator AgrA in a TCS. Upon AIP-dependent activation of the system, AgrA induces expression of the *agrBDCA* promoter (P2), initiating a positive feedback loop. In addition, AgrA induces expression from the promoter P3 (downstream and divergent to P2) as well as other target promoters (e.g. controlling PSM genes) [46]. It is thought that AgrA's primary contribution to *S. aureus*

pathogenicity is mediated via the effector transcript RNAIII, which is under control of promoter P3 [47, 202]. RNAIII itself is a 'pleiotropic effector' [203] that not only encodes for δ-hemolysin [204] but also acts as a regulatory RNA either directly, or indirectly through its action on the regulator Rot. For its direct regulatory roles, RNAIII binds its target mRNAs - virulence factors like protein A [205], coagulase [206], SA1000 and SA2353 [207] - to prevent translation and induce degradation of the transcripts (reviewed by [208]). Perhaps the most thoroughly studied RNAIII interaction is with the *rot* transcript, which was first identified by McNamara and colleagues [209] during a transposon screen for compensatory mutations in an *agr*-null strain. The mechanistic significance of this interaction is that RNAIII-mediated translational repression of the *rot* transcript, via binding and degradation, prevents Rot-dependent repression of virulence factors (Rot: **r**epressor **o**f **t**oxins) [207, 210]. This clarified why **i)** an *agr* null strain is severely impaired in virulence factor expression [47] and **ii)** an *agr rot* double mutant displays increased production of these factors when compared to an *agr* single mutant [209, 211]. However, it is also noteworthy that Rot can also act as a positive regulator. Its overall regulon includes 146 genes, of which 60 are negatively and 86 positively regulated [212]. Therefore, the name Rot, though historically justified, is partially misleading in the context of its overall function.

In addition to this well-characterized system, there are myriad other systems involved in the regulation of *S. aureus* virulence. The Rot protein, for example, belongs to the family of SarA proteins, which encompasses at least eleven members in *S. aureus* and was defined based on homology of the proteins to the *S. aureus* staphylococcal accessory

regulator A (SarA) regulator [213, 214]. The SarA protein was first described by Cheung and colleagues [215] in connection to *agr* [216]. Since these early investigations, this regulator has been the topic of extensive research (reviewed by [203]) describing both, the direct and indirect, effects of the protein. SarA has been shown to **i)** induce expression of genes controlled by promoters P2 and P3, thereby feeding into the action of the Agr system [217-219]; **ii)** facilitate the binding of AgrA to P2 and P3 [220]; and **iii)** directly bind to sites upstream of its target genes to induce their expression [219, 221, 222]. Each of these actions directly affects the expression of virulence determinants, establishing SarA as one of the master regulators of *S. aureus* pathogenicity [213]. The control of *sarA* itself is multifactorial (reviewed by [203]), but peak expression has been reported during later growth phases [223] and thus approximately coincides with Agr-dependent activation of virulence determinants. Additional post-translational regulatory mechanisms have also been proposed [224, 225], adding further nuance to this already complex system.

Another protein with homology to SarA is MgrA (formerly referred to as Rat, [226]), which has a tripartite effect on virulence factor expression, by either **i)** direct interaction with Agr, **ii)** interaction with the transcription factor SarS or **iii)** direct induction of its target transcripts [227]. In these ways, the regulator positively and negatively controls a large regulon, which, amongst others, includes numerous virulence factors and surface proteins [228]. Other systems in turn control MgrA, e.g. the TCS ArlRS [229] as well as the small regulatory RNA RsaA [230]. RsaA is of particular interest, as (translational) regulation by RNA-RNA interaction has been found to be a ubiquitous mechanism of

gene regulation (reviewed by [208, 231]), as discussed in chapter 4 of this dissertation. RsaA thus interacts with the *mgrA* transcript and represses translation of the transcript [230]. Notably, RsaA itself is controlled by the alternative σ factor σ^B [230, 232], which has been previously shown to modulate virulence of *S. aureus* [233-237]. This creates a rather interesting situation where an alternative σ factor (σ^B) controls a regulatory RNA (RsaA), which in turn controls a transcription factor (MgrA), that interacts with the major regulatory system (AgrBDCA), that activates another regulatory transcript (RNAIII), which suppresses the (negative) regulator (Rot), allowing the expression of proteins that promote invasive behavior and pathogenicity of *S. aureus*. Furthermore, even this presentation belies the true complexity of these systems: at each junction, secondary (direct or indirect) effects on other genes or regulators are also present, producing an even more involved regulatory network. Several other transcription factors (e.g. other members of the Sar family [203]), two-component systems (e.g. SaeRS [238-240]) and regulatory RNAs (e.g. SSR42 [241], SprD [242] and SprC [243]) have been found to influence pathogenic behavior, but will be not discussed in further detail here.

The intricacy, redundancy, and interconnectivity of these regulatory networks are no mere coincidence but nothing less than an absolute requisite for survival. The delicate modulation of energy-consuming virulence factor production under different micro- and macronutrient conditions is exemplary of the economic allocation of energy resources in this bacterium. Therefore, all indications support the case that no encoded regulatory factor presents an unnecessary burden, but that every convergence and divergence, every layer of regulation, and every additional regulator integrated in the network that

guides *S. aureus* lifestyle serves an integral purpose for fine-tuning the conditions that allow the bacterium to successfully colonize or invade its host.

S. aureus plays its hand

The environments where *S. aureus* must survive differ significantly on every possible level, from an undisturbed colony in the human nose to the peril of engulfment by a macrophage. Moreover, the organism must account for host-dependent, large-scale variations (e.g. the immune response of carriers vs. non-carriers) as well as specific interactions with other microorganisms in the polymicrobial communities where it resides. In simpler terms, the living conditions of *S. aureus* are exceedingly complex; the bacterium's ability to quickly adjust to new conditions is thus the cornerstone of its success as a pathogen. These changes are governed by elaborate regulatory networks that dictate whether to hide or attack, to lie dormant or proliferate, to save or expend energy. This complexity may well be the explanation for the failure of research across several decades to find 'the holy grail' of *S. aureus* research, the single master regulator that controls virulence; it is likely there is no such regulator, as the intricacy of environmental cues and stimuli cannot be accommodated by one major switch, but rather a finely adjusted system of interconnected relays. The shortcomings of this pursuit are perhaps best exemplified in the fact that the most significant regulator of virulence, the Agr quorum sensing system in concert with RNAIII, is inactive in numerous clinical isolates, not only during invasive infection, but also prior to colonizing the nose [134-136]. Therefore, it is imperative to consider the regulatory networks in their entirety - every switch and gear that they encompass - for minor changes in these

systems can propagate to major alterations in the final outcome (i.e. severe bacterial infection). To do so, we must take into account not only 'classical' transcription factors (TFs), two-component systems (TCSs) and σ factors, but other proteins and molecules that have historically been underappreciated in terms of their regulatory capabilities. These would include, for example, both regulatory small RNAs (sRNAs) and active components of the transcription machinery, and will be referred to as 'non-classical' regulators. Both groups of alternative regulators will be discussed in this dissertation. Specifically, the research described here aims to **i)** understand how under-investigated components of the transcription machinery can have both global as well as gene-specific effects on the transcription process (chapter 2 and 3), and **ii)** identify and catalogue undiscovered regulatory elements (sRNAs) in *S. aureus* (chapter 4). This latter approach is of particular interest, as horizontal gene transfer and strain-specific differences have been shown to play an integral role in the evolution of this bacterium.

It is imperative to understand the precise role of these regulatory systems in the adjustment of *S. aureus* to ever-changing environments, be it in a battle with the immune system or other bacteria in its natural niche, in a quest for nutrients during infection, or during exposure to antibiotics in the hospital. When performed in meaningful backgrounds (e.g. the USA300), research directed to fill in the blanks of our existing regulatory maps will produce invaluable information about the adaptive mechanisms of *S. aureus*, an organism that must be constantly 'running to stand still' [112] in order to secure its success as a pathogen.

SMALL THINGS CONSIDERED: THE SMALL ACCESSORY SUBUNITS OF RNA POLYMERASE IN GRAM-POSITIVE BACTERIA

Note to reader

This chapter was previously published as a manuscript [244], and has been included with permission from the publisher (Appendix I).

CHAPTER 2: THE δ SUBUNIT OF RNA POLYMERASE GUIDES PROMOTER SELECTIVITY AND VIRULENCE IN *STAPHYLOCOCCUS AUREUS*

NOTE TO READER

This chapter was previously published as a manuscript [245], and has been included with permission from the publisher (Appendix II).

CHAPTER 3: THE ω SUBUNIT GOVERNS RNA POLYMERASE STABILITY AND TRANSCRIPTIONAL SPECIFICITY IN *STAPHYLOCOCCUS AUREUS*

NOTE TO READER

This chapter was previously published as a manuscript [246], and has been included with permission from the publisher (Appendix III).

CHAPTER 4: GENOME-WIDE ANNOTATION, IDENTIFICATION, AND GLOBAL TRANSCRIPTOMIC ANALYSIS OF REGULATORY OR SMALL RNA GENE EXPRESSION IN *STAPHYLOCOCCUS AUREUS*

NOTE TO READER

This chapter was previously published as a manuscript [247], and has been included with permission from the publisher (Appendix IV).

CHAPTER 5: CONCLUDING REMARKS AND FUTURE DIRECTIONS

CHECKS…

The variation and hostility of the environments where *S. aureus* resides have demanded an unparalleled versatility to secure bacterial survival. This adaptation involves the ability to quickly adjust physiological processes via differential expression of regulatory components governing these pathways. As discussed in this dissertation, a multitude of individual regulatory elements (including the ones identified in chapters 2-4) guide alterations in gene expression patterns. Nevertheless, our knowledge of regulators individually must be complemented by an understanding of these factors in their native context within global regulatory networks. Only this will allow us to ultimately develop an understanding of how *S. aureus* (and bacteria in general) makes complex decisions in an ever-changing environment [248].

Evidence suggests that at least some aspects of *S. aureus* gene expression follow temporal patterns, perhaps driven by proliferation and subsequent increase in bacterial culture density over time. The importance of these factors is reinforced by the central role of the Agr quorum sensing system in the lifestyle of *S. aureus* (as discussed in chapter 1). With regards to temporally resolved expression patterns, it has been repeatedly demonstrated that different adhesins are expressed during the consecutive

stages of nasal colonization [116, 117, 128]. Similarly, temporal patterns have long been known to be present during the 'normal' bacterial growth curve under laboratory conditions, wherein gene expression is regulated depending on culture density, amongst other factors (as discussed by us recently [249]). Nevertheless, growth in a laboratory setting presents a rather crude representation of the situation *in vivo*, since (external) stimuli like nutrient alterations/limitations, sudden exposure to antibacterial host molecules, and/or onset of an immune response, are entirely absent (although nutrient and/or oxygen deprivation are present at later phases of growth). Furthermore, bacteria in the nose fail to reach densities found in laboratory culture (though permanent carriers have been connected to higher bacterial loads [92]), therefore limiting the potential influence of quorum sensing signals under these conditions. In line with these findings, Burian and colleagues [127, 130] showed that the *agr* locus (as well as *saeRS*) is transcriptionally inactive during nasal colonization, while the essential *walKR* TCS is highly transcribed, supporting the conclusion that the latter controls nasal adhesion. Nevertheless, the precise stimulus for activation of the WalKR system is not known, and it is therefore unresolved which signals guide temporal expression patterns during nasal colonization. Such a shortcoming in understanding the adaptation to an important and, at the same time, easily accessible, niche highlights the need to further investigate the decision making process in *S. aureus*.

Regardless of these obvious limitations, the scientific community has made significant advancements in unraveling the function as well as the signals governing numerous regulators in *S. aureus*. Despite this progress, most studies that aim to understand a

particular regulatory factor are restricted to only characterizing specific in- and outputs, e.g. CodY (indirectly) senses the absence of branched chain amino acids and in turn reduces expression of genes involved in central metabolism as well as virulence factor production [167, 168, 250]. However, as indicated previously, no circumstance exists in which only one input determines the cell's fate. Rather, multiple signals are sensed at any given time and the receiving factors/pathways intersect and interact synergistically or antagonistically in turn. Therefore, bacterial gene regulation cannot be framed as a linear series of events but rather as a three-dimensional network with a multitude of nodes and switches that integrate these inputs to fine-tune major processes of the cell, e.g. the Agr system's role as a 'nexus' [25] for virulence.

Seshasayee and colleagues eloquently discuss these regulatory networks on the basis of results from the model organism *Escherichia coli* [248], though their conclusions are nonetheless valid for other bacteria, including *S. aureus*. In order to model how various signals influence cellular behavior, three primary classes of transcription factors are outlined: **i) Exogenous regulators** are often TCSs, which consist of two distinct functional units: a membrane-bound sensor (histidine kinase) that responds to an external stimulus by transmitting a signal to an intracellular TF, which then affects the transcription of the system's target genes [251]. External stimuli can also be sensed when extracellular molecules are imported and bound by regulatory enzymes within the cell. A primary example of this type of TF is the Fur regulator, which reacts to cellular levels of the essential transition metal iron in *S. aureus* and other (pathogenic) bacteria [252]. Another system able to sense external signals is the TCS SaeRS (described

above) that adjusts virulence factor expression in response to salt stress, low pH, subinhibitory clindamycin concentrations and exposure to proteins/peptides produced by human neutrophils [253-255]. In contrast to exogenous TFs that connect cellular physiology to the environment, **ii) Endogenous TFs** only bind molecules within the cell. These are commonly key molecules of major metabolic pathways that indicate the general energy and physiological state of the cell. **iii)** The last category, **hybrid regulators**, can sense signals (e.g. amino acids) that can be of exo- or endogenous in origin. This bifunctionality of such regulators prevents a definitive assignment to one of the two previous groups.

Information about the abundance of each class of regulators can give general information about the extent of interaction of a bacterium with its environment. In *E. coli*, it was shown that ~24% of the 120 regulators with known targets are endogenous, 48.5% exogenous and 27.5% hybrid, meaning that ~76 % have the ability to respond to external stimuli [256]. Although only speculative at this point, a similar relationship could also be described for *S. aureus*, supported by the comparable numbers of TCSs and TFs between *S. aureus* and *E. coli* (TCSs: 16 vs. 28, TFs: 115 vs. 120) [185, 186, 248, 257]. These findings corroborate the extensive interplay observed between *S. aureus* and its immediate surroundings. Here, the bacterium must be able to integrate external and internal signals that provide information about the activity (e.g. antibacterial) of the host and the cell's own physiological status, respectively. These inputs have to be simultaneously processed so that the subsequent cellular responses can be

orchestrated in a coordinated and logical fashion, securing survival of the single cell as well as the population.

Although significant advances have been made in our understanding of regulatory networks in general (with *E. coli* as the primary model) and TFs in *S. aureus* specifically, our knowledge of the minutiae involved in global regulation is still rudimentary. This is due to a variety of shortcomings, though a defining bottleneck is the identification and characterization of large numbers of yet undescribed or uncharacterized regulators. As discussed, regulatory active components of RNAP as well as regulatory RNAs are prime examples of this shortcoming, which we attempted to resolve in this dissertation.

…AND BALANCES

In our effort to understand how and when a bacterium infects its host, we are inevitably confronted with the questions ‘What defines virulence?’ and ‘Which bacterial properties unlock its pathogenicity?’. Historically, we have characterized a bacterium’s pathogenic capability as the sum of its encoded proteins that injure the host or avoid its immune responses. Only recently has the scientific community started to appreciate that virulence is not solely defined by encoded virulence determinants, but that virulence is largely dependent on the susceptibility of the host and the nature of general host-pathogen interactions [258]. Opportunistic pathogens like *Acinetobacter baumannii* or *S. epidermidis* exemplify the advantage of this perspective. While *A. baumannii* is responsible for devastating infections [259, 260], it is only sparsely equipped with

pathogenic factors, and it has even been proposed that the bacterium does not encode a single *bona fide* virulence factor [261]. Similarly, *S. epidermidis* has been referred to as an 'accidental' pathogen [262], as it is ubiquitously found as a commensal on the human skin and lacks the vast majority of virulence factors found in related pathogens (e.g. *S. aureus*) [263, 264]. Nevertheless, this commensalism can quickly escalate to life-threatening invasive infections (e.g. blood stream infections in ICUs), particularly in connection to indwelling implants [265, 266]. These examples highlight how largely benign bacteria can, under certain conditions, cause disease and emphasize that a bacteria-centric view, focusing only on encoded virulence markers, is indeed too simplistic. Instead, the complex interplay at the host-pathogen interface must be considered to understand a bacterium's pathogenic potential.

For *S. aureus*, several lines of research have shown that the interaction of host and bacterium is particularly host-specific. A prime example is the process of nasal colonization, where a large number of studies identified, amongst other aspects, genetic predisposition and gender to be risk factors for *S. aureus* colonization (comprehensively reviewed in [267]). We now appreciate that the intricate relationship of *S. aureus* with its human host is a result of co-evolution [268-270]. Both the host immune system and bacterial virulence have evolved to survive at their interface in what has been described as an 'arms race' by Dawkins and Krebs in 1979 [271]. Although a plethora of battlegrounds for pathogen and host have been described, perhaps the best characterized is the fight for iron (chapter 1). Here, the host has adapted to sequester most available iron and even reduce iron levels upon recognition of an infection [172,

173]. In response, *S. aureus* has developed sophisticated means to recover these hidden treasures by ‘cheating, thievery, and piracy’ [170, 272]. Another important example is the fine balance between commensalism and infection at the skin barrier, where the (innate) immune system and bacterial factors (e.g. ACME-encoded SpeG [52]) are engaged in a constant, counteractive effort [273]. Although this balance has developed over millions of years, the equilibrium is frequently disturbed. An example of this relationship is the start of the antibiotic era when the host prevailed and, for a very brief window in the 60’s/70’s, we had the hubris to believe that we would be able to eradicate bacterial infections altogether [274]. In response to the ‘many millions of metric tons of antibiotic compounds… [that] have been released into the biosphere’ [275] since the beginning of the antibiotic era, bacteria have rapidly developed resistance mechanisms to circumvent their negative actions [275]. Through these antibiotic resistance patterns, in combination with the spread of newly emerging CA-MRSA strains in the healthy population, the bacterium has once again gained significant momentum. As these examples show, the host-pathogen balance is fragile, and small changes, e.g. host-specific differences or evolutionary adaptation of the pathogen, can have a strong influence on the overall outcome of any given interaction.

The delicacy of this interplay is now appreciated, and antimicrobial strategies, rather than attempting to eradicate the pathogen, aim to disarm the organism or boost host defense to increase the immune system’s chances of gaining the upper hand [276]. Historically, bacteriostatic antibiotics have followed this model, in which bacteria are hindered in their plan of action and the eradication itself is driven by the immune system

(reviewed by [277]). In this vein, interference with the host-pathogen balance can be far subtler than our current approaches and still move the needle in favor of the host, in turn promoting clearance of the infection. To date, significant process has been made in this area, and numerous immunomodulatory candidate molecules have been identified [278-285].

During the 'arms race' between bacteria and host, *S. aureus* strongly relies on virulence factors that it utilizes in hiding from the immune system (e.g. various surface factors of *S. aureus* [286]) and/or interference with the bodies defense mechanisms (e.g. cytolytic toxins [287]). However, virulence factors expression is thought to be energetically costly [288]; similar to antibiotic resistance expression (e.g. from larger SCC*mec* elements in HA-MRSA strains [21]), this expression program is considered a fitness burden that forces the bacterium to balance toxicity and energy homeostasis [289]. Ultimately, the cell spends only **i)** as much as it can afford and **ii)** as much as is necessary [132]. In terms of pathogenic organisms, this temporal thriftiness is closely related to the idea that a given (virulence) gene can be beneficial under certain conditions, e.g. during invasive disease, while being disadvantageous during others, e.g. colonization of the host (a concept referred to as 'antagonistic pleiotropy' [290]). Bliven and Maurelli [269] have thus speculated that pathogens circumvent this dilemma by 'evolv[ing] mechanisms to neutralize the deleterious effects arising from antagonistic pleiotropy, while at the same time conserving the beneficial ones'. One way, for example, to prevent the deleterious effects while maintaining the advantages is to use regulatory systems that only use genes under certain conditions.

In order to understand the interplay between host and pathogen and, more importantly, to intervene with infection in a targeted manner, we must investigate those factors that guide bacterial (virulence factor) gene expression. Bacteria not only gain new weapons during their 'arms race' with the body, but also learn how to use them in the most efficient way without disturbing the (energy) homeostasis of the cell. In the context of *S. aureus*, it is necessary to elucidate how and why virulence is induced and how certain genes (not only regulators, but effectors as well) influence the host-pathogen balance. Once we have a better understanding of the regulatory processes and the signals that influence the interaction between host and bacterium, we can utilize this information to revise and extend treatment therapies.

THE IMPORTANCE OF NON-CLASSICAL REGULATORS

As discussed, it is of utmost importance to characterize regulatory circuits in their entirety in order to understand and, in turn, predict bacterial pathophysiology. Although significant progress has been made in the field of *S. aureus* gene regulation, our picture is far from complete. These shortcomings are particularly salient in our current understanding of 'non-classical' gene regulators.

For 'classical' proteinaceous regulatory molecules, including TFs, TCSs and σ factors, an abundance of information is available, facilitating the identification [185] and prediction of their function based on protein domain conservation [291] and homology to proteins in other bacteria, e.g. ones deposited to the Protein Data Bank [292]. Similarly, putative targets of TFs, TCSs or σ factors can be identified based on the known

consensus sequences for their binding sites on promoter regions (e.g. Fur box [293] or the consensus sequence for the alternative σ factor σ^B [294]). For other regulatory molecules, like small regulatory RNAs or proteins that exert their regulatory behavior via alternative mechanisms (e.g. small accessory RNAP subunits), such endeavors are significantly more challenging. Ultimately, the current limitations in the integration of 'non-classical' regulators into existing networks can be attributed to: **i)** not yet having identified all such elements, **ii)** a lack of information about the signals that trigger a specific response in these regulators, and **iii)** an unavailability of experimental data identifying possible transcriptional targets of these regulators. In the following, we will further discuss each of these challenges and the contribution of this dissertation in overcoming them:

i) RNAP is one of the best-studied enzyme complexes due to its central role in organismal function. Consequently, it has long been known that RNAP contains several small subunits in addition to its four main subunits $\alpha_2\beta\beta'$, and closely associated σ factors [295-298] (though the presence of these subunits can differ between Gram-positives and Gram-negatives [244]). Within the complex, several proteins have already been assigned regulatory roles. It is generally appreciated that the α subunits are able to make contact to DNA-sequences upstream of a promoter or bind additional regulatory factors, while σ factors guide transcription machinery by recognizing specific promoter sequences. In contrast, other subunits, i.e. δ, have been less appreciated for their regulatory properties: while δ was initially studied in the context of phage-infection [296], it was not until several decades later that the protein was established as a

permanent RNAP component [299], which plays a role in the maintenance of stringency in the transcriptional process (demonstrated in a number of studies and summarized by us [244]). Although this general regulatory role was known, no gene-specific effects had been assigned until recently, leading to its exclusion from 'classical' regulatory networks. Through the research described in this dissertation, we have extended the existing knowledge base and suggested potential selective effects for δ. Our data show that abrogation of the subunit's activity results in broadly altered virulence factor expression. As we detected no large-scale alterations in expression of known *S. aureus* virulence regulators, it is likely that δ interacts directly with its targets. Though precise mechanisms for this phenomenon will need to be elucidated, these findings, together with various observations from others ([300-303] and discussed by us [244]), imply a potentially important role for this subunit within the regulatory circuits of *S. aureus* and other bacteria. This is particular intriguing as δ resides 'at the core' of the transcriptional process, pointing towards a very basic role in regulating intrinsically important processes, e.g. as described for σ factors.

With respect to sRNAs, these molecules have been known to function as global regulators of bacterial physiology [304], and have already been demonstrated to play central roles in controlling virulence [305-308], stress response [309] and metabolism [310]. They exert their roles by acting as important sensors, connectors or effectors (transcriptional, post-transcriptional and at the protein level) in the regulatory network of the cell, and it is thus crucial to include these elements in our future regulatory roadmaps [311]. Unfortunately, the available information about encoded sRNAs was

rudimentary for a long time, mainly due to technical limitations (to be discussed further). Nevertheless, driven by the establishment of next generation sequencing (NGS) as a standard lab technology, this picture is changing quickly [312, 313]. As a result of this extensive use of NGS, the number of sequenced genomes in publicly available databases steadily increases: at the time of this dissertation, >13k complete bacterial genome sequences were available in the NCBI Microbial Genomes database (https://www.ncbi.nlm.nih.gov/genome/). In order to transfer these raw sequences into a usable format, adequate tools for reliable data processing and genome annotation must be developed alongside these advances. Although automated annotation has significantly improved [314], the sheer amount of available data still 'makes bioinformatics the bottleneck' [315]. While prediction software can not only identify the presence of protein-coding sequences but also certain conserved noncoding RNA (ncRNA) species (i.e. tRNA and rRNA), the identification of genes (e.g. sRNAs) that are not widely conserved, missing ribosomal binding sites, and/or with unclear open reading frames commonly presents itself as a hurdle. Despite these challenges, automated sRNA prediction is now a valuable tool to supplement proteinaceous as well as tRNA and rRNA annotations [316-318]. As with all computational approaches, these predictions must be complemented by experimental data to prevent erroneous identification and allow for the confident integration of sRNAs into existing genome files (and subsequently into regulatory networks). Likewise, a unified nomenclature system for identified transcripts should be implemented, as discrepancies can lead to the redundant identification of sRNAs.

In the study described in chapter 4, we addressed each of these problems. For the first time, we introduced an annotation system for *S. aureus* that supports the classification and organization of all previously identified sRNAs. This was used as a platform for the identification of 39 as yet undescribed transcripts, increasing the combined number of currently known sRNAs in the USA300 background to 303 transcripts (~10% of all encoded genes). This large number highlights the importance of this relatively unexplored group of regulatory factors in *S. aureus*: while the bacterium encodes for 135 known proteinaceous regulatory factors (TFs, TCSs and σ factors combined), there are at least twice as many sRNAs transcribed. Our optimized RNAseq [319] and sRNA identification/annotation procedure can now be used as a blueprint for future studies in other organisms. Consequently, we performed similar studies for three additional organisms, *S. epidermidis, S. carnosus* [320] and *A. baumannii* [321]. The latter bacterium was investigated due to its ability to cause devastating infections, including ventilator-associated pneumonia, blood stream infections or SSTIs [322]. In our study of this organism, we identified 78 uncharacterized sRNA transcripts [321], including a group of conserved and highly expressed phage-derived sRNAs, as well as conservation patterns amongst these sRNAs that suggest a modular arrangement similar to protein domain architecture.

In order to further demonstrate the utility of our sRNA identification and annotation approach, we employed our genome annotation files, now including currently known sRNAs, to study the evolutionary implications of these transcripts [320]. Here, we cross-compared the sRNA content between a pathogenic bacterium (e.g. *S. aureus*),

opportunistic pathogen (e.g. *S. epidermidis*) and non-pathogenic organism (e.g. *S. carnosus*) [320]. In so doing, we were able to gain insight into orthologous elements that pointed to both a set of core sRNAs, which are found in a wide number of strains, as well as elements only present in virulent lineages, i.e. those that possibly contribute to pathogenicity.

Though the identification and classification of these sRNAs is an important first step toward a more complete understanding of bacterial gene regulation, the role of these data is first and foremost the production of a solid foundation upon which further experimental efforts can investigate the precise roles of these transcripts in the cell. Indeed, these experiments have only further expanded the horizon of this field of research, providing us with a greater appreciation for the magnitude of the task ahead.

ii) After initial identification and cataloguing, the next logical next step is exploration of the precise conditions and signals that trigger a response by these elements. Unfortunately, these factors are not yet known for many (if not most) sRNAs; largely as a result of their absence in genome annotation files, and their consequent omission from many high throughput transcriptomic analyses. Our unfamiliarity with such signals is not only a problem for 'non-classical' regulators but also for known proteinaceous elements, as discussed earlier in this dissertation. Only when we understand which inputs govern and alter specific cellular processes can we create maps of networks and predict the outcomes of host-pathogen interplay, which in turn will lead to more successful attempts to interfere with the pathogen's plan of action. As our ultimate goal

is to identify the contribution of these *S. aureus* transcripts to its regulatory network (and ultimately to its ability to cause disease), we need to decipher which of the transcripts are active (or inactive) under conditions that mirror those found in the host. Herein we extend current knowledge by performing RNAseq in human serum (chapter 4), as well as during adaptation to stationary phase growth [249]. Serum was chosen for examination because blood stream infections present an increasing problem [56-60], and serum contains (natural) antibodies [323], antimicrobial peptides [324, 325], and low iron levels [326], presenting an appropriate model for conditions and challenges that the bacterium might encounter during host invasion. After initial examination, we recorded decreased expression of RNAIII in line with previous studies [327, 328], therefore validating our approach. In addition to RNAIII, 83 other sRNAs (~27% of the total sRNA content) displayed differential expression in serum vs. TSB, with the two most strongly affected transcripts showing a ~600-fold and ~1200-fold increase and decrease in abundance, respectively. The decrease of *agr*/RNAIII expression itself could be a result of AIP sequestration by the serum component apolipoprotein B [325], or via a more active mechanism, as high virulence factor expression could be a disadvantage during growth in serum [288]. Independent of the ultimate cause of altered RNAIII expression, it is interesting to speculate as to whether the other differentially expressed sRNAs are controlled in an *agr*-dependent manner, influenced by other serum factors or react to the cell's overall physiological state. Regardless of the nature of the stimulus, these transcripts are certainly highly responsive to the environments they encounter in the human body. Further work should aim to deconvolute which

stimuli in human serum (i.e. as a system radically different from TSB) trigger the activation of these transcripts and which other regulators mediate this response.

For any given sRNAs, the change observed in its expression pattern under certain environmental conditions/stimuli is a good indicator for its role within the cell's regulatory networks. The role of proteinaceous factors, too, can often be evaluated for activity and specificity of their response in this way (e.g. Agr system, σ^B). However, expression-activity relationships are not always so straightforward; TCSs, for example, are often constitutively expressed (although additional autoinduction can be present) [329]. Contrary to sRNAs and in line with TCS expression, we were able to show a relative, stable and robust expression of small RNA polymerase subunits δ and ω throughout the growth of *S. aureus* (chapter 2 and 3, as well as [249]). This finding is surprising given the gene-specific regulatory roles of the δ subunit, and the likely non-constitutive expression of most of its target genes (e.g. virulence determinants). We thus expect alternative mechanisms to mediate activation/deactivation similarly to TCSs. Our studies into the ω subunit of RNA polymerase (chapter 3) offered the first glimpse of such an alternative control system for the δ subunit. The function of ω was found to be mainly of a structural nature and deletion of the subunit induced structural stress within the RNAP complex accompanied by release of δ. Since δ can only perform its function when bound to the core RNAP subunits, this release would be an effective way to regulate activity of δ itself. Although this hypothesis is rather novel and will require further evaluation, this would indicate an additional layer of transcriptional regulation, comparable to a previously described change in the affinity of alternative σ factors to ω-

less RNAP in *E. coli* [330] and cyanobacteria [331]. In this type of regulatory setup, no specific biochemical signals would be required; rather, it would allow the cell to respond to structural damage of the RNAP complex, e.g. due to heat stress of other physical signals (in the context of chapter 3, due to depletion of ω). This mode of activation would conceptually resemble that of RNA thermometers, in which temperature-sensitive RNA structures, e.g. in the 5' untranslated region, control mRNA translation [332]. For both RNAP subunits as well as RNA thermometers, the 'signal' would therefore cause structural changes, which in turn alter regulator activity and transcriptional outcomes.

In summary, our studies have identified conditions of high/low activity of alternative regulators of interest, and therefore present an important first step towards the integration of sRNAs and small RNAP subunits into 'classical' regulatory networks.

iii) The last of the initial steps towards the incorporation of newly identified regulatory elements into established networks is the characterization of their physiological effects.

In the context of small RNA polymerase subunits, in particular δ, our transcriptomics approaches showed not only a general loss of stringency in the transcriptional process, but also a decreased expression of various virulence determinants, which was additionally confirmed by extensive phenotyping. This strong shift in expression of one particular set of genes in the absence of any notable changes in expression of typical regulators of *S. aureus* virulence implies that δ may in fact act as a gene-specific regulator itself. If so, the subunit must be able to distinguish between genes while bound

to RNAP via either specific DNA-sequences and/or DNA-modifications. Each of these has been previously shown to be recognized by proteins carrying a specific (HARE) helix-turn-helix (hth) domain [300] that we found to be highly conserved amongst δ proteins across various Firmicutes [244]. During our investigation of possible δ-specific promoter features, we were able to identify differences in abundance of the presence of a consensus [HAATTWD] motif in promoter regions of putative δ-regulated genes (unpublished observation). This sequence was found in the upstream regions of 76.5% of genes that showed decreased expression in the δ-less strain, while only 24.5% of the upregulated genes in this strain carried the motif in their extended promoter region. These data are further supported by Prajapati *et al*, [303], who described that replacement of certain AA repeats (as modeled in the consensus) by CC in AT-rich regions diminished the positive transcriptional effects of δ on a model promoter. Although somewhat preliminary at this point, these data combined suggest that the selectivity of δ for its target genes is driven by the recognition of specific AT-rich promoter features by a conserved hth domain of the subunit.

In contrast to target identification for RNAP subunits, where the search is limited to particular DNA-sequences or modification, the pinpointing of sRNA targets is decidedly more challenging. Accordingly, of the 303 sRNAs in *S. aureus* USA300, less than 10% have been functionally characterized (often lacking the exact mechanism of action). Although reverse genetic screens have been useful in the past in the identification of the precise function of genes in *S. aureus* [333], this strategy proves to be recalcitrant for sRNA study. The non-coding RNA SSR42, a strong effector of virulence in *S. aureus*

[241], exemplifies these difficulties. This sRNA has been shown to be required for cytolytic activity, immune evasion and pathogenesis during infection of a murine SSTI model. Though the cell's transcriptome is dramatically altered in a ΔSSR42 strain, no specific cellular targets could be identified [241]. This case is particularly notable, as loss of the sRNA results in clear phenotype, affecting one of the best-studied aspects (virulence factor production) of *S. aureus*. This author, too, has spent significant energy on identifying a target for SSR42 interaction (data not shown) to no avail. These examples are not meant to belittle such efforts, but rather indicate how easy it can be to miss even the biggest target when the system is as challenging as for sRNAs.

Overall, our studies serve as a model for the three steps required toward integration of 'non-classical' regulators into the 'classical' regulatory landscape of *S. aureus*: i) We were able to describe the potentially gene-specific activity of a resident component (δ) of RNAP. In addition, we identified 39 unknown sRNAs in the *S. aureus* USA300 background and made information about previously known sRNAs more accessible for further investigations by creating the first publicly available genome files to contain sRNA annotations. ii) These files were used to catalogue differential sRNA expression under pathophysiologically relevant conditions (human serum), thereby establishing relevance of these transcripts to the infection process. Furthermore, we reported the release of δ from the structurally disturbed transcription machinery, explaining how this regulatory active component of the RNAP complex could be controlled itself. iii) With regard to target identification, evaluation of the diminished virulence factor production in

δ-depleted strains led to the identification of putative targets and DNA features that may mediate target recognition by this subunit.

In the future, each of these elements will need to be fully integrated into our existing regulatory maps of the cell. Before then, there is significant amount of work ahead of us. However, with the first steps behind us, further studies can evaluate the cross-talk of these 'non-classical' regulators with known (and yet to be identified) elements and sub-networks. Such an endeavor will be a herculean task for the coming decades, but is nonetheless paramount in the construction of a holistic view of the cell's complex machinery that will pave the way for more effective development of antimicrobial treatments.

FUTURE DIRECTIONS

The experiments in this dissertation have advanced our knowledge about regulation in *S. aureus*, and we hope that our contributions will bolster future efforts to combat this pathogen that has for too long plagued patients across the globe. Nevertheless, our studies have to be seen as just a starting point, and follow-up experiments are required to fully elucidate the role of the investigated regulatory elements in *S. aureus* disease causation. Experiments that could supplement this knowledge base are described in the following two sections, with regard to either **i)** small RNAP subunits or **ii)** sRNA-related projects.

i) In the studies described in this dissertation, we were able to identify a role of δ in gene-specific regulation of *S. aureus* and its effect on the production of numerous virulence determinants. As this activity implies regulatory control by δ itself, we propose that the physical state of the RNAP complex and δ release upon structural disturbance may govern the activity of this subunit. Further characterization of promoter features that result in δ subunit dependency will be required to adequately describe its effect on target virulence factors. In order to characterize these features, DNA-sequences or modifications of directly δ-dependent promoters need to be identified. In such circumstances, where limited information is available about specific targets, chromatin immunoprecipitation sequencing (ChIPseq) is often the method of choice. The method was first introduced in 2007 [334] and is widely used today [335-337] in both prokaryotic and eukaryotic studies. Briefly, ChIPseq is based on the use of tagged DNA-binding proteins, which, after cross-linking, are enriched upon precipitation along with their bound DNA-fragments. Sequencing of these fragments then provides information on the target sequences of the regulator in question. Such an approach has previously been successfully employed to study genomic RNAP distribution in *E. coli* [338]. In the context of δ, a similar study could be conducted in the presence and absence of the subunit, with precipitated promoters then correlated with existing RNAseq data to generate a list of elements that are specific δ targets.

Ultimately, target identification for regulatory elements must be accompanied by information concerning the conditions that prompt this regulation. Our experiments suggested that, under certain conditions, i.e. due to loss of ω, δ can be released from

the RNAP, abolishing its influence on transcription. Nevertheless, it is unclear as to whether this “stress-model” is an artifact of the depletion of ω or if the release of ω (and subsequently δ) actually occurs *in vivo*. This uncertainty highlights the future need to study RNAP complex composition under different stressors to establish an improved structure-function relationship. In the end, only this information can contextualize our data to relevancy in the sophisticated regulatory machinery of *S. aureus*.

ii) As discussed earlier, expression and activity of sRNAs, as with bacterial regulators in general, has to be investigated under meaningful conditions, preferably during infection. Three studies which are prime examples of such *in vivo* systems include RNAseq experiments with RNA isolated from immunodeficient versus healthy mice during systemic infection [132], from a murine model of osteomyelitis [339] as well as from human nasal *S. aureus* carriers [131]. Studies like these have the potential to greatly advance the field and the publicly available datasets should be thoroughly investigated for sRNA activity. Although all three studies are pushing the boundaries of our field, they are limited to either easily accessible niches (e.g. nose) or cohorts of high *S. aureus* abundance during infection (e.g. kidney). In comparison, one important niche stands out as unexplored territory: though heavily guarded by the immune system, blood is the medium that allows dissemination to distal sites. Therefore, gaining further insight into the host-pathogen interaction at this particular juncture could prove beneficial to future efforts to intervene with SAB and infection in general. Unfortunately, technical limitations severely complicate this progress. While kidneys commonly bear the highest bacterial loads in murine systemic infection models of *S. aureus*, bacterial loads in the blood are

significantly lower, making RNA preparation from these samples challenging. Nevertheless, these limitations may soon be overcome by recent advances in sequencing technologies, of which a prime example is single cell RNAseq [340]. In this technique, problems of low input are circumvented and additionally heterogeneity within the sample appreciated. However, in order to reflect a bacterial community in its entirety, information from several such experiemnts needs to be combined.

Despite a steadily increasing pool of information about *S. aureus* encoded sRNAs and their expression patterns, identification of direct targets is indispensable and one of the rate limiting steps in understanding the precise functions of these transcripts. Pulldown procedures to pinpoint the interacting partners of sRNAs have been proposed and tested, but reliable methods are still sparse. We are currently in the process of examining an approach where an oligo $d(A)_{25}$ tail is added to a plasmid-encoded version of an sRNA in question along with its native promoter. As such long, homogenous strings of a particular nucleotide are irregular in bacteria, the tagged sRNA is easily distinguished from the remainder of the cell's RNA content. Using a eukaryotic mRNA cleanup kit (based on oligo $d(A)_{25}$ coupled to magnetic beats), this RNA, as well as the corresponding interacting elements (RNAs or proteins), can be identified via transcriptomic or proteomic approaches.

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Type of Use	Thesis/Dissertation
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Title of your work	Non-classical regulators in Staphylococcus aureus
Publisher of your work	n/a
Expected publication date	May 2017
Permissions cost	0.00 USD
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FEMS Microbiology Reviews, fuv005, 39, 2015, 541–554

doi: 10.1093/femsre/fuv005
Advance Access Publication Date: 15 April 2015
Review Article

REVIEW ARTICLE

Small things considered: the small accessory subunits of RNA polymerase in Gram-positive bacteria

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One sentence summary: This review details over four decades of research on the small, accessory subunits of the Gram-positive transcriptional machinery.

Editor: Grzegorz Wegrzyn

ABSTRACT

The DNA-dependent RNA polymerase core enzyme in Gram-positive bacteria consists of seven subunits. Whilst four of them ($\alpha_2\beta\beta'$) are essential, three smaller subunits, δ, ε and ω (~9–21.5 kDa), are considered accessory. Both δ and ω have been viewed as integral components of RNAP for several decades; however, ε has only recently been described. Functionally these three small subunits carry out a variety of tasks, imparting important, supportive effects on the transcriptional process of Gram-positive bacteria. While ω is thought to have a wide range of roles, reaching from maintaining structural integrity of RNAP to σ factor recruitment, the only suggested function for ε thus far is in protecting cells from phage infection. The third subunit, δ, has been shown to have distinct influences in maintaining transcriptional specificity, and thus has a key role in cellular fitness. Collectively, all three accessory subunits, although dispensable under laboratory conditions, are often thought to be crucial for proper RNAP function. Herein we provide an overview of the available literature on each subunit, summarizing landmark findings that have deepened our understanding of these proteins and their function, and outline future challenges in understanding the role of these small subunits in the transcriptional process.

Keywords: RNA polymerase; delta subunit; RpoE; omega subunit; RpoZ; epsilon subunit; RpoY; transcriptional regulation

INTRODUCTION

The ability of bacterial cells to precisely adjust and adapt to their environment is crucial for survival. Accordingly, the expression of genes, and their products that facilitate adaptation to changing conditions, is a highly controlled and organized process. Transcription in all forms of life is performed by DNA-dependent RNA polymerase (RNAP), with enzymes from the different branches of life showing a high degree of similarity (Ebright 2000; Sekine, Tagami and Yokoyama 2012). With respect to eubacteria, core RNAP consists of two α subunits, one β and one β' subunit, all of which are essential for a viable cell. Besides these well-studied and essential components, several additional, smaller subunits, δ, ε and ω, have been described and intensively researched over a number of decades (Burgess 1969; Pero, Nelson and Fox 1975; Keller *et al.*, 2014). Additional to this, bacteria possess σ factors (housekeeping or alternative) that assist RNAP with promoter recognition, and the initiation of transcription (Helmann and Chamberlin 1988; Feklistov *et al.*, 2014). While $\alpha_2\beta\beta'$ RNAP and the σ factor together are able to perform all of the required steps for transcription, the additional, smaller subunits support the complex by various means, reaching from coordinating RNAP folding and assembly to increased transcriptional specificity, influencing RNAP recycling and possibly protecting the cell against phage infection.

Received: 19 December 2014; **Accepted:** 19 January 2015

As ω is found in Gram-positive as well as Gram-negative bacteria (Minakhin *et al.*, 2001), it has been more heavily investigated than the other subunits (for a thorough review, see Mathew and Chatterji 2006). With regard to the δ subunit, it is seemingly confined to the Firmicutes; although proteins with some sequence homology can be found in certain Tenericutes as well (our unpublished observation). Collectively, despite many years of research on this protein in low G + C Gram-positive bacteria, the literature documenting it has never been collated in a single place. As such, it forms a major component of the work detailed herein. Nevertheless, we also seek to also update the literature surrounding ω, as well as introduce newly published data on ε. We believe that of these subunits, δ is particularly interesting, since we still lack a complete understanding of how this enigmatic protein mediates its function, despite a history of more than 40 years of research. Accordingly, we present the progress of δ research over four decades, summarizing key milestones in our understanding of this subunit, and highlight future challenges in dissecting its role in transcription, as well as cellular fitness and survival.

THE δ SUBUNIT OF RNAP

Structure, abundance and interaction of the δ subunit with RNAP

The interaction of δ with RNAP

The presence of δ as an integral part of RNAP was first described in 1975 when Pero, Nelson and Fox (1975) identified and named a 21.5 kDa protein that co-purified with the RNAP complex from phage-infected *Bacillus subtilis*. Here the group reported for the first time the effects of δ on transcriptional specificity, using *in vitro* transcription assays that demonstrated it was required to maintain the strand-specific transcription of phage genes characteristically observed during *in vivo* infection. These experiments provided not only a structural but also a functional link between RNAP and δ. Subsequently, several groups simultaneously described the co-purification of δ with RNAP in *B. subtilis* (Halling, Burtis and Doi 1977; Plevan *et al.*, 1977; Tjian *et al.*, 1977). In a more recent approach, Doherty *et al.* (2010) used fluorescent-labeled δ in *B. subtilis* to observe its interaction with RNAP *in vivo*, showing the subcellular co-localization of this subunit with other RNAP proteins (β' and ω). Lately, our group demonstrated the interaction of δ with the β and β' subunits of RNAP under native conditions in *Staphylococcus aureus* (Weiss *et al.*, 2014). This interaction is most likely facilitated by the N-terminus of the protein, since a C-terminally truncated version of the enzyme still co-purifies with core RNAP (Lopez de Saro, Woody and Helmann 1995). Stoichiometrically, it appears that the subunit binds in an approximate 1:1 ratio with other subunits of core-RNAP (the α subunit excepted) (Halling, Burtis and Doi 1977). Interestingly, early studies were unable to purify RNAP containing a σ factor as well as δ at the same time. This led to the idea of temporally separated binding of these two subunits, and questions about when and how δ interacts with core RNAP. In order to understand the association of δ with core RNAP in a time-dependent manner, and in correlation to DNA-binding and transcriptional initiation, sedimentation experiments were used, demonstrating that excess amounts of the subunit lead to release of the σ factor from *B. subtilis* RNAP (Williamson and Doi 1978). This seemed plausible since δ had been shown in early studies to decrease activity of core RNAP at certain promoters, while σ is well known to have stimulatory effects, suggesting a competitive relationship between these two subunits. These findings, to a certain extent, were overruled when experiments showed the need for both subunits in the context of promoter selection (Achberger and Whiteley 1981), leading to key studies revealing that δ and σ factors are in fact able to bind to RNAP at the same time (Hyde, Hilton and Whiteley 1986). This ultimately excluded the hypothesis of a completely competitive δ vs σ relationship. Instead, the decreased binding ability of σ after δ subunit association with RNAP indicates 'negative cooperativity' in which both proteins are able to bind to the RNAP simultaneously, but where binding of δ possibly weakens the binding of σ and leads to increased release of the factor. Such a scenario suggests a model in which both subunits are required for directed binding to promoter regions, and initiation of transcription.

δ expression and protein levels within the cell

Various studies have explored the conditions that affect δ abundance within bacterial cells, using a variety of different strains and backgrounds. In general, there is agreement that *rpoE* (the δ encoding gene) is highly expressed during exponential phase (mid and late) and, to varying degrees, during stationary phase, in standard laboratory conditions. In *S. aureus*, peak expression appears to be during exponential phase under standard conditions and when grown in amino acid- or glucose-limiting media (Watson, Antonio and Foster 1998; Weiss *et al.*, 2014). In *B. subtilis*, *rpoE* is most highly expressed during the transition between exponential and stationary phase, as well as in extracts from spores (Lopez de Saro, Yoshikawa and Helmann 1999). For *Streptococcus agalactiae*, findings from reporter constructs mirror that seen in *S. aureus* and *B. subtilis*, with *rpoE* being expressed during all growth phases, peaking during lag and (late) log, before dropping slightly when reaching stationary phase (Seepersaud *et al.*, 2006). In *S. mutans*, it was shown that *rpoE* is expressed maximally during late exponential phase, and subsequently declines during stationary phase (Xue *et al.*, 2010). One study suggested that δ accounts for 0.3% ($\pm$ 0.1%) by weight of the total soluble protein of *B. subtilis* cells (10^4 molecules cell^{-1}), representing a 5:1 molar excess compared to RNAP during exponential and stationary growth phase (Lopez de Saro, Yoshikawa and Helmann 1999). In contradiction, Doherty *et al.* (2010) found a total abundance of 2.1×10^4 ($\pm$ 580) molecules per cell for the β' subunit and 2.3×10^4 ($\pm$ 900) molecules for δ in exponentially growing cells, resulting in an approximate 1:1 ratio of the subunit to RNAP. Although these studies show variation in comparison to each other, it is clear that δ is present in relatively equal amounts to other components of core RNAP, therefore suggesting a permanent interaction of this subunit with the transcription machinery. In terms of factors that influence its expression, it has been reported for *B. subtilis* (Lopez de Saro, Yoshikawa and Helmann 1999), *S. agalactiae* (Seepersaud *et al.*, 2006) and *S. aureus* (Weiss *et al.*, 2014) that *rpoE* transcription is driven from a σ^A-dependent promoter. Beyond this, *rpoE* does not appear to be autoregulated in *B. subtilis*, but is at least partially repressed by acid and H_2O_2 stress (Lopez de Saro, Yoshikawa and Helmann 1999). Collectively, the strong presence of δ during most/all growth phases, in multiple bacterial species, highlights its potential importance for Gram-positive cells.

Structure, domains and active sites of the δ subunit of RNAP

When studying the primary amino acid (aa) sequence of δ proteins from numerous organisms (Fig. 1), it is apparent that they all possess two distinct regions, as first described by Lopez de Saro, Woody and Helmann (1995): an ordered and structured N-terminus, and a flexible and unstructured C-terminus. The C-terminus is characterized by highly acidic and repetitive aa

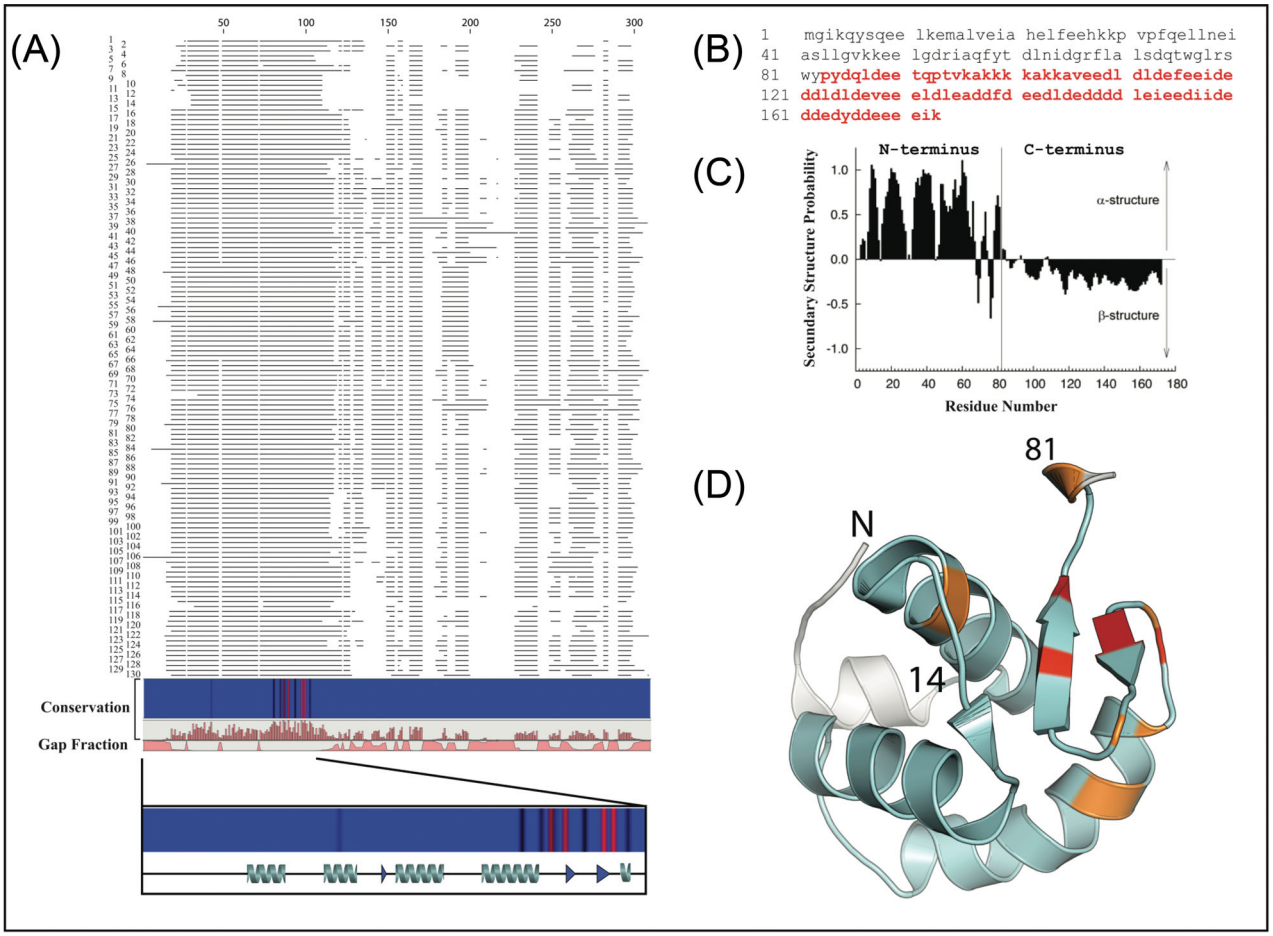

Figure 1. Structural features of the δsubunit of RNAP. **(A)** Alignments of 130 representative δ proteins from different Gram-positive species (using CLC Main Workbench software). Shown are (from top to bottom) broken black lines next to numbers from 1 to 130 (corresponding to bacterial species and IMG Gene ID numbers (Markowitz *et al.*, 2014), see Table S1, Supporting Information) represent the alignment of δ proteins. Sequence conservation is highlighted by two graphs: the upper one marks residues that are at least 95% (black) and 99% (red) conserved. The one below highlights the amount of conservation (0–100%) for each amino acid. The gapped fraction visualizes the amount of divergence in each region of the protein. At the bottom, an enlargement of the N-terminus is shown and highlights the conservation in regions that harbor specific protein domains (α-helices or β-sheets). **(B)** Amino acid sequence of the *B. subtilis* δ subunit. Black letters mark the N-terminus and red letters the C-terminus of the protein. **(C)** Secondary structure prediction of the ordered N-terminus and the disordered C-terminus of the δ protein, showing a bias towards α and β structures, respectively. This figure is reproduced from Papouskova *et al.* (2013). **(D)** Structure of *B. subtilis* δ from the protein database (PDB ID: 2M4K). The blue region corresponds to the HARE-HTH domain (residue 14–81); residues in red (≥99%) and orange (≥95%) highlight amino acids that are conserved from the alignment.

residues; together, the N- and C-termini have a significantly acidic pI of 3.6. A wealth of additional structural information for δ was generated by Motackova *et al.* (2010) using NMR technology and the *B. subtilis* protein, specifically focusing on the N-terminal 100 aa. Four α-helices were identified; each being 5 to 12 aa in length (residue Q8–K12, L16–H27, F33–L44, G52– N63). In addition, a β-sheet, consisting of three β-strands (residues V31–P32, F68–A70, T75–L78), was reported. A concern with these approaches was the inclusion of a His-tag to facilitate purification, possibly interfering with NMR analysis. To assess this, the same group later conducted structural analyses on the full-length protein lacking such a tag, again using NMR (Papouskova *et al.*, 2013). This approach proved to be especially challenging because of the acidic C-terminus; however, these studies confirmed previous work with the His-tagged N-terminal variant. Relaxation experiments in these works showed that the C-terminal tail transiently contacts the ordered N-terminal domain, leading the authors to suggest that folding of the N-terminus may be influenced by interactions with its C-terminal partner. In addition, interdomain interactions (between the C- and N-terminus, and the C-terminus with itself), as well as the formation of possible β-sheet structures, were predicted for the flexible C-terminal region.

An attempt to identify functional domains found only weak homology to DNA/RNA-binding proteins for the N-terminus of δ, and therefore initially no nucleic acid binding properties of the subunit were assumed (Motackova *et al.*, 2010). A more recent study then compared sequence and structural features of a variety of elements to the human ASXL protein, identifying similarities of the ASXL N-terminus and δ (Aravind and Iyer 2012). The N-terminus of this protein is characterized by a winged helix-turn-helix (wHTH) domain that was named after its presence in the group of **H**B1, **A**SXL and **r**estriction **e**ndonuclease proteins (HARE-HTH). Other than the δ subunit, HARE-HTH domains in prokaryotes are mainly found in restriction endonucleases that are associated with DNA-modifying methylases. Based on this study, a role in the recognition of modified DNA sequences for δ has been suggested, although it is not yet experimentally

proven. Nevertheless, the presence of a HARE-HTH motif in δ presents the interesting possibility that not only does it interact with RNAP but that it also contacts DNA itself, allowing it to discriminate between sequences that differ, for example, in their degrees of DNA modification. As such, it is tempting to speculate that, similar to alternative σ-factors, δ potentially mediates directed transcriptional effects by recognizing specific promoter features, rather than solely having global effects on a large number of genes through modulation of RNAP activity.

To understand the importance and conservation of key δ regions, our group recently published alignments using various different species that carry a δ subunit protein (Weiss *et al.*, 2014) (Fig. 1). We demonstrate that HARE-HTH domains show a higher degree of conservation than other portions of the protein, emphasizing its likely importance for function. In contrast, the C-terminus of δ shows a large degree of variation. Further to this, there is a clear degree of divergence in the context of protein length for δ from various species (Table S1, Supporting Information), largely resulting from variations in the C-terminal domain. This suggests that the C-terminus either plays species specific roles or is at least partially dispensable for full function of the subunit. In line with this latter idea, we have shown that a δ variant of *S. aureus* containing a truncated C-terminus (missing ~25% of the protein) is still able to successfully interact with components of the transcription machinery, and complement, at least in part, the phenotypes of an *rpoE*-null strain (Weiss *et al.*, 2014). This is in line with earlier findings in *B. subtilis*, also describing that interaction of δ with RNAP is mediated by the N-terminus of the subunit (Lopez de Saro, Woody and Helmann 1995).

The δ subunit and its impact on transcriptional selectivity

Early work highlighting the influence of δ on transcription

The majority of early information regarding δ function was generated by studies exploring transcriptional patterns of phage genes in *B. subtilis*. Upon infection, phages go through a predictable pattern of early, middle (4–5 min) and late (8–10 min) gene expression (Gage and Geiduschek 1971). Phage genes transcribed in this temporal pattern are located on different strands (heavy or light) of the phage genome. Whilst these specific temporal and strand-specific transcriptional patterns are governed by phage-encoded proteins (Gage and Geiduschek 1971; Fox 1976; Giacomoni 1981), it has been shown that δ, as an innate part of RNAP, is also involved in maintaining their specificity and order of transcription (Pero, Nelson and Fox 1975; Achberger, Hilton and Whiteley 1982a; Dobinson and Spiegelman 1987). These experiments initially demonstrated that δ has a supportive role for transcriptional selectivity, acting alongside and in concert with other transcription factors (e.g. phage-encoded σ factors and regulatory proteins). This was projected to not only be true for phage-related genes but for the transcriptional process in general.

The δ subunit enhances RNAP fidelity and selectivity

Other early studies on the function of δ centered not only around phage genes but also on its effects on transcription from synthetic templates (poly(dA-dT)) (Tjian *et al.*, 1977). It was reported that whilst δ had only a limited effect on the expression of phage genes, it strongly decreased RNAP activity towards synthetic templates, suggesting a general function in selectivity. This led to studies focused on a wider spectrum of transcriptional targets, employing a variety of templates, including different phages, *B. subtilis* chromosomal DNA, plasmids and synthetic targets (Dickel, Burtis and Doi 1980). These *in vitro* transcription experiments revealed varying effects after the addition of purified δ to reaction mixtures. Specifically, in the case of one phage tested (Φe), δ had no negative effects on RNAP activity, whilst for *B. subtilis* chromosomal DNA, the synthesis of RNA was decreased by up to 87% for some regions in the presence of this subunit. Moreover, transcription from DNA fragments that did not contain specific and known promoter sequences was almost entirely repressed by the presence of δ. This suggests that the δ subunit affects different promoters in different ways, thus supporting promoter specificity of the transcriptional machinery, whilst at the same time suppressing transcription from loci that do not contain δ-specific promoter elements. Similar effects for δ on the transcription of non-coding-regions were also described by Achberger and Whiteley (1981). Their studies, using *in vitro* transcription employing endonucleolytic fragmented DNA from *B. subtilis* bacteriophage SP82, revealed that in the absence of δ, the RNAP holoenzyme binds non-specifically to fragments that do not have promoters, resulting in transcription from these sites. The addition of δ to *in vitro* transcription reactions restored binding of the complex exclusively to promoter regions and produced transcription patterns (mid vs late phage genes) that are characteristic of those seen *in vivo*. Along the same lines, UV cross-linking experiments reveal that the addition of δ to core-RNAP or the RNAP holoenzyme leads to decreased binding at non-promoter sequences (Hilton and Whiteley 1985).

In a study by Dobinson and Spiegelman (1987), features that guide the variable effects of different promoters were explored by assessing the expression of two early Φ29 promoters in run-off experiments (initiation complex assays). In these works, it was shown that the influence of δ on transcription appears to be dependent on the strength of the promoter in question. Specifically, varying effects on transcriptional repression were observed, with the stronger of the two promoters showing no decrease in expression upon δ addition, whilst the weaker promoter resulted in a 50% decrease in transcription after supplementation with the subunit. These results gave early insight into the selective effects of δ, and suggested that promoter strength may be a driving force of its activity. This model has been supported by the findings of Juang and Helmann (1994b) in their work with the *B. subtilis ilv-leu* operon. In this study, it was shown that δ negatively influences open complex formation and has stronger effects towards the transcription of weaker promoters, and less of an influence on medium strength promoters. Consequently, it was suggested that δ decreases promoter melting, which leads to decreased transcription from non-promoter sequences, as well as at weaker promoters. In contrast, promoters that are considered closer to optimal are relatively independent of the effects of δ. Thus, a model describing three general types of promoters and their susceptibility to δ was proposed as follows (Juang and Helmann 1994b): (i) at weak promoters, δ prevents RNAP binding and therefore transcription; (ii) at medium strength promoters, δ allows the binding of RNAP, but decreases open complex formation; and (iii) at strong promoters, δ seemingly has little effect on gene expression.

δ interferes with open complex formation

Initial Studies by Spiegelman, Hiatt and Whiteley (1978) and Achberger, Hilton and Whiteley (1982a) indicated that δ functions in the window between promoter recognition and initiation, rather than being directly involved in initiation itself. Subsequently, experiments by Chen and Helmann (1997) furthered this notion, showing that δ is important for open complex

formation. This is in support of Juang and Helmann, who used, amongst other approaches, footprinting analysis to describe the inhibitory effects of δ on open complex formation (Juang and Helmann 1994a, 1994b, 1995). Furthermore, they demonstrated that loss of δ specifically interfered with promoter melting, rather than impacting the stability of the open promoter complex, which is somewhat in contrast to Rabatinova *et al.* (2013), who suggested that the longevity of open complexes is diminished by δ. Importantly, each of these studies again support the notion that δ influences transcription prior to initiation.

RNAP recycling

Whilst the majority of δ-related studies describe its negative effects on transcription of specific promoters, several groups have demonstrated that δ also has the capacity to increase overall transcriptional activity (Spiegelman, Hiatt and Whiteley 1978; Achberger and Whiteley 1981; Juang and Helmann 1994b). These positive effects are suggested to be a result of a decrease in non-specific transcription, as well as increased core-RNAP recycling in the presence of δ. The specific mechanism by which this latter process occurs is not fully understood; however, a more efficient release of RNAP after termination of transcription has been suggested (Juang and Helmann 1994b). This effect is thought to parallel the manner by which δ prevents RNAP from binding to non-promoter sequences, thereby leading to decreased product inhibition, and increasing the speed at which the transcription complex is available to initiate new rounds of gene expression. Structurally, it has been hypothesized that this process is facilitated by the C-terminus of δ (Lopez de Saro, Woody and Helmann 1995), where the negatively charged part of the protein competes with DNA/RNA for RNAP binding, thereby causing increased release of the transcriptional machinery. In more recent experiments investigating the function of HelD, another RNAP-associated protein, it was confirmed that δ is able to increase transcriptional cycling by faster release of RNAP after termination; and is also able to release stalled RNAP from DNA, increasing transcriptional activity (Wiedermannova *et al.*, 2014). Both effects have also been found to be true for HelD, and interestingly, both proteins together show a strong synergistic effect. Whilst the precise reason for this synergy is incompletely understood, the results highlight the complex and multifactorial regulation of RNAP activity.

The role of the initiating nucleotide on δ-dependent regulation

Although the contention that δ-dependent transcriptional effects are influenced by individual promoter strength is well established, it fails to completely explain all changes in gene expression mediated by this subunit. Therefore, a more recent study in *B. subtilis* has centered on delineating the exact role of δ in promoter melting and open complex formation, with a specific focus on the transcription initiating NTP (iNTP) (Rabatinova *et al.*, 2013). This idea was shaped by previous works, showing that promoter activity is not only determined by pure binding efficiency of RNAP to promoter sequences but also by the ability to initiate transcription. While high levels of iNTPs ensure efficient transcription, lower amounts cause collapse of the open promoter complex, and prevent transcriptional initiation. In *Escherichia coli*, transcriptional initiation from rRNA promoters, which have inherently unstable open promoter complexes, is dependent on the availability of transcript specific iNTPs (ATP or GTP) (Gaal *et al.*, 1997; Murray, Schneider and Gourse 2003). A similar scenario was also shown for *B. subtilis*, where nutritional starvation and onset of the stringent response results in decreased levels of GTP and therefore negatively influences transcription of iNTP-sensitive rRNA promoters (Ochi, Kandala and Freese 1982; Krasny and Gourse 2004). Subsequently, it was shown that such regulation is not only true of rRNA promoters but other genes as well, thus presenting a general regulatory concept (Krasny *et al.*, 2008). Accordingly, Rabatinova *et al.* (2013) explored the influence of iNTP on δ-mediated function in *B. subtilis*, revealing that the δ subunit influences transcription by enhancing the effects of iNTP on open complex formation. Promoters that possess relatively unstable open complexes require higher amounts of iNTP to initiate transcription. δ destabilizes open complex formation, thus increasing the amount of iNTP required for successful transcription initiation; supporting the notion of iNTP-dependent transcriptional regulation. This appears to be particularly true for promoters that are iNTP sensitive (e.g. rRNA promoters), whilst other promoters, which display stable open complexes, are less likely to be affected by the influence of the δ subunit.

The impact of abrogated δ activity on fitness and virulence

Phenotypic effects of δ deletion

Although the specific effects of δ on transcription have been well documented, uncovering the phenotypic effects of *rpoE* deletion was, at least initially, more challenging. A *B. subtilis* mutant lacking the δ subunit of RNAP was shown to be viable, able to form spores and did not display obvious growth defects under standard laboratory conditions (Lampe *et al.*, 1988). Although unexpected at first, these results are not surprising for an accessory subunit. They do, however, raise the question about under which conditions the transcriptional effects of δ result in altered behavior of the cell, and, thus, under which stimuli the δ subunit is beneficial for cellular survival. An early indication about possible δ function came from a study in *S. aureus* investigating starvation survival. Watson, Antonio and Foster (1998) identified *rpoE* during a transposon screen targeted towards genes that are important for survival and recovery from prolonged stationary phase starvation. A transposon insertion within the early portion of *rpoE* caused impaired survival under amino acid-limiting conditions and acid stress. Similar *rpoE*-dependent stress phenotypes were later shown in *S. mutans*, where a δ subunit mutant is impaired in acid and H_2O_2 stress survival (Xue *et al.*, 2010). Additionally, the mutant was described as having a clumping phenotype, and to reach lower final densities in liquid cultures when compared to the wild type. Another common phenotype described for various bacterial species, including *B. subtilis* (Lopez de Saro, Yoshikawa and Helmann 1999), *S. aureus* (Weiss *et al.*, 2014) and *S. agalactiae* (Jones, Needham and Rubens 2003), is the extended lag phase of *rpoE*-depleted strains when subcultured into fresh media from stationary phase growth. Thus, it would appear that δ is required for adaption to, and survival during, stress and changing environmental conditions, as well as growth phase transition. It has also been documented that *B. subtilis rpoE* mutants are characterized by elongated cell morphology, the propensity to clump during log phase and the appearance of rough-edged colonies when grown on solid media (Lopez de Saro, Yoshikawa and Helmann 1999). Interestingly, and despite earlier studies that described normal sporulation of *B. subtilis* Δ*rpoE* strains (Lampe *et al.*, 1988; Lopez de Saro, Yoshikawa and Helmann 1999), it has also been shown that a transposon insertion in this gene suppresses the effects caused by mutations (*pdhB* or *pdhC*) that block sporulation (Gao and Aronson 2004). The exact mechanism by which this occurs is unclear; however, these findings emphasize the widespread effects

of δ on transcription, and its importance in maintaining cellular homeostasis. Considering the widespread defects displayed by *rpoE* mutants in a wealth of species, it is perhaps expected that *rpoE* mutants of *B. subtilis* are readily outcompeted by the wild-type strain when cultured together over prolonged periods (Rabatinova *et al.*, 2013).

The role of δ in pathogenic organisms

Several groups have examined the effects of *rpoE* deletion on more complex processes, such as during the interaction of pathogenic bacteria with their hosts. Since disease causation is a process that involves the precise timing of expression for a variety of different genes, including those required for interaction with the immune system, virulence factor expression, biofilm formation and nutrient acquisition, it is perhaps no surprise that δ has been shown to play and important role in virulence. In *S. agalactiae*, a study employing signature-tagged transposon mutagenesis in a neonatal rat sepsis model identified an *rpoE* mutant as displaying the most impaired virulence from the entire library (Jones, Knoll and Rubens 2000). In a subsequent study by the same group, it was suggested that the virulence defect was linked to increased killing by phagocytes, explaining the lower survival rates observed for *rpoE* mutants in whole human blood; despite not showing altered survival in human plasma and chemically defined media (Jones, Needham and Rubens 2003). An important consideration with these findings is that the *rpoE* transposon mutant is not a complete knockout, but is in fact only a low *rpoE* expressing strain due to the location of the insertion. As such, the strain possesses around a 10-fold decrease in *rpoE* transcript compared to the wild type, which is seemingly insufficient to maintain full δ-function, leading to the decrease in virulence described. Therefore, it appears that in *S. agalactiae*, and perhaps other pathogens (see below), there is a minimal concentration of δ required in the cell to ensure transcriptional specificity and facilitate virulence.

The pathogenic role of δ has also been explored in another streptococcus, namely *S. mutans*. Interestingly, and in contrast to the negative effects of *rpoE* loss on virulence that were obtained for *S. agalactiae*, it was described that deletion of *rpoE* leads to increased expression of virulence-related traits (Xue *et al.*, 2011). Specifically, increased co- and self-aggregation, and an altered extracellular matrix, have been reported for *rpoE* mutants, which may be caused by differentially expressed surface proteins and polysaccharides. Furthermore, *S. mutans* Δ*rpoE* strains also display elevated attachment to human extracellular matrix components, such as collagen and fibronectin. Despite these seemingly enhanced aggregative properties, *rpoE* deletion causes an inability to attach to human epithelial cells (HEp-2) compared to the wild-type strain; likely due to increased clumping of the mutant. Beyond these findings, loss of *rpoE* in *S. mutans* results in the derepression of enzymes that facilitate the metabolism of a number of sugars, allowing the mutant to utilize a larger array of carbon sources than the parental strain. Additionally, a panel of antibiotics and compounds that are toxic to the wild type were ineffective against the mutant in phenotypic microarray studies. This highlights the remarkably broad impact that δ has on bacterial physiology and metabolism, each of which influence cellular fitness, and thus pathogenesis.

In a more recent study by our group, we explored the effects of δ in the major human pathogen, *S. aureus* (Weiss *et al.*, 2014). Deletion of *rpoE* resulted in decreased expression and abundance of a variety of virulence factors, leading to decreased fitness of the strain and impaired virulence. This latter point is manifested by a diminished ability to survive in whole human blood, and resist phagocytosis by human leukocytes. These *in vitro*/*ex vivo* phenotypes were shown to correlate with *in vivo* findings, where the mutant strain was found to be significantly impaired in its ability to cause disease in a murine model of sepsis and dissemination.

Global transcriptional and translational effects of δ

Exploring the influence of δ on global gene expression has been aided by cutting edge high-throughput methodologies, including transcriptomics and proteomics. These techniques have provided insight into the role of δ in maintaining transcriptional specificity, and how this links to the various phenotypes observed. To explore why an *rpoE* mutant of *S. mutans* displays alterations in virulence-related behaviors, as well as impaired ability to survive during stress (Xue *et al.*, 2010, 2011), gene expression (Xue *et al.*, 2010) and protein abundance (Xue *et al.*, 2012) were assessed for the wild type and mutant under several conditions. Transcriptomic analyses conducted via microarray revealed that, independent of the growth condition, more than 50% of the upregulated transcripts in an *rpoE* mutant were non-coding regions (Xue *et al.*, 2010). In comparison, only 20–30% of non-coding transcripts displayed increased expression in the mutant strain. These findings corroborate previous *in vitro* studies that demonstrate non-specific transcription of non-promoter regions when δ is removed from core RNAP (see above).

In addition to its effects on non-coding regions, coding sequences were also shown to be influenced by δ in *S. mutans*, in a manner partially independent of growth phase or condition (Xue *et al.*, 2010). Specifically, 24 genes were identified as being downregulated in the *rpoE* mutant under all conditions. These genes were involved in malolactic fermentation, histidine metabolism, biofilm formation, adherence, virulence, and resistance to antibiotics and other inhibitory compounds. In addition, during growth under standard laboratory conditions, a large number of transcripts were differentially expressed when comparing the mutant and parental strain, including those involved in the transport of a wealth of different compounds, metabolism and energy production/conversion. Following H_2O_2 or acid stress, a number of protective genes were not induced in the mutant strain, indicating that an incomplete stress response exists upon *rpoE* loss; however, the general stress response still appeared to be intact in the mutant strain. The authors ultimately concluded from these works that diminished survival of the mutant strain when exposed to stress results from a general deregulation of the *S. mutans* transcriptome, leading to an impaired, slower and less directed attempt to adapt to changing environments and stress conditions.

In follow-up studies, the same group compared previous transcriptomic data with new proteomic data of the *rpoE* mutant and wild type, using identical growth conditions (Xue *et al.*, 2012). It was noted that two proteins, phosphoglucomutase (PGM) and phosphopentomutase (DeoB), were decreased in abundance either during all (PGM) or most (DeoB) growth phases and conditions. Both were identified as displaying this same pattern in microarray analysis as well, suggesting distinct and very gene-specific regulatory effects of δ. In the context of growth phase, chaperones, stress-related factors and enzymes known to be involved in protein turnover were altered in the mutant during exponential phase; whilst multiple sugar transporters and metabolism (MSM) systems demonstrated fluctuations during stationary phase. It was suggested that these changes in protein abundance could be an indicator of internal cell stress, rather than direct *rpoE*-guided effects. When comparing protein

composition of the *rpoE* mutant and parental strain after exposure to external stressors, it was noted that both strains were able to induce protection-specific pathways, which was again largely consistent with transcriptomic data. However, despite general adaptation patterns being similar between mutant and wild type, it was observed that the upregulation of proteins required to adapt to environmental change was weakened upon the loss of δ, again indicating a slower or dampened response to stress.

Using RNAseq technologies, our own group revealed similar effects of *rpoE* deletion in *S. aureus* as compared to *S. mutans* (Weiss *et al.*, 2014). Specifically, our *rpoE* mutant displayed differential expression of 191 genes compared to the wild type during exponential growth, including a significant decrease in many known or implicated virulence factors, such as toxins, hemolysins and secreted proteases. While global regulators were largely unaffected, the clustered alteration of virulence gene expression again suggests gene specific effects, such as those seen for PGM and DeoB in *S. mutans* (Xue *et al.*, 2012). Beyond reduced virulence gene expression, we notably observed that under standard conditions weakly expressed genes were generally derepressed upon loss of *rpoE*. Interestingly, we also saw a large increase in non-core genome elements, including prophage-encoded genes. This parallels the effects seen for non-coding sequences in *S. mutans*, and further highlights that loss of the δ subunit results in decreased transcriptional selectivity, and an overall deregulation of the transcriptional process.

When one considers all of the available transcriptomic/proteomic studies with *rpoE* mutants, three general conclusions can be made. (i) Certain genes and gene groups are changed in expression/protein abundance independent of growth phase or stress condition. This suggests a gene-specific mechanism of regulation that involves δ, opening the possibility that there may be promoter features other than strength (for example iNTP-sensitivity) at work. (ii) During exponential and stationary phase growth (as seen for *S. aureus* and *S. mutans*), loss of *rpoE* leads to a complex deregulation of transcription, causing differential expression patterns, and the upregulation of typically lowly expressed genes and non-coding elements. (iii) Finally, it has been shown that cells lacking *rpoE* still adapt to changing environmental conditions, but in a less rapid and less robust manner than the wild type. This would seem to account for the decreased survival of *rpoE* mutants under stress conditions, and also explain the extended lag phase observed when mutants are subcultured into fresh media. Accordingly, the effects of *rpoE* disruption on survival and adaptation can be viewed as a fitness defect, resulting from diminished transcription of specific pathways and traits due to relaxed transcriptional specificity. With this being said, the specific and direct regulation of certain genes (e.g. PGM and DeoB in *S. mutans*) and traits (virulence determinants in *S. aureus*) still occurs. Collectively, each of these facts and findings highlight the multifactorial influence of δ, ranging from gene-specific to global effects, leading to a lack of fitness, and alterations in key cellular processes.

Summary of the δ subunit

Collectively, all of the information contained herein leads to a model for δ function (Fig. 2): A major feature of the δ subunit is that it decreases the stability of RNAP-DNA interaction at non-coding regions and weak promoters that possess inherently unstable open promoter complexes. Consequently, δ biases the transcriptional machinery towards those open-reading frames that have strong promoters/stable open promoter complexes, thus favoring expression from such sites. In addition to this, features beyond promoter strength (e.g. iNTP requirements) likely result in promoter-specific effects of δ in addition to its more general role. Furthermore, δ increases RNAP recycling and causes overall enhanced transcriptional activity. In

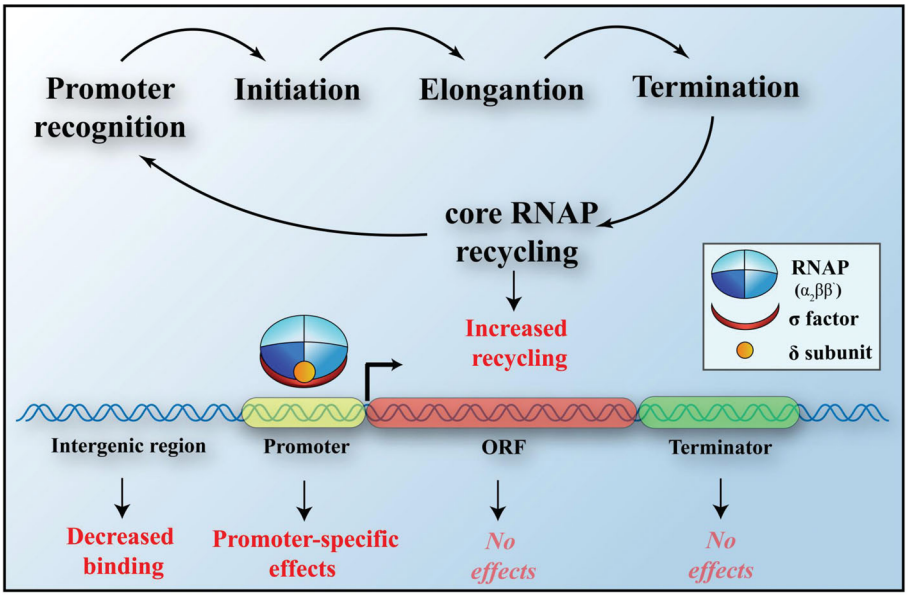

Figure 2. A model describing the effect of δ on different phases of transcription. The available literature suggests that δ has varying effects on each stage of the transcriptional process. The subunit prevents non-specific binding and transcription of RNAP from intergenic and non-promoter-containing regions. This influence on open complex formation and the initiation of transcription vary from promoter to promoter, thereby increasing transcriptional specificity of RNAP. Whilst elongation and termination are seemingly unaffected, δ does limit the binding of RNAP to DNA at terminators, allowing faster recycling of the enzyme, and increasing overall transcription. Shown are the stages of transcription and the corresponding gene regions in black letters and δ-dependent effects in red. Note that the ε and ω subunits are omitted from this figure for reasons of simplicity.

general, δ weakens the binding of RNAP to DNA and negatively influence open complex formation and stability; which appears to be mediated by structural features of the δ protein. Specifically, its N-terminus mediates interaction with core RNAP, whilst the negatively charged and intrinsically disordered C-terminus is thought to interfere with the RNAP-DNA interaction. Ultimately, δ acts as a guide for RNAP, increasing its transcriptional specificity. Loss of this factor causes deregulation, and results in a decreased ability to adjust to changing environments, general fitness defects and the reduced virulence of pathogenic species. All of these consequences highlight the central importance of δ as a regulatory factor directing gene expression in Gram-positive bacteria.

ADDITIONAL, SMALL ACCESSORY RNAP SUBUNITS IN GRAM-POSITIVE BACTERIA

In addition to the δ subunit, Gram-positive bacteria possess two other accessory RNAP subunits, omega, ω (RpoZ) and epsilon, ε (RpoY). Whilst ε is unique to Gram-positives, ω is found in RNAP complexes from archea to eukaryotes. Both of these subunits and their roles within the RNAP complex is discussed in the following sections.

The ω subunit

The ω subunit of RNAP is an intensively studied protein that has been widely described in the literature. With this being said, the majority of studies have been performed using the Gram-negative model organism *E. coli*, and appear to be only partially translatable to Gram-positive species, as discussed below. An extensive and comprehensive review on the history of ω research, including Gram-positive as well as Gram-negative bacteria, has been published by Mathew and Chatterji (2006) where the authors summarize structural and functional roles of the subunit. In the context of this review, we will offer an overview of data that is available and valid for Gram-positive organisms, as well as highlight functional differences compared to Gram-negative bacteria.

The ω protein is widely conserved across all forms of life

With an increasing number of bacterial, archaeal and eukaryotic genomes available, it has become clear that the ω subunit is conserved in all branches of life. In eukaryotes, a sequence, structural and functional homolog of ω (RPB6) exists that is associated with RNAPI, II and III, whilst archaeal genomes harbor a sequence homolog termed RpoK (Minakhin *et al.*, 2001). RPB6 has been shown to be essential for RNAP assembly in eukaryotes (Nouraini, Archambault and Friesen 1996); however, in bacteria, deletion of the ω subunit in a variety of different species does not result in cell lethality (Gentry and Burgess 1989; Kojima *et al.*, 2002; Mathew, Ramakanth and Chatterji 2005; Doherty *et al.*, 2010; Jie, JiLiang and DongJie 2010; Santos-Beneit *et al.*, 2011; Gunnelius *et al.*, 2014). This is particularly intriguing, since the subunit is conserved in all of the sequenced genomes of free-living bacteria (Minakhin *et al.*, 2001), suggesting an important evolutionary role for it in cellular survival

The binding of ω to RNAP

The ω protein, which ranges in size from ∼9 to 11.5 kDa depending on the bacterial species in question, was first shown to be a dedicated subunit of *E. coli* RNAP, rather than just a tightly bound factor, when cross-linking experiments demonstrated its specific binding to the β′ subunit (Gentry and Burgess 1993). Subsequently, it was found to be an integral part of the transcription machinery *in vivo* when the association of ω directly with RNAP was shown (Dove and Hochschild 1998). In these studies, ω was translationally fused to a DNA-binding protein (the cI repressor from bacteriophage λ) and was found to activate transcription of promoters adjacent to the corresponding λ operator, demonstrating interaction of the chimeric λcI-ω fusion protein with RNAP. Both of these studies have since been supported by more recent work solving the structure of ω-containing RNAP from *Thermus aquaticus* and *E. coli* (Zhang *et al.*, 1999; Murakami 2013). Although no such crystallization studies for RNAP have been performed in Gram-positive bacteria, several experiments in *B. subtilis* demonstrate co-purification of ω with RNAP, highlighting its association with other subunits of the transcriptional machinery (Spiegelman, Hiatt and Whiteley 1978; Achberger, Tahara and Whiteley 1982b). These results were confirmed and extended in a recent approach in which Delumeau *et al.* (2011) were not only able to co-purify ω with the essential RNAP subunits (α, β, β′) but also determined a 2:1:1:1 ratio for $\alpha_2\beta\beta'\omega$ during exponential and stationary growth. These results clearly show that, as for Gram-negative bacteria, the ω subunit is an integral part of the Gram-positive transcriptional machinery.

The role of ω for structural integrity of RNAP

Extensive research has been performed on the involvement of ω in folding of the β′ subunit, and RNAP assembly. Its importance for the assembly process was first considered when RNAP purified from *Rhodobacter capsulatus* and *E. coli* was shown to require ω, or GroEL and ω, respectively, to remain active *in vitro* (Mukherjee and Chatterji 1997; Richard *et al.*, 2003). This requirement for the GroEL chaperone was considered puzzling, but this factor had been shown to co-purify with RNAP, and was thus thought to be important for the folding of the transcriptional complex (Mukherjee and Chatterji 1997; Mukherjee *et al.*, 1999). Interestingly, deletion of ω in *E. coli* results in a massive increase of GroEL recruitment to RNAP, suggesting that GroEL might be employed to overcome misfolding due to ω loss (Mukherjee *et al.*, 1999). This was confirmed when *in vivo* GroEL substrates in *E. coli* were determined (Houry *et al.*, 1999), showing that other than β′, all of the essential RNAP subunits, as well as ω, require GroEL for proper folding. Surprisingly, these results were found to be only partially true in *B. subtilis*, where GroEL does not appear to be associated with RNAP subunits (Endo and Kurusu 2007). With this being said, the Gram-positive study identified considerably fewer GroEL substrates than the study performed in *E. coli* (28 vs ∼300). Therefore, either there are indeed fewer substrates for this chaperone in *B. subtilis* or experimentation in the Gram-positive organism had lower sensitivity in comparison with the *E. coli* study.

Interestingly, an earlier study in *E. coli* described *in vitro* cross-linking of ω and β′ (Gentry and Burgess 1993), whilst at the same time showing that β′ was not a substrate of GroEL and that the ω subunit increases (together with GroEL) the *in vitro* activity of RNAP. As such, it was suggested that ω had a specific function in supporting the folding of β′. This hypothesis was reinforced by a structural study that investigated the precise interplay of ω and β′, demonstrating interaction of conserved regions for these two proteins (Minakhin *et al.*, 2001). Consequently, it was proposed that ω acts as a molecular latch, guiding the interaction of β′ with the $\alpha_2\beta$ complex. Studies by Ghosh, Ishihama and Chatterji (2001) supported this notion by demonstrating that the interaction of ω with β′ was able to prevent aggregation of the latter protein during *in vitro* renaturation. Furthermore, this same group was able to show that native folding of β′ requires ω, and

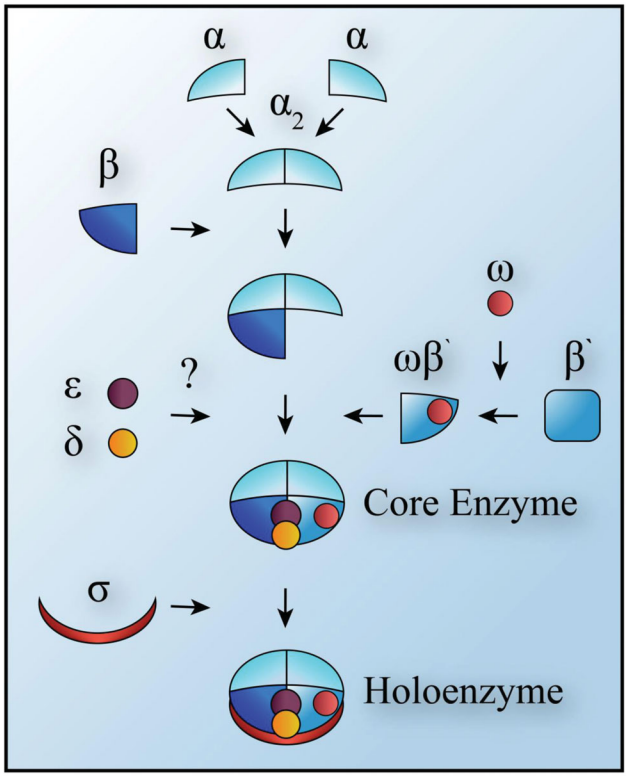

Figure 3. Model for RNA polymerase assembly in Gram-positive bacteria. After association of two α subunits, the dimer binds to the β subunit. The β' protein is held by the ω subunit (Minakhin *et al.*, 2001) in order to prevent misfolding (Ghosh, Ramakrishnan and Chatterji 2003) and aggregation (Ghosh, Ishihama and Chatterji 2001), before the β'-ω complex docks with $\alpha_2\beta$ (Mathew and Chatterji 2006; Ganguly and Chatterji 2011). Thus far, it is unknown when the ε and δ subunits bind to the complex; however, it is believed that ε interacts at the region of the β' jaw and secondary channel (Keller *et al.*, 2014), whilst δ is thought to interact with the β and β' subunits (Weiss *et al.*, 2014). Finally, and in order to enable RNAP to perform promoter-specific transcription, the σ factor docks with core RNAP resulting in holoenzyme formation.

that folding of the β' subunit in the absence of ω results in an intermediate state between a denatured and native conformation (Ghosh, Ramakrishnan and Chatterji 2003). These results were supported by work in *Bacillus cereus*, where expression of ω increased by as much as 10-fold after heat stress (42°C), suggesting a chaperone-like role, where larger amounts of the subunit can counteract RNAP denaturation (Periago *et al.*, 2002). Other than a function in β'-folding, it was also shown that the removal of ω leads to increased proteolytic cleavage of β' during RNAP isolation in *Mycobacterium smegmatis*, suggesting an additional protective role of the ω subunit (Mathew, Ramakanth and Chatterji 2005). Based on these studies, the involvement of ω in RNAP assembly was described, whereby an α-dimer and the β subunit assemble before docking to ω-bound β' occurs (as visualized in Mathew and Chatterji 2006 and shown in Fig. 3). Both the sequential assembly of the subunits themselves, as well as the importance of ω for the assembly process, was recently confirmed by studies that investigated active RNAP reconstitution at air–water interfaces (Ganguly and Chatterji 2011).

Further to this, the comparison of different RNAP crystal structures revealed that ω seems to have varying interactions with β' and within the RNAP complex in diverse organisms (Murakami 2013). For example, in *T. aquaticus* and *E. coli*, ω displays only partially overlapping conformations, and thus has varying interactions with β'. This is especially interesting, given that no complete Gram-positive RNAP structure is available so far, and therefore no definite assumptions about $\omega - \beta'$ interaction can be made. These differences in conformation and interaction between the subunits could account for the various functional properties of ω in different bacterial species, which are discussed in the following section.

ω and the stringent response: structural and functional variability in different bacterial species

In *E. coli*, the ω-encoding *rpoZ* gene is located in the same operon as *spoT* (Gentry and Burgess 1989; Sarubbi *et al.*, 1989). SpoT is a bifunctional enzyme that facilitates the degradation and synthesis of guanosine tetraphosphate and pentaphosphate (ppGpp and pppGpp), thus regulating the levels of these alarmones, and onset of the stringent response (reviewed in Cashel *et al.*, 1996; Chatterji and Ojha 2001). In such a scenario, ppGpp/pppGpp are produced as a result of nutritional stress and cause global changes in transcription. Extensive work has centered on the mechanism of ppGpp/pppGpp recognition by ω, largely in *E. coli* (reviewed in Mathew and Chatterji 2006). Such studies recently led to identification of the 'magic spot', the binding site of ppGpp on RNAP (Kahrstrom 2013). Together three publications by different groups described that the binding of ppGpp to RNAP occurs at the interface of the β' and ω subunits (Mechold *et al.*, 2013; Ross *et al.*, 2013; Zuo, Wang and Steitz 2013), and that deletion of key residues negatively influences growth under nutrient-limiting conditions via loss of ppGpp binding to RNAP (Ross *et al.*, 2013). Interestingly, homology analysis shows that key β' and ω residues important for ppGpp binding are not conserved within the Firmicutes (or the *Thermus* and *Aquificales* genera), therefore suggesting alternative mechanisms of ppGpp recognition by RNAP exist (Ross *et al.*, 2013). This is in line with findings from *B. subtilis*, highlighting that although ppGpp is used as a signaling molecule to react to changing nutrient availability, ppGpp does not change transcriptional specificity by directly binding to ω and RNAP; thus, ω deletion does not interfere with stringent response induction (Krasny and Gourse 2004; Doherty *et al.*, 2010; Kriel *et al.*, 2012). Instead, this alarmone causes changes in intracellular GTP concentrations by modulating the activity of GTP synthesis proteins, which subsequently leads to altered activity of GTP (iNTP) sensitive promoters, such as ribosomal RNA genes (Krasny and Gourse 2004; Kriel *et al.*, 2012). Together these findings show that, although ω and the utilization of ppGpp to signal and react to nutrient limitation are conserved, the mechanism for alarmone recognition is variable. This emphasizes the need to examine and understand the function of ω in species beyond the standard model organism, *E. coli*. Interestingly, these possible functional differences between genera are also mirrored in the cellular abundance of the subunit itself. Doherty *et al.* (2010) were able to show that in *E. coli* almost every RNAP polymerase includes a ω subunit (85%), whilst in *B. subtilis* the amount is significantly lower, with only 48% of RNAP complexes containing the subunit.

ω is involved in σ factor recruitment

RNAP purified from *E. coli rpoZ* mutants displays an altered composition, with the amount of σ^{70} associated diminished, and a concomitant increase in bound σ^{38} (Geertz *et al.*, 2011). These changes in RNAP composition result in altered gene expression and DNA relaxation. Interestingly, the wild-type phenotype (increased supercoiling) is restored by overexpressing σ^{70} in ω-null strains. Similar results were obtained in the cyanobacterium *Synechocystis* sp. PCC 6803 (Gunnelius *et al.*, 2014a), wherein an

rpoZ knockout had impaired recruitment of the primary σ factor, resulting in decreased expression of genes under the control of this element. Inversely, a large number of typically lowly expressed genes were upregulated, suggesting activity of one or more alternative σ factors. These data indicate a role for ω in transcriptional specificity, and are supported by later publications describing an altered response to heat stress upon deletion of *rpoZ*, leading to impaired survival at higher temperatures (Gunnelius *et al.*, 2014b). Importantly, such findings correlate with early studies showing that purification of ω-deficient RNAP in *E. coli* is depleted for σ^{70}, leading to the conclusion that ω is required for correct RNAP folding and σ-factor binding (Mukherjee *et al.*, 1999). Overall, this line of research describes how the structural effects of ω are required for RNAP integrity and functionality, which leads to a role in fine-tuning transcription by adjusting affinity for central transcriptional regulators such as σ factors. These results were all obtained in Gram-negative organisms, thus highlighting the need for further investigation in Gram-positive species.

The ω subunit of Gram-positive bacteria

As detailed above, in *E. coli* the ω subunit mediates folding of the β' subunit, influences assembly of RNAP, orchestrates gene expression during the stringent response and plays a role in ordering transcriptional specificity. In Gram-positive bacteria, the functional role of ω is much less clear. Since ppGpp does not bind RNAP in *B. subtilis*, the ω subunit is unlikely to be directly involved in the stringent response of this organism. This raises the question of additional functions for the Gram-positive ω subunit outside of ppGpp binding and β' folding. Whilst studies have been conducted for various Gram-negative bacteria beyond *E. coli*, including *Sinorhizoboium meliloti* (Krol and Becker 2011), *Xanthomonas campestris* (Jie, JiLiang and DongJie 2010) and cyanobacteria species (Gunnelius *et al.*, 2014a, 2014b), thus far the role of ω in Gram-positive bacteria has only been extensively examined in two *Streptomyces* species and *M. smegmatis*.

When exploring the role of the ω subunit in *Streptomyces kasugaensis*, it was found that an *rpoZ* mutant had diminished synthesis of the antibiotic kasugamycin, and that the strain was also deficient for aerial hyphae generation (Kojima *et al.*, 2002). A genetic basis for this latter phenotype was not reported; however, the kasugamycin biosynthetic genes were found to have decreased expression in the mutant strain. This decrease in expression appears to be mediated by reduced production of the KasT regulator, which is known to positively regulate kasugamycin production (Ikeno *et al.*, 2002, 2006). Interestingly, when *kasT* was expressed under the control of a different promoter (*ermEp*), it was transcribed at wild-type levels in the *rpoZ* mutant, and resulted in expression of the kasugamycin-producing operon (Kojima *et al.*, 2002, 2006). Although the underlying mechanism for this regulation is not currently understood, these results do indicate that ω is capable of having promoter-specific transcriptional effects.

Subsequent to this, the role of ω was also analyzed in *Streptomyces coelicolor*. Interestingly, the ω proteins of *S. kasugaensis* and *S. coelicolor* display 98% sequence similarity, with the protein from the latter being able to complement *rpoZ* loss in *S. kasugaensis* (Kojima *et al.*, 2002). Santos-Beneit *et al.* (2011) have detailed pleiotropic phenotypes for a *S. coelicolor rpoZ* mutant, including slow growth, absent spore-pigmentation and increased sensitivity to heat, with temperatures above 40°C leading to an aerial hyphae-null phenotype. Additionally, the mutant displayed altered expression of two antibiotics, actinorhodin (ACT) and undecylprodigiosin (RED). Whilst ACT was produced earlier during growth in an *rpoZ*-depleted strain, the mutant failed to produce the antibiotic in later growth phases, showing overall reduced levels after 70 h. For RED, it was reported that production was essentially abolished in the mutant strain, akin to kasugamycin synthesis in *S. kasugaensis*. While there is no clear explanation for how deletion of *rpoZ* simulatiously modulates expression of these structurally unrelated metabolites, or leads to pleiotropic phenotypic alterations in *S. coelicolor*, it was shown that *rpoZ* has a PhoP-binding site (PHO box) (Sola-Landa *et al.*, 2005) upstream of its transcriptional starts site (Santos-Beneit *et al.*, 2011). The repressor PhoP is known to inversely regulate the expression of secondary metabolites in response to the availability of phosphate (Sola-Landa, Moura and Martin 2003). For *rpoZ*, it was shown that PhoP does indeed bind to its promoter region, and that deletion of *phoP* results in an increased abundance of the subunit. Whilst these experiments suggest that ω itself can be regulated in an environment-depenent manner, it is not completely understood how the subunit exerts its effects on a plethora of different genes and cellular processes. Furthermore, the notion that *rpoZ* expression is controlled by an environment-responsive regulator is challenged, at least in part, by findings in *B. subtilis*. In this organism, Delumeau *et al.* (2011) described that during sporulation, or following stress in vegetative cells, no changes in RNAP subunit stoichiometry are observed. This is in line with findings from Nicolas *et al.* (2012), who report that expression of the subunit is stable under 104 different growth conditions in *B. subtilis*, suggesting constant and environment-independent abundance of ω. These opposing findings may represent species-specific differences, highlighting not only the need to investigate how ω mediates its effects on gene expression but also how its production within the cell is controlled.

In *M. smegmatis*, it was first shown that *rpoZ* deletion causes changes in colony morphology, and a slower growth phenotype similar to that reported for *E. coli* (Mukherjee *et al.*, 1999; Mathew, Ramakanth and Chatterji 2005). Further studies described that ω-depleted strains present additional pleiotropic alterations, including a decrease in sliding motility, and diminished biofilm formation due to an altered biofilm matrix and cell surface resulting from shorter chain mycolic acids (Mathew *et al.*, 2006). With regard to this latter point, ω-deficient strains of *M. smegmatis* are unable to manipulate their mycolic acid and glycopeptidolipide profiles to that required for biofilm maturation; indeed, even after the onset of biofilm formation, *rpoZ* mutants maintain mycolates only found in the wild type during planktonic growth (Mathew *et al.*, 2006; Mukherjee and Chatterji 2008). Two possible explanations for these findings have been suggested: firstly, the onset and maturation of biofilms requires significant changes in transcription (Sauer 2003), which are facilitated by various regulatory mechanisms, including alternative σ factors (Rachid *et al.*, 2000; Bateman *et al.*, 2001; Knobloch *et al.*, 2001, 2004). Secondly, such events are governed by stress adaption through the stringent response and ppGpp (Balzer and McLean 2002; Taylor *et al.*, 2002; Lemos, Brown and Burne 2004; Gjermansen, Ragas and Tolker-Nielsen 2006; He *et al.*, 2012). Both of these processes are controlled by ω in Gram-negative species, although, as suggested above, there appears to be disparate function for this subunit between the two classes of bacteria. Indeed, an alignment of the ω protein between *M. smegmatis* and *E. coli* reveals that the conserved residues required for ppGpp binding are, as with *B. subtilis* (Ross *et al.*, 2013), not conserved (our unpublished observation). As such, it seems more probable that, as with *E. coli* and cyanobacteria, the binding of ω to RNAP in *M. smegmatis* results in an altered affinity for different σ factors, and therefore negatively influences the ability to express genes required for

biofilm formation. This highlights the variable nature and, more importantly, the need to study ω in a wider range of Gram-positive bacteria, before drawing general conclusions about its function.

The ε subunit

Besides the α_2, β, β', δ and ω, an additional seventh RNAP subunit was recently identified in Firmicutes. Early studies described a protein that co-purifies with RNAP in different *Bacillus* species (Spiegelman, Hiatt and Whiteley 1978; Achberger, Tahara and Whiteley 1982b), but not in *E. coli* (Achberger, Tahara and Whiteley 1982b). Since it had a similar size to the 9 kDA ω subunit (first termed ω_2), this additional 11 kDa protein was originally named ω_1. Subsequent studies in *B. subtilis* showed that the protein was expressed (Nicolas *et al.*, 2012) and associated with RNAP during different growth phases, and during environmental stress (Delumeau *et al.*, 2011). This protein was also shown to remain tightly bound when overexpressing the $\alpha_2\beta\beta'\omega$ RNAP complex (Yang and Lewis 2008), suggesting that it is indeed a real subunit, rather than just an RNAP-associated factor. This hypothesis was further supported by the finding that ω_1, as well as ω, is present in the cell at equal molecular amounts to β (Delumeau *et al.*, 2011).

Notably, since its initial description in the literature in 1978, no significant advances have been made to functionally and structurally characterize this protein, until recently. Acknowledging this lack of focus, Keller *et al.* (2014) investigated ω_1 in *B. subtilis* and confirmed its interaction with RNAP by examining co-localization of the protein with RNAP, proving that it actually functions as a true subunit. Furthermore, sequence analysis revealed that it is conserved only in the Firmicutes and shows no sequence similarity to the ω subunit. Therefore, the protein was recently renamed the epsilon subunit (ε), and its encoding gene termed *rpoY*. Interestingly, in Firmicute genomes sequenced to date, *rpoY* is located in a bicistronic operon with the gene encoding RNase J1 (*rnjA*). This RNase has been extensively described as being required for post-transcriptional regulation, including RNA degradation and maturation (Britton *et al.*, 2007; Deikus and Bechhofer 2011; Linder, Lemeille and Redder 2014). Although a connection for these two gene products has yet to be established, it is tempting to suggest that a functional importance may exist for both elements in posttranscriptional processes and/or transcript maturation.

When analyzing ε structure by X-ray crystallography, a $\beta\beta\alpha\beta$ motif that may putatively influence protein–protein interaction was described (Keller *et al.*, 2014). Interestingly, the structure of ε shows homology to Gp2 proteins, which are found in bacteriophages that infect Gram-negative bacteria (Hesselbach and Nakada 1977), and have been shown to block RNAP function by preventing open complex formation (Nechaev and Severinov 1999; Camara *et al.*, 2010; James *et al.*, 2012; Bae *et al.*, 2013). Indeed, similar to Gp2, it was shown that ε binds to the β' jaw and secondary channel of RNAP, potentially preventing access of phage proteins to the transcriptional machinery (Keller *et al.*, 2014). Although Gp2 is known to be present only in phages that infect Gram-negative bacteria, Keller and colleagues hypothesize that the ε subunit could represent a protective factor against thus far unidentified Gp2-like proteins of Gram-positive bacteriophages. This suggestion is in line with the increasing number of mechanisms that are being documented which protect bacteria against phage infection (Labrie, Samson and Moineau 2010). Even though the structural data derived by Keller *et al.* (2014) support such a hypothesis, no experimental evidence, including phage infection assays, has yet been established. Additionally, no phenotypes or effects on transcription *in vitro* or *in vivo* were reported upon ε deletion. This makes the ε subunit of RNAP an interesting target for future experimentation in *B. subtilis* and other Gram-positive bacteria in general.

CONCLUDING REMARKS

With the wealth of information available on small RNAP subunits in Gram-positive bacteria, it is clear that these proteins present an important part of the transcriptional machinery. Studies describing the role of δ, ε and ω have been conducted over several decades, and considerable progress has been made. These subunits have been shown by countless groups to possess vital functions, ranging from guiding the assembly and structural integrity of RNAP, σ factor selectivity, influencing promoter specificity and potential roles in protecting cells against phage infection. Their significance is mirrored by their presence and conservation within nearly all of the Gram-positive genomes thus far sequenced. With modern high-throughput transcriptomic and proteomic approaches, as well as structural studies, information has been brought to light that allows explanation of the numerous phenotypes observed for null mutants of the subunits in various bacterial species. Nevertheless, the underlying mechanism by which these proteins mediate their function is still incompletely understood. It will be fascinating to observe how new approaches and studies, combined with this existing body of knowledge, will guide future work to reveal the precise role of these subunits within bacterial cells.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSRE online.

ACKNOWLEDGEMENTS

We would like to thank William Broach, Christina Krute and Halie Miller for their review of the manuscript, as well as Galen Madden for help designing the illustrations.

FUNDING

This study was supported in part by grant AI080626 (LNS) from the National Institute of Allergies and Infectious Diseases.

Conflict of interest. None declared.

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Supplemental Table S1: Species names, IMG Gene IDs and protein length for δ sequences contained with the Figure 1A alignment. All protein information was obtained from the Integrated Microbial Genome (IMG) system (https://img.jgi.doe.gov) (Markowitz *et al.*, 2014).

# in Figure 1A	IMG Gene ID	Species	Protein length (aa)
1	645482284	*Selenomonas flueggei*	109
2	650788107	*Carnobacterium sp.*	205
3	2511470775	*Pelosinus sp.*	122
4	640209387	*Thermosinus carboxydivorans*	121
5	651578560	*Acetonema longum*	125
6	2509275160	*Desulfosporosinus orientis*	136
7	641688320	*Exiguobacterium sibiricum*	174
8	644183400	*Catenibacterium mitsuokai*	111
9	642323807	*Clostridium spiroforme*	114
10	642191922	*Clostridium ramosum*	113
11	646240522	*Mollicutes bacterium*	110
12	643184683	*Eubacterium biforme*	113
13	650272364	*Clostridium sp.*	106
14	2514580865	*Eubacterium sp.*	107
15	646852895	*Bacillus selenitireducens*	174
16	2523747826	*Jeotgalicoccus marinus*	170
17	643658815	*Macrococcus caseolyticus*	173
18	2517443193	*Staphylococcus massiliensis*	176
19	643727220	*Staphylococcus carnosus*	184
20	2520306012	*Staphylococcus sp.*	179
21	647470149	*Staphylococcus epidermidis*	178
22	646636532	*Staphylococcus lugdunensis*	173
23	637888727	*Staphylococcus aureus*	176
24	642562690	*Staphylococcus haemolyticus*	172
25	2518442252	*Allofustis seminis*	180
26	644194367	*Listeria grayi*	182
27	639707453	*Listeria welshimeri*	176
28	2514158098	*Listeria monocytogenes*	178
29	2520299204	*Catellicoccus marimammalium*	188
30	2514553789	*Dolosigranulum pigrum*	172
31	651443943	*Fructobacillus fructosus*	180
32	651429677	*Leuconostoc fallax*	186

33	644429341	*Leuconostoc mesenteroides*	192
34	641604257	*Leuconostoc citreum*	193
35	651010218	*Leuconostoc sp.*	193
36	2520072467	*Leuconostoc gelidum*	193
37	650764460	*Aerococcus urinae*	213
38	644363720	*Catonella morbi*	225
39	2514611694	*Facklamia languida*	221
40	650258718	*Eremococcus coleocola*	227
41	651114493	*Lactococcus lactis*	187
42	2511651893	*Lactococcus garvieae*	186
43	650788107	*Carnobacterium sp.*	205
44	646090746	*Granulicatella adiacens*	204
45	2523707725	*Bavariicoccus seileri*	214
46	2514413459	*Lactobacillus versmoldensis*	213
47	650345384	*Streptococcus vestibularis*	191
48	638493971	*Streptococcus pneumoniae*	197
49	650333312	*Streptococcus infantis*	191
50	2515219321	*Streptococcus massiliensis*	196
51	650086157	*Streptococcus anginosus*	195
52	2512471342	*Streptococcus constellatus*	195
53	2515263509	*Streptococcus ovis*	196
54	2519825720	*Streptococcus minor*	194
55	2523205322	*Streptococus plurextorum*	197
56	650855100	*Streptococcus parauberis*	187
57	2523948014	*Streptococcus castoreus*	197
58	648821780	*Streptococcus pyogenes*	202
59	2515275679	*Streptococcus didelphis*	188
60	2515731753	*Streptococcus mutans*	194
61	2515297213	*Streptococcus caballi*	193
62	648815525	*Streptococcus bovis*	190
63	650330852	*Streptococcus equinus*	190
64	2512479078	*Streptococcus macacae*	193
65	2515262707	*Streptococcus ferus*	194
66	2511692614	*Tetragenococcus halophilus*	203
67	2515485516	*Enterococcus columbae*	201
68	650845241	*Melissococcus plutonius*	210
69	2519529468	*Enterococcus faecalis*	208
70	2514448739	*Enterococcus saccharolyticus*	201
71	2522028557	*Enterococcus hirae*	203
72	651430688	*Weissella cibaria*	197

73	651432316	*Lactobacillus coryniformis*	186
74	643927141	*Lactobacillus paracasei*	223
75	2514683090	*Lactobacillus zeae*	224
76	2523925542	*Lactobacillus harbinensis*	228
77	2524573453	*Lactobacillus saerimneri*	190
78	644905590	*Lactobacillus plantarum*	202
79	646291449	*Lactobacillus coleohominis*	190
80	2514646519	*Lactobacillus suebicus*	195
81	641713578	*Lactobacillus fermentum*	195
82	650245840	*Lactobacillus oris*	165
83	2500070755	*Lactobacillus reuteri*	185
84	648250028	*Lactobacillus salivarius*	208
85	2514643970	*Lactobacillus mali*	191
86	651443154	*Lactobacillus animalis*	189
87	2514648593	*Lactobacillus malefermentas*	206
88	644307024	*Lactobacillus hilgardii*	211
89	637796229	*Lactobacillus sakei*	202
90	644358292	*Lactobacillus acidophilus*	184
91	639672085	*Lactobacillus gasseri*	186
92	2514731674	*Lactobacillus sp.*	181
93	637622251	*Bacillus clausii*	168
94	637060510	*Bacillus halodurans*	164
95	641380263	*Bacillus weihenstephanensis*	175
96	2519373257	*Bacillus cereus*	176
97	637508443	*Bacillus anthracis*	175
98	639751904	*Bacillus thuringiensis*	176
99	642460509	*Bacillus anthracis*	175
100	637326668	*Oceanobacillus*	173
101	2514400189	*Ornithinibacillus sp.*	187
102	2514123921	*Halobacillus halophilus*	178
103	2520240165	*Salimicrobium sp.*	183
104	639313077	*Bacillus licheniformis*	171
105	642878827	*Bacillus pumilus*	179
106	643431281	*Anoxybacillus flavithermus*	199
107	644800105	*Geobacillus sp.*	186
108	650287269	*Planococcus donghaensis*	188
109	2520841753	*Bacillus isronensis*	194
110	641573981	*Lysinibacillus sphaericus*	184
111	2520890491	*Lysinibacillus fusiformis*	183
112	2514700644	*Bacillus smithii*	182

113	642982695	*Bacillus coahuilensis*	177
114	2529279564	*Sporosarcina sp.*	188
115	2506240325	*Sulfobacillus thermosulfidooxidans*	105
116	2506612322	*Sulfobacillus acidophilus*	97
117	645028913	*Alicyclobacillus acidocaldarius*	174
118	651560222	*Caldalkalibacillus thermarum*	165
119	643788320	*Brevibacillus brevis*	186
120	646752012	*Bacillus tusciae*	167
121	651607961	*Desmospora sp.*	147
122	2512925420	*Saccharibacillus sacchari*	186
123	2511571733	*Paenibacillus terrae*	182
124	2516613897	*Paenibacillus sp.*	187
125	650065345	*Paenibacillus vortex*	188
126	2507047969	*Paenibacillus lactis*	185
127	650134438	*Paenibacillus larvae*	179
128	2508845954	*Thermobacillus composti*	193
129	2516584637	*Cohnella panacarvi*	200
130	646320341	*Bacillus subtilis*	173

APPENDIX II

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Infection and Immunity

The δ Subunit of RNA Polymerase Guides Promoter Selectivity and Virulence in Staphylococcus aureus

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Infect. Immun. **2014, 82(4):1424. DOI: 10.1128/IAI.01508-14. Published Ahead of Print 3 February 2014.**

IAI Journals.ASM.org

The δ Subunit of RNA Polymerase Guides Promoter Selectivity and Virulence in *Staphylococcus aureus*

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In Gram-positive bacteria, and particularly the *Firmicutes*, the DNA-dependent RNA polymerase (RNAP) complex contains an additional subunit, termed the δ factor, or RpoE. This enigmatic protein has been studied for more than 30 years for various organisms, but its function is still not well understood. In this study, we investigated its role in the major human pathogen *Staphylococcus aureus*. We showed conservation of important structural regions of RpoE in *S. aureus* and other species and demonstrated binding to core RNAP that is mediated by the β and/or β′ subunits. To identify the impact of the δ subunit on transcription, we performed transcriptome sequencing (RNA-seq) analysis and observed 191 differentially expressed genes in the *rpoE* mutant. Ontological analysis revealed, quite strikingly, that many of the downregulated genes were known virulence factors, while several mobile genetic elements (SaPI5 and prophage ϕSA3usa) were strongly upregulated. Phenotypically, the *rpoE* mutant had decreased accumulation and/or activity of a number of key virulence factors, including alpha toxin, secreted proteases, and Panton-Valentine leukocidin (PVL). We further observed significantly decreased survival of the mutant in whole human blood, increased phagocytosis by human leukocytes, and impaired virulence in a murine model of infection. Collectively, our results demonstrate that the δ subunit of RNAP is a critical component of the *S. aureus* transcription machinery and plays an important role during infection.

Bacterial gene transcription is a complex, multifactorial process that involves several key enzymes and regulatory elements. It is driven by the activity of DNA-dependent RNA polymerase (RNAP) and its associated proteins, which form a multisubunit enzyme consisting of one β subunit, one β′ subunit, two identical α subunits, and one ω subunit (reviewed in reference 1). Together these form the RNAP apoenzyme, which is able to perform RNA elongation and termination; however, initiation requires the involvement of a σ factor. Typically, most bacterial species harbor several different σ factors, a primary one (σ^A or σ^{70}) that mediates housekeeping gene transcription and a variety of alternative sigma factors, which aid in the response to unfavorable environmental conditions and stress.

In certain Gram-positive species and particularly the *Firmicutes* (see Fig. S1 in the supplemental material), an additional RNAP subunit is present, termed the δ factor, or RpoE (2). In *Bacillus subtilis*, the 173-amino-acid delta subunit has been shown to reduce nonspecific binding of RNAP to DNA and lead to an elevated preference for DNA regions that include promoter sequences (3, 4). In early experiments, it was shown that RpoE has a role specifically confined to promoter selection and influences the ability of RNAP to form open promoter complexes (5, 6). Full-length RpoE has also been described as displacing nucleic acids from RNAP-DNA or RNAP-RNA complexes *in vitro*, which may explain the decreased affinity of RNAP-RpoE for nonpromoter regions. This feature of RpoE activity has additionally been attributed to its ability to stimulate RNAP recycling by enhancing the release of the complex from terminator sequences (6).

The effects of RpoE are far from arbitrary, since it has been shown that the δ subunit of RNAP not only distinguishes between promoter and nonpromoter sequences but also is important in the recognition of individual promoter features. A comparison of two ϕ29 promoters by transcription runoff revealed selective, promoter-dependent effects of RpoE (7). While the stronger of these two promoters was largely unaffected by RpoE, the weaker promoter showed significantly decreased expression in the presence of this factor. In order to explain the effect of RpoE on promoter selection, Rabatinova et al. (8) investigated the role of initiating nucleotide triphosphate (iNTP) recognition by RNAP and RNAP-RpoE complexes. iNTP is required to stabilize open complex formation and therefore to initiate transcription. It is thought that by destabilizing DNA-RNAP complexes, RpoE increases the amount of iNTP required to transcribe from certain promoters. Finally, in an approach aiming to identify functional regions of RpoE, Lopez de Saro et al. (9) created several truncated variants of the protein. In doing so, they showed that the N-terminal portion is required for binding to RNAP and also guides the correct orientation of the disordered and polyanionic C-terminal region. A limitation of these studies regarding RpoE function, however, is that they were performed *in vitro*, using only single promoters. Therefore, the true extent of δ-subunit influence on genome-wide transcriptional effects is still somewhat restricted at this point.

Phenotypically, *rpoE* mutants of *B. subtilis* have an extended lag phase upon subculturing of stationary-phase cells in fresh medium, as well as a moderately changed cell morphology (10). Furthermore, competition experiments revealed that when cocul-

Received 24 January 2014 **Accepted** 26 January 2014

Published ahead of print 3 February 2014

Editor: A. J. Bäumler

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.01508-14.

doi:10.1128/IAI.01508-14

tured with the wild type, δ-factor-lacking strains show decreased fitness and are outcompeted during growth over several days (8). Finally, a role for RpoE in sporulation has been suggested, since *rpoE* loss was shown to suppress a mutation (*pdhC*) that negatively influences sporulation (11).

The role of RpoE has also been addressed for a small number of pathogenic organisms, most notably in streptococcal species. RpoE in *Streptococcus mutans* is most abundant during exponential and early stationary phases of growth, and *rpoE* deletion causes an extended lag phase. *S. mutans rpoE* mutants are also more sensitive to environmental stresses (H_2O_2 stress and acid stress) and show alterations in biofilm formation and virulence (12). Interestingly, *rpoE* deletion was also reported to increase self-aggregation, coaggregation with other oral microorganisms (13), and an elevated ability to bind human extracellular matrix proteins. For *Streptococcus agalactiae*, there have been a limited number of studies which show greatest *rpoE* expression during exponential growth (14) and attenuated virulence of *rpoE* mutants in a rat sepsis model (15, 16) and human whole-blood survival assays.

In *Staphylococcus aureus*, the only information thus far regarding RpoE is that strains lacking this factor display defects in starvation-induced stationary-phase survival/recovery and slightly increased acid sensitivity (17). Notably, however, no investigation of its role in cellular physiology and virulence has been performed. Therefore, in this study, we investigated the role of RpoE in promoter selectivity using transcriptome sequencing (RNA-seq) technology and determined that in *S. aureus*, the δ subunit specifically guides RNAP toward strongly expressed promoters. Deletion of this factor results in a normalizing of transcriptional activity across genes, guiding RNAP away from key virulence-affecting loci. Such effects strongly impair the pathogenic potential of *S. aureus*, leading to diminished infection using a systemic model of sepsis and increased phagocytosis by human leukocytes. Collectively, our results suggest that *rpoE* is involved in orchestrating the ability of *S. aureus* to react and adapt to environmental changes and thus plays a critical role during virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used for experimental procedures are listed in Table 1. An *rpoE* transposon mutant in *S. aureus* USA300 strain JE2 was acquired from the Nebraska transposon library (NARSA). This mutation was transferred via ϕ11 transduction to our USA300 Houston wild-type strain (18). Successful transduction of this mutation was confirmed by PCR, using gene-specific (OL1709 and OL1710) and transposon-specific (OL14721 and OL1472) primers. Cultivation of bacteria was performed in tryptic soy broth (TSB) at 37°C. Where required, erythromycin (5 mg/ml), lincomycin (10 mg/ml), or chloramphenicol (10 mg/ml) was added to the medium. Synchronous cultures were obtained as described by us previously (19).

Construction of an *rpoE* complemented strain. The *rpoE* gene and its promoter were amplified via PCR, using primer pair OL1973 and OL1975, which are located 200 bp upstream from the *rpoE* translational start codon and at the 3′ end of the *rpoE* coding region, respectively. In addition to the native *rpoE* sequence, a hexahistidine (His_6) tag was included in the reverse primer (OL1975) to create a fusion protein that could be used for downstream purification. For the cloning of truncated *rpoE*-His_6 fragments, primers 2931 (half of the N terminus only, amino acids 1 to 159), 2932 (the N-terminal half of the protein, amino acids 1 to 264), and 2933 (the N-terminal half of the protein and half of the C terminus, amino acids 1 to 396) were used instead of 1975. The PCR products were cloned into shuttle vector pMK4 and transformed into chemically competent *Escherichia coli* DH5α. Clones were confirmed via PCR using the same set of oligonucleotides used for cloning. Additionally, Sanger sequencing using primers for the pMK4 multiple cloning site (M13Fw and M13Rv) was performed to confirm fidelity of the construct. The plasmids were transformed into *S. aureus* RN4220 by electroporation and confirmed by PCR. Correct clones were used to generate a ϕ11 lysate for transduction into the *S. aureus* USA300 Houston *rpoE* transposon mutant, which was again confirmed as described above.

Mapping the *rpoE* promoter. Rapid amplification of cDNA ends (RACE) was used to identify the *rpoE* transcriptional start site. For this approach, we used the 5′RACE core set (TaKaRa) and primers OL2018, OL2019, OL2020, OL2021, and OL2022 (Table 1). PCR products were TA cloned using the StrataClone PCR cloning kit (Agilent). The transcriptional start site was identified by sequencing (MWG operon) 10 plasmids that originated with the TA cloning using the universal primers M13Fw and M13Rv.

qPCR. Quantitative real-time PCR (qPCR) analysis was conducted as described previously (20) using the *rpoE* gene-specific primers OL1976 and OL1978 and OL2981 and OL2982 and 16S rRNA primers OL1184 and OL1185. Three independent replicates were used to calculate final values.

Zymograms and Western blot analysis. *S. aureus* strains were grown as described above, with samples taken at the time points specified. Zymograms were performed with culture supernatants as described by us previously (21). Intracellular and secreted protein fractions were harvested and subjected to SDS-PAGE and Western blot analysis as described by us previously (21). Immunoblotting was performed with a mouse monoclonal anti-His_6 (Covance) or anti-LukS (IBT) antibody at a 1:1,000 or 1:20.000 dilution, respectively, overnight at 4°C. Secondary antibodies were affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Cell Signaling Technology).

RpoE pulldown assay. A coprecipitation was performed to identify proteins that interact with RpoE. Briefly, *rpoE*-negative strains carrying pMK4::*rpoE*-His_6 or pMK4::*rpoE*trunc1-His_6 were harvested after 3 h of growth, and intracellular fractions were isolated as described previously (22). Cellular extracts were mixed with nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen), and samples were incubated with agitation at 37°C for 1 h. Beads were then washed 5 times (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) while rotating for 10 min at 37°C. After each wash step, beads were allowed to settle for 10 min, the supernatant was removed, and fresh washing buffer was added. RpoE and any interacting proteins were recovered by the addition of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). Isolated proteins were then used either to perform SDS-PAGE with subsequent silver staining or for mass-spectrometric analysis, as described by us previously (23).

Transcriptomic analysis via RNA-seq. For both the wild-type and mutant strains, three independent cultures were grown as described above. Synchronized cultures were harvested after 3 h, and RNA was isolated using a Qiagen RNeasy kit (Qiagen). To ensure complete removal of genomic DNA, RNA was treated with DNase I (Turbo DNA free; Ambion). The concentration and purity of RNA were determined using an Agilent 2100 bioanalyzer. Following quantification, equimolar amounts of RNA, from each of the three replicate preparations, were pooled, and rRNA was removed using the MICROBExpress (Invitrogen) and Ribo-Zero (Epicentre) kits. Removal of rRNA was confirmed, again employing the Agilent 2100 bioanalyzer. rRNA-depleted samples were then used for RNA-seq analysis using the IonTorrent Total RNAseq kit v2 according to the manufacturer's instructions (Life Technologies). Templated ion sphere particles were generated using the Ion OneTouch 200 template kit, v2. Sequencing was performed on an IonTorrent 318 chip using an Ion PGM 200 sequencing kit. Data generated were exported to the CLC Genomics Workbench software package for analysis. Reads were aligned to the USA300 FPR genome, and expression values for each gene were determined as RPKM (reads per kilobase per million reads) values. An RPKM threshold of detection value of 10 was imposed as a lower-level cutoff, and data were normalized using the quantile normalization ap-

TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype and/or description or sequence[a]	Reference or source
E. coli		
DH5α	Cloning strain	47
S. aureus		
RN4220	Restriction-deficient strain	Lab stocks
USA300 HOU	USA300-HOU MRSA isolate cured of pUSA300-HOU-MRSA	19
NE 646	USA300 JE2 *rpoE*::Bursa, *rpoE* mutant	NARSA
JAI1287	USA300 HOU *rpoE*::Bursa, *rpoE* mutant	This study
JAI1607	USA300 HOU *rpoE*::Bursa, pMK4::*rpoE*-His_6, $rpoE^+$	This study
JAI1569	USA300 HOU *rpoE*::Bursa, pOS1sGFP-P_{sarA}-*sod*, *rpoE* mutant	This study
JAI1570	USA300 HOU pOS1sGFP-P_{sarA}-*sod*	This study
Plasmids		
pMK4	Gram-positive shuttle vector	48
pJAI101	pMK4::*rpoE*-His_6	This study
pAW102	pMK4::*rpoE*-His_6trunc1, truncated RpoE, N terminus and half-C terminus	This study
pAW103	pMK4::*rpoE*-His_6trunc2, truncated RpoE, N terminus	This study
pAW104	pMK4::*rpoE*-His_6trunc3, truncated RpoE, half-N terminus	This study
pOS1sGFP-P_{sarA}-*sod*	*sarA* promoter controlling expression of *gfp*	25
Primers		
OL1471	TTTATGGTACCATTTCATTTTCCTGCTTTTTC	49
OL1472	AAACTGATTTTTAGTAAACAGTTGACGATATTC	49
OL1709	ATGAAAATTCAAGATTATACAAAAC	This study
OL1710	AATAGTTGGTGCGATTTTCTCTTC	This study
OL1184	AGCCGACCTGAGAGGGTGA	50
OL1185	TCTGGACCGTGTCTCAGTTCC	50
OL1973	CGCGGATCCAAGGACCAATTGGCAAAGAACGAC	This study
OL1975	ACCGGAGTCGACTTAATGGTGATGGTGATGGTGATCGTTGAAGTCTTCTTCGTCTTC	This study
OL1976	GAGCGTTAGGTGATTATGAGTACG	This study
OL1978	CACTTCTTCGTCTTCGTCTAGTTC	This study
OL2018	TTAATCGTTGAAGTC	This study
OL2019	GTTTTGTATAATCTTGAATTTTC	This study
OL2020	CAATAAATGATTTTTCATCAACC	This study
OL2021	CAATCATTCGGATATAGTCATTG	This study
OL2022	GATGAAGATGAACTAGACGAAG	This study
OL2931	ATGGTCGACTTAGTGGTGATGGTGATGATGATTTTCAATTTCTTCGTACTC	This study
OL2932	ATGGTCGACTTAGTGGTGATGGTGATGATTTCAATATCATCTACCGAATACC	This study
OL2933	ATGGTCGACTTAGTGGTGATGGTGATGATGTTGATCATCTGTTTGAGCTGG	This study
OL2981	TCGTACTCATAATCACCTAAC	This study
OL2982	GGCGAAACAATGAACTTAT	This study

[a] Underline denotes restriction enzyme site.

proach (24). Genes demonstrating changes in expression lower than 2-fold were excluded from further analysis.

Assay to detect alpha-hemolysin activity. The activity of alpha-hemolysin was determined as described by us previously (25) using pooled, whole human blood (Bioreclamation). Hemolysin activity (Ha) was calculated using the following equation: Ha = $(OD_{543} \times 1{,}000)/(\text{vol. of sample} \times 15 \text{ min} \times OD_{600} \times 0.5)$.

Coinfection of whole human blood. The ability of strains to survive in human blood was determined by seeding equal amounts of exponentially growing wild-type and *rpoE* mutant bacteria into pooled, whole human blood (Bioreclamation). Pooled blood was used to minimize effects that result from single-donor samples, such as being preconditioned by *S. aureus* infection or variation due to the immune status of the donor. Samples were incubated with shaking at 37°C, with aliquots withdrawn hourly to determine the bacterial burden by serial dilution and plating on TSA and TSA supplemented with erythromycin. Bacteria growing on TSA reflect the total number of cells in the sample, while TSA containing erythromycin allows only *rpoE* mutants to grow.

Analysis of differential phagocytosis by human leukocytes using flow cytometry. Phagocytosis of wild-type and mutant cells was determined using pooled, whole human blood (Bioreclamation) and flow cytometry, as described by us previously (25). Experiments comparing the wild type and mutant were conducted using the same batch of blood in order to exclude variations resulting from different blood samples. These studies were facilitated by the generation of green fluorescent protein (GFP)-expressing variants of the USA300 Houston wild type and its *rpoE* mutant using plasmid pOS1sGFP-P_{sarA}-*sod* RBS (26). Cells were analyzed using forward and side scattering in a fluorescence-activated cell sorting (FACS) Excalibur cytometer (BD Biosciences). FACSDiva version 6.1.3 software (BD Biosciences) was used to analyze the data. Results represent the averages for three independent experiments and are presented as the percentages of GFP-positive cells plus or minus standard errors of the means (SEM).

Coinfection model of murine sepsis and dissemination. In order to determine the virulence of strains, a murine coinfection model of sepsis was employed. These experiments were conducted as described previ-

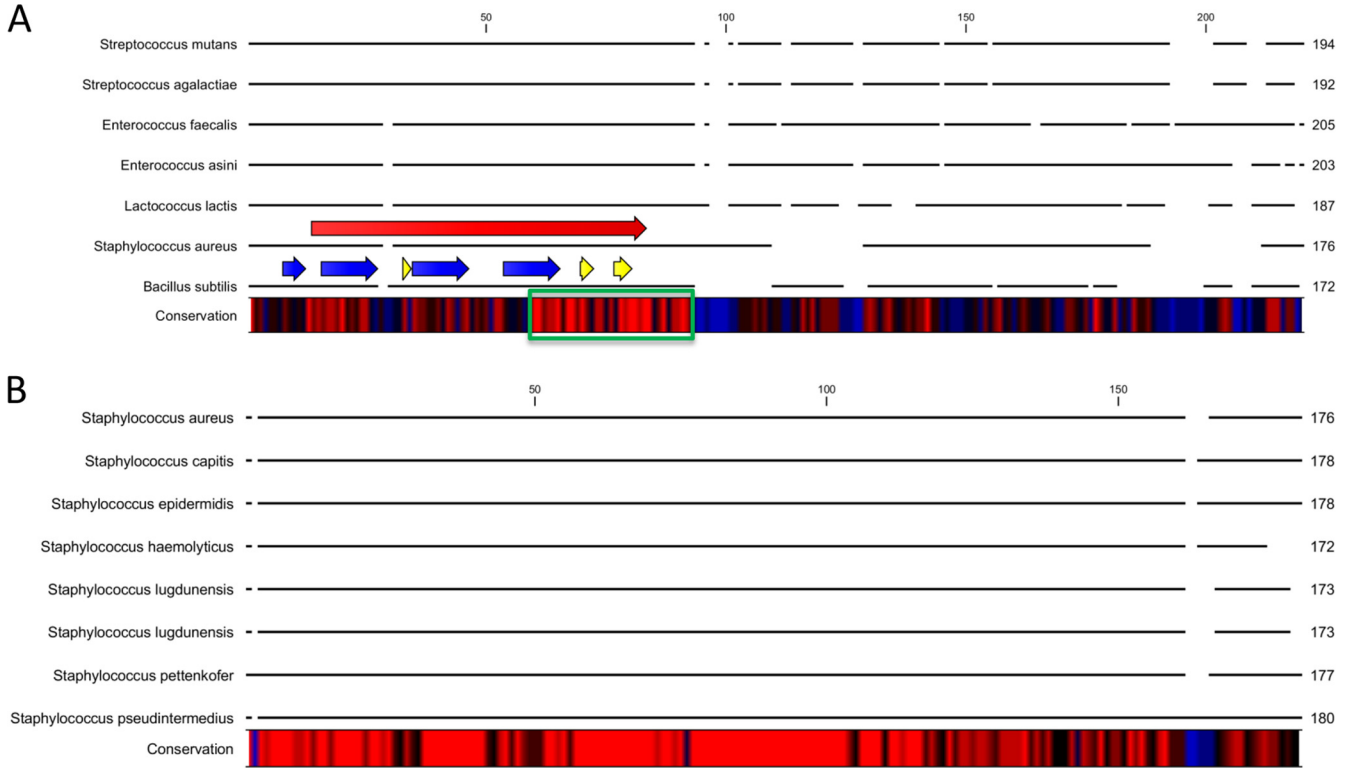

FIG 1 Protein alignment of the δ subunit of RNAP. Alignments of representative RpoE proteins from different species were prepared using CLC Main Workbench software. (A) Comparison of RpoE sequences between *S. aureus* and other Gram-positive organisms. Colors represent the degree of similarity, with red being the most identical and blue/black the most divergent. Broken black lines next to the species names represent alignment of proteins to the consensus sequence. The green rectangle denotes the region of highest conservation among different species; the red arrow marks the HARE-HTH motif identified by Pfam search using the *S. aureus* protein; blue and yellow arrows represent α-helices and β-sheets, respectively, identified previously in the *B. subtilis* protein (28). (B) Comparison of RpoE between staphylococci.

ously (27), with the following modifications. Briefly, 6-week-old, female CD-1 Swiss mice were purchased from Charles River Laboratories and housed in the vivarium at the College of Medicine, University of South Florida. Ten mice were inoculated by tail vein injection with 100 μl bacterial suspension (1×10^8 CFU/ml), containing the wild type and the *rpoE* mutant in a 1:1 ratio. The infection was then allowed to proceed for 7 days before mice were euthanized and the kidneys were collected. Each organ was homogenized in 3 ml sterile PBS, and the numbers of CFU/kidneys for the wild type and its *rpoE* mutant were determined via serial dilution, as described above. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of South Florida (permit number A-4100-01).

RESULTS

***S. aureus* RpoE shows homology to delta subunit proteins of other Gram-positive bacteria.** The majority of information regarding the δ subunit of RNAP comes from studies in *B. subtilis* and streptococcal species. We therefore compared the primary structure of the *S. aureus* counterpart to those of its homologs in other organisms to determine if differences in sequence were apparent (Fig. 1A). Interestingly, the proteins analyzed show significant variation across their sequences yet retain homology in the N terminus, with a conserved region between amino acid residues 14 and 91 (*S. aureus* amino acid numbering). A sequence search (pfam.sanger.ac.uk) revealed this region as containing a predicted helix-turn-helix (HARE-HTH) DNA-binding motif, which is also found in the *B. subtilis* version of the protein. Notably, the N-terminal region of the HTH motif (residues 14 to 57) shows decreased similarity between proteins from various species, while the C-terminal portion of the motif (residues 58 to 91) displays a high degree of homology. The presence of such an α-helix-rich region has previously been discussed for *B. subtilis* (28); however, its biological function is currently unknown. We also analyzed the similarity of RpoE between various staphylococci (Fig. 1B). As expected, we observed a much larger degree of similarity for protein sequences within the genus, although again, the C terminus displayed less conservation than the N terminus.

***rpoE* is transcribed from a single σ^A-dependent promoter during exponential growth.** To investigate the role of *rpoE* in *S. aureus*, we first began by defining its promoter. Using 5′ RACE, we detected a single transcriptional start site 68 bp upstream of the translation initiation codon, which is preceded by a strong σ^A-type promoter, comprised of a −35 sequence of TTGcgA (lowercase letters represent divergence from the consensus), followed by a perfect 17-nucleotide (nt) spacer and a consensus −10 sequence (TATAAT). Additionally, a strong ribosome binding site (AGGAaG) 9 nt upstream of the ATG initiation codon was observed. To track the growth phases and situations in which the *S. aureus* cell deploys RpoE, we next analyzed its transcription using quantitative real-time PCR (Fig. 2). We detected strongest

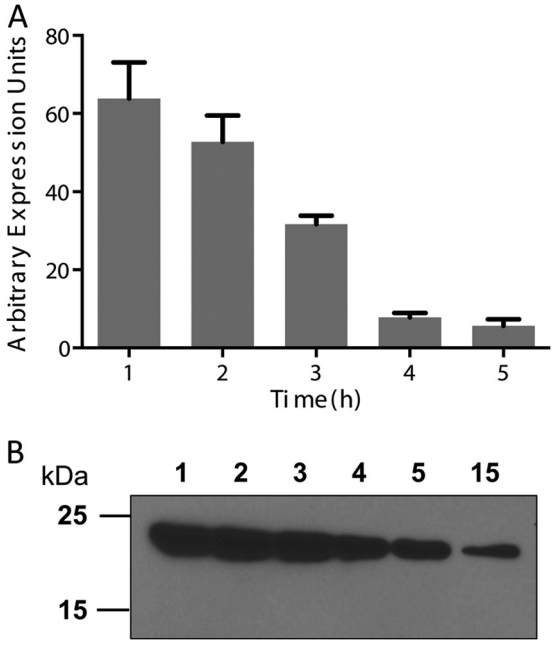

FIG 2 *rpoE* expression in *S. aureus* is maximal during exponential growth. (A) Quantitative real-time PCR analysis was used to measure *rpoE* expression from 3 independent cultures. Error bars ± SEM are shown. (B) Western blot analysis using an anti-His antibody. Samples were standardized using the Bradford assay, and an equal amount of protein for each time point was loaded onto gels. Shown is a representative image of 3 independent experiments.

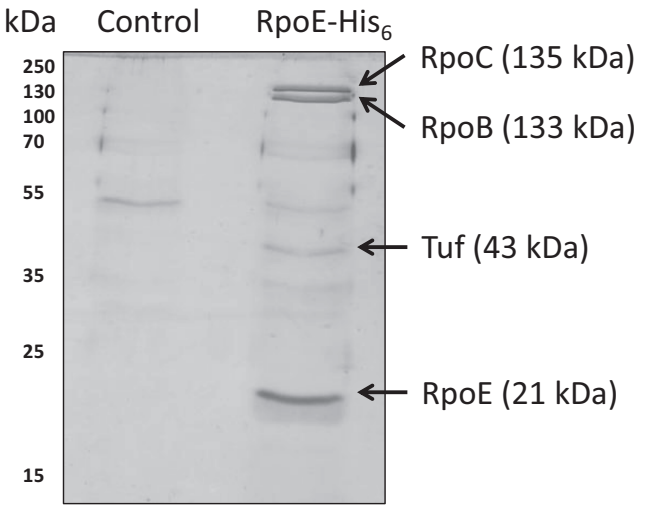

FIG 3 RpoE interacts with the *S. aureus* RNAP complex. A His-tagged version of RpoE was purified by pulldown assay from *S. aureus* cell lysates, alongside empty vector control samples, and run on an SDS-polyacrylamide gel (silver stained). Samples were subjected to mass-spectrometric analysis for protein identification. Shown are the identities of bands, alongside their molecular masses.

expression of *rpoE* during exponential growth, with a peak between 1 and 2 h and a subsequent decline at later time points. To correlate these findings with protein stability, we assessed the abundance of RpoE using a His_6-tagged variant expressed from its own promoter on the shuttle vector pMK4. Consistent with our transcriptional findings, we identified high protein levels during exponential growth, which again declined over time.

RpoE interacts directly with RNA polymerase. To determine whether the δ subunit binds to core RNAP in *S. aureus*, we performed a pulldown assay using a plasmid-encoded, His-tagged variant of the RpoE protein. Intracellular lysates were generated from exponentially growing *rpoE* mutants bearing either the pMK4::*rpoE*-His_6 construct or pMK4 alone (control). RpoE-His_6 and its interacting partners were then purified by pulldown assay and analyzed by SDS-PAGE and mass spectrometry. We identified 3 major protein bands on SDS-PAGE gels compared to control samples with molecular weights that correlate to those expected for RNA polymerase subunit β (RpoB), β′ (RpoC), and δ itself (Fig. 3). Using mass spectrometry, we confirmed that the identities of these bands did indeed correspond to the suggested components of RNAP. A fourth protein was also identified in test samples and was found to be elongation factor Tu (Tuf). Collectively, our results demonstrate that RpoE is part of the *S. aureus* transcription machinery and most likely interacts directly with the β and/or β′ subunit of RNAP, although we cannot rule out that weaker interactions with other subunits of this complex also occur.

To explore which components of RpoE mediate this interaction, we generated 3 His_6-tagged, truncated forms of the protein on the shuttle vector pMK4 in the *rpoE* mutant background. These contained the following: (i) the N terminus and half of the C terminus (amino acids 1 to 369), (ii) only the N terminus (amino acids 1 to 264), or (iii) half of the N terminus only (amino acids 1 to 159). Abundances of the truncated proteins were first measured by Western blotting (see Fig. S2A in the supplemental material). Interestingly, only the longest of the truncated versions was detected in these studies. To identify if this lack of protein resulted from a lack of truncated protein expression, we performed qPCR analysis (see Fig. S2B in the supplemental material). Importantly, we observed robust expression for all variants of *rpoE*. Since the proper construction of all plasmids was confirmed by Sanger sequencing, we concluded that the shortest variants of RpoE are likely characterized by protein instability. We next repeated our pulldown experiments using the longest of the RpoE truncated proteins to examine RNAP binding. Upon doing so, we observed results identical to those for the full-length RpoE protein (data not shown). As such, we were able to show that the C terminus of RpoE, at least in part, is dispensable for interaction with RNAP, further supporting the hypothesis that δ subunit-RNAP interactions are mediated by the RpoE N terminus. Interestingly, we also showed that in *S. aureus*, deleting the entire RpoE C terminus results in inherent instability for the entire protein and a lack of functionality for the *S. aureus* δ subunit.

The *S. aureus* δ subunit of RNAP strongly influences expression of virulence determinants and genes encoded on mobile genetic elements. Given that RpoE functions as a component of the transcriptional complex, we next set out to assess its role in influencing gene expression in *S. aureus*. This was achieved using RNA sequencing (RNA-seq) technologies and our *rpoE* mutant strain. Samples of the wild type and mutant were taken during exponential growth (3 h), and the changes in gene expression were compared. Upon analysis, we observed 191 differentially expressed genes in the *rpoE* mutant strain compared to expression in the parent (Fig. 4), with 83 positively regulated and a further 108 that are repressed (see Table S1 in the supplemental material). To

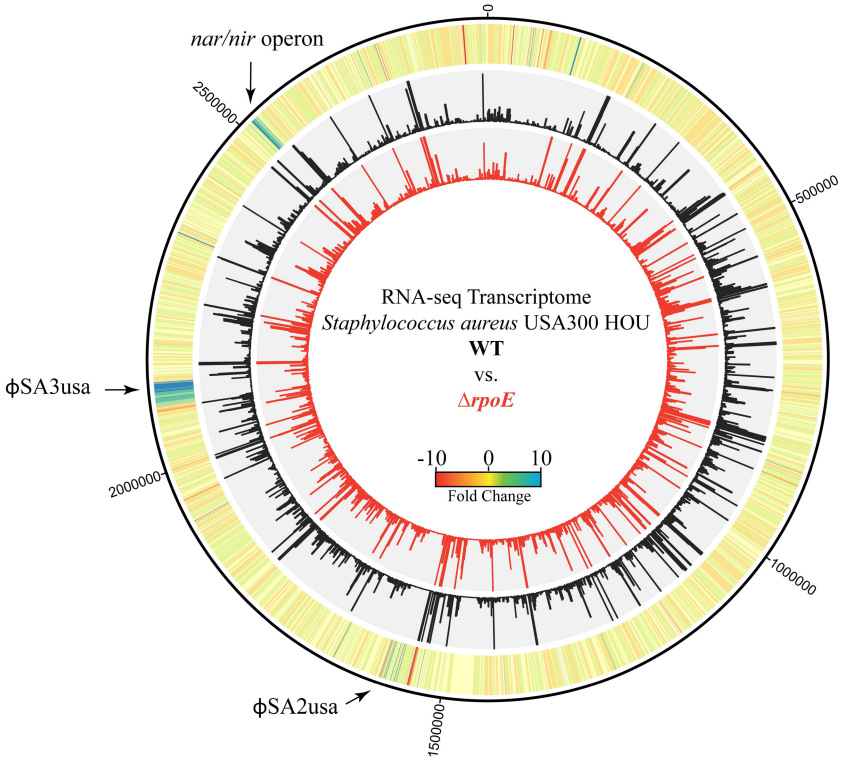

FIG 4 Genomic map of altered transcription profiles upon *rpoE* deletion. The outermost circle (yellow) represents a heat map comparing changes in gene expression for the *rpoE* mutant compared to results for the wild type. The middle circle (black) depicts RPKM values for the wild type, while the inner circle (red) represents RPKM values for the *rpoE* mutant (see Materials and Methods for an explanation). ϕSA3usa and ϕSA2usa denote both prophages found in *S. aureus* USA300.

confirm data derived from our RNA-seq experiments, we performed qPCR with a representative subset of genes, which confirmed the direction and fold change of expression observed (see Fig. S3 in the supplemental material).

When genes with decreased expression were reviewed by ontology (Fig. 5), one of the most striking observations was that the transcription of a very large number of known virulence factors is reduced in the *rpoE* mutant. These include 9 of the 10 major se-

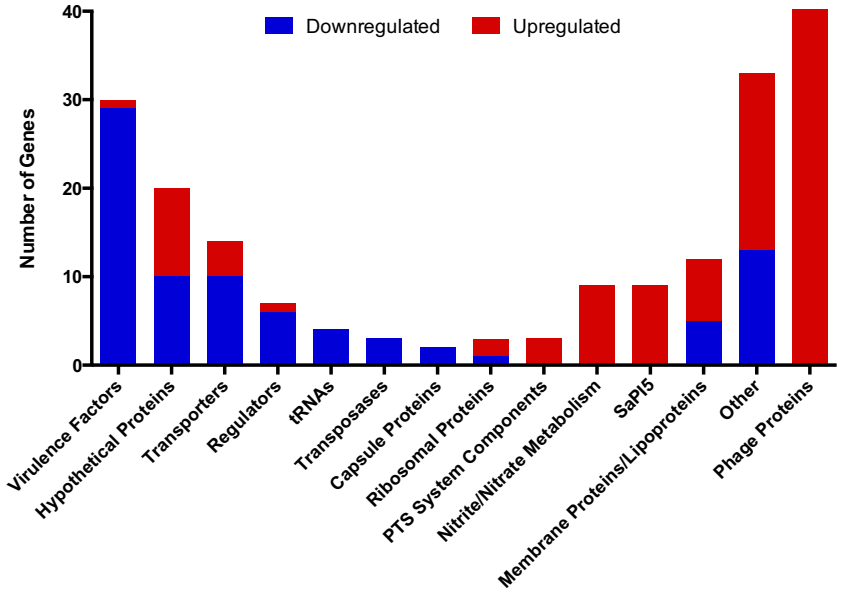

FIG 5 Ontological grouping of genes influenced by RpoE activity in *S. aureus*. A total of 191 genes showed at least a 2-fold alteration of expression in the *rpoE* mutant compared to that in the parental strain. Genes are grouped according to known or predicted ontologies.

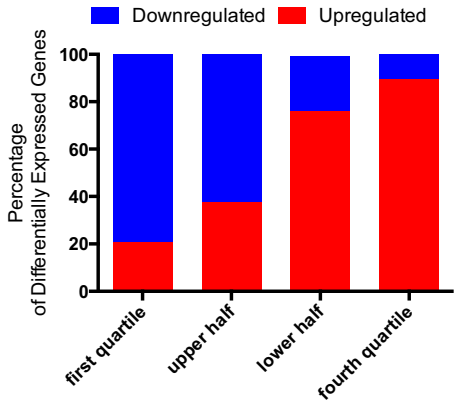

FIG 6 RpoE influences RNAP promoter selectivity based on promoter strength. The 191 genes identified as being differently expressed in the *rpoE* mutant were evenly separated into four groups based on strength of expression in the wild type. Specifically, the first quartile represents the 47 genes most highly expressed under standard conditions (3 h of growth in TSB) from the pool of 191. Conversely, the fourth quartile refers to the 47 genes expressed at the lowest levels from the pool of 191. Upon *rpoE* disruption, a normalizing affect is observed whereby highly expressed genes are overwhelmingly downregulated and low-expression genes are significantly upregulated.

creted proteases, components of 3 of the 4 bipartite toxins, nuclease, several phenol-soluble modulins (PSMs), alpha-hemolysin, beta-hemolysin, and a number of known immune evasion factors. To ensure that changes in gene expression for virulence factors was not due to unintended mutations in global regulatory systems, we sequenced the *agr* operon, RNAIII, and the *sae* operon. No alteration in gene sequence was found between the *rpoE* mutant and the wild-type strain, indicating that the observed changes were due to ablation of RpoE activity. Changes in the expression of key virulence regulation loci were noted (RNAII and RNAIII, ~1.4-fold; *saeR*, 2.8-fold), although to a significantly lesser extent than that observed for most virulence factors (4- to 8-fold). This suggests that the changes in expression of *agr* and *sae* do not completely explain the major alterations observed in virulence factor expression upon *rpoE* deletion.

Analysis of genes with increased expression in the mutant revealed, quite remarkably, that almost the entire ϕSA3usa prophage is upregulated (39 genes; increases ranging from 2.9- to 29-fold). Additionally, for the SaPI5 pathogenicity island, another mobile genetic element, expression of 9 genes was increased. Further, we observed upregulation of the *nar-nir* operon, as well as a variety of membrane and lipoproteins.

***rpoE* disruption leads to a normalizing of gene expression in *S. aureus*.** When a heat map of the changes in gene expression is reviewed (Fig. 4), it is apparent that those genes with altered expression in the mutants are for the most part evenly distributed over the entire genome (with the exception of phage ϕSA3usa, SaPI5, and the *nar-nir* operon). Interestingly, however, upon *rpoE* disruption, a normalizing of expression appears to occur. Specifically, the majority of genes that had decreased expression in the mutant are typically highly transcribed in the wild-type strain. Furthermore, genes that are typically expressed at a low level in the parent are significantly upregulated in the *rpoE*-lacking strain (Fig. 6). Therefore, the difference in expression between strongly and weakly transcribed genes becomes dampened, and due to the normalizing effect of *rpoE* disruption, growth-phase-specific expression patterns are less pronounced. These data support the suggestions from other organisms that RpoE influences promoter selectivity of RNAP based on promoter strength (6), whereby RpoE enhances the transcription of strong promoters and limits expression from weaker elements. In *S. aureus* such a function appears to have evolved to include the favoring of virulence factor promoters, which are commonly among the most highly expressed genes.

RpoE disruption leads to the decreased accumulation and activity of key virulence factors. Given that virulence factors were among the most profoundly affected upon *rpoE* disruption, we next sought to determine if these changes led to alterations in protein abundance and activity. As such, we first assessed Panton-Valentine leukocidin (PVL) protein levels via Western blotting (Fig. 7A). We determined that, much like the case with RNA-seq data, a significant decrease in the LukS signal was detected in culture supernatants from the *rpoE* mutant compared to results for the parental and complemented strains. Following this, we next performed activity profiling for alpha-hemolysin using whole human blood. Upon analysis of the hemolytic capacity of culture supernatants, we observed a 3-fold decrease in hemolytic activity upon *rpoE* disruption, which was largely restored by supplying *rpoE* in *trans* (Fig. 7B). Finally, given that 9 of the 10 secreted proteases had diminished expression in the mutant strain, we performed protease activity assays. When the mutant strain was cultured on casein nutrient agar, we observed a decrease in clearing around the *rpoE* mutant in comparison to results for the parent (Fig. 7C). Using zymography with gelatin as a substrate, we observed even more pronounced effects, with a significant reduction in proteolytic activity and with several key proteolytic bands proving to be absent in the mutant strain (Fig. 7D). Collectively, these findings confirm not only the RNA-seq observations that RpoE preferentially influences RNAP toward highly expressed virulence factor genes but that *rpoE* ablation leads to the diminished transcription, protein synthesis, and activity of central pathogenic determinants.

An *rpoE*-defective strain demonstrates decreased virulence in both human and murine models of infection. To determine if these global alterations in virulence factor production led to measurable outcomes for pathogenesis, we next investigated the ability of an *rpoE* mutant to survive and compete against the wild type in whole human blood. Accordingly, exponentially growing wild-type and *rpoE* mutant cells were inoculated into whole human blood in a 1:1 ratio. Aliquots were then withdrawn every hour for 3 h, and the number of CFU/ml of each strain was determined via plating on TSA alone or TSA containing erythromycin (which selects for the antibiotic resistance cassette in the mutant strain). Over the infection period, the percentage of *rpoE* mutant cells continually decreased (Fig. 8A), with a 1:0.46 ratio observed at 2 h (31.6% recovery of the mutant inoculum) and a 1:0.2 ratio observed at 3 h (17% recovery of the mutant inoculum). By comparison, identical studies performed with TSB over a similar 3-h period revealed no changes in viability for the *rpoE* mutant strain (see Fig. S4 in the supplemental material). This suggests that a virulence defect, rather than decreased fitness, mediates the impaired survival in whole human blood.

In order to explore this survival defect in human blood more closely, we next assessed interaction of the *rpoE* mutant and wild-type strains with human leukocytes. Accordingly, GFP-expressing variants of each strain were used to separately infect whole human

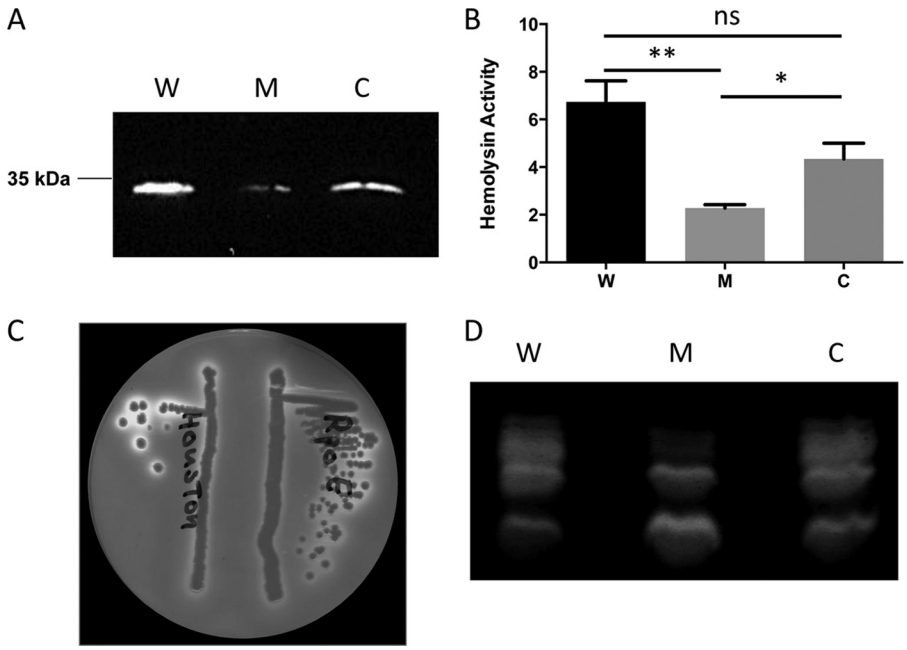

FIG 7 RpoE ablation leads to decreased accumulation and activity of key virulence factors. (A) LukS (33 kDa) Western blot using supernatant (15 h) from an *rpoE* mutant and its parental and complemented strains. The amount of protein loaded was standardized using a Bradford assay. (B) Hemolytic activity assay using culture supernatants and pooled, whole human blood. Data presented are from at least three independent replicates, with error bars ± SEM shown; *P* values were determined using Student's *t* test *, $P < 0.05$; **, $P < 0.005$; ns, not significant). (C and D) Protease activity assay using casein nutrient agar (C) or gelatin zymography (D). W, wild type; M *rpoE* mutant; C, complemented *rpoE* mutant.

blood. Samples were withdrawn at 2 h and 4 h, and the percentage of each strain present in granulocyte cells was determined using FACs analysis, as described by us previously (25). We determined that at both time points, there were significantly more *rpoE* mutant cells within human granulocytes than cells of the parental strain (Fig. 8B). These increased rates of phagocytosis are likely mediated by decreased virulence factor expression seen in the δ subunit mutant and explain the survival defect observed in whole human blood.

To determine if these *ex vivo* human findings were recapitulated *in vivo*, we assessed the infectious capacity of an *rpoE* mutant using a murine model of sepsis. Accordingly, we coinfected mice with 1×10^7 CFU/ml of the wild-type and *rpoE* mutant strains in a 1:1 ratio. After 7 days, mice were euthanized, the kidneys were harvested, and the bacterial burdens of the wild-type and mutant were determined. Three mice died before the end of the infection period and were excluded from analysis. When determining bacterial loads within the kidneys of surviving mice (Fig. 8C), we found a 10-fold decrease in *rpoE* mutant cells from the inoculum, with only 10% of the total bacterial burden resulting from the δ subunit mutant (Fig. 8D). These findings demonstrate that RpoE-mediated guidance of RNAP toward highly expressed promoters, including virulence factors, is required for successful *S. aureus* infection.

DISCUSSION

In this study, we sought to explore the role of the delta subunit of RNAP in the major human pathogen *S. aureus*. Despite being first identified as a unique, Gram-positive specific component of the RNAP apoenzyme more than 30 years ago (2), very little is understood about its role and function. Herein we show that in *S. aureus*, it is highly expressed early in growth and seemingly interacts with the β and β′ subunits of RNAP, suggesting they might be its binding partners *in vivo*. Interestingly, other subunits of RNAP were not copurified, leading to the conclusion that a direct interaction with the α, ω, and σ subunits is perhaps unlikely. Importantly, these data are the first evidence from any organism that points to putative interaction partners for RpoE, providing much-needed understanding of its functional role.

As a component of the RNAP complex, the role of the δ subunit is clearly confined to activities concerning gene expression. Therefore, we sought to understand and analyze its target genes in *S. aureus*, using RNA-seq technologies, during exponential growth (a time when RpoE is highly abundant in the *S. aureus* cell). We identified almost 200 genes that were changed in expression upon *rpoE* disruption. Interestingly, these genes displayed a strong pattern in terms of RpoE influence, with typically highly expressed genes in the wild type being strongly downregulated in the mutant, while many low-expression genes were upregulated. As such, loss of the δ subunit in *S. aureus* appears to lead to a normalization of gene expression, abrogating the more variable, temporal expression patterns typically observed. Such a finding is in line with the hypothesis that RpoE weakens binding of RNAP and DNA and therefore influences weaker promoters more than stronger ones (7). In support of this, Juang and Helmann (6, 29) distinguished 3 different kinds of promoters affected by RpoE: (i) weak promoters that are repressed by RpoE, (ii) moderate-strength promoters that show decreased open complex formation in the presence of the delta subunit, and (iii) strong promoters that are resistant to RpoE inhibition.

Such a role for the delta subunit of RNAP also appears to be conserved in *S. aureus* based on our findings, and this opens up an

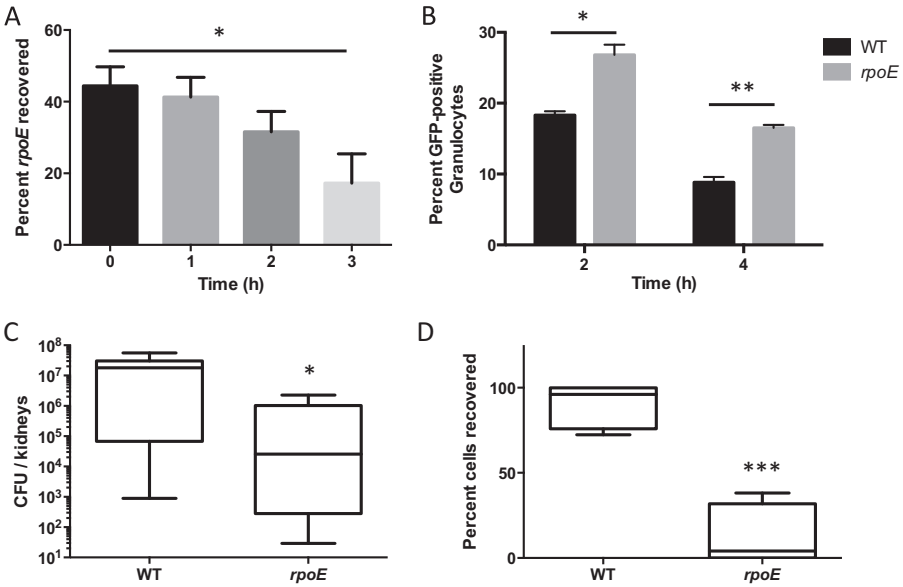

FIG 8 The δ subunit of RNAP is required for virulence in both human and murine models of infection. (A) Both the WT and mutant strains were inoculated into pooled (5 donors) whole human blood at a 1:1 ratio and incubated for 3 h at 37°C. Aliquots were withdrawn at the times specified, and the numbers of CFU/ml were determined by plating on either TSA or TSA containing erythromycin (to select for the mutant strain). Data are generated from three independent replicates; error bars ± SEM are shown. (B) Wild-type and *rpoE* mutant strains harboring a constitutively expressing GFP gene were separately incubated in whole human blood. Samples were withdrawn, and the percentage of GFP^+ granulocytes was measured by FACs. Data are generated from three independent replicates; error bars ± SEM are shown. (C and D) Mice were infected with an equal ratio of *rpoE* mutant and wild-type cells. After 7 days, mice were euthanized and bacterial loads in the kidneys were assessed. Box-and-whisker plots represent the minimum and maximum values (whiskers), as well as the 25th to 75th percentiles (boxes). The median for each group is indicated as a solid black line. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$; determined using Student's *t* test.

interesting point of evolution. It is believed that the influence of RpoE in selectively guiding RNAP toward important promoters is to mediate rapid changes in gene expression, and thus survival, during stress. Such a notion is supported by observations from both *B. subtilis* (8) and *S. mutans* (30), which demonstrate that *rpoE* ablation leads to an impaired ability to recover from stresses. Specifically, in *S. mutans* (30), it has been shown that an *rpoE* mutant has decreased accumulation of oxidative-stress defense proteins upon hydrogen peroxide challenge and a concomitant reduction in survival. These lower levels of protective proteins are not caused by downregulation in the mutant *per se* but rather a stronger, RpoE-influenced stress response in the parent, leading to increased accumulation of key protective proteins. This demonstrates that in the absence of RpoE, cells are less primed to respond transcriptionally to unfavorable conditions and mount a sufficiently protective response.

In *S. aureus* a lone study that references the δ subunit of RNAP, where an *rpoE* mutant was identified as having a markedly decreased ability to recover following prolonged starvation, exists (17). Herein we show (see Fig. S5 in the supplemental material) that such an effect is true even after overnight growth, since we observe an extended lag phase of our *rpoE* mutant similar to those described for other low-G+C Gram-positive organisms. It seems that such a defect is confined to the initial adaptation to new environments, since we observed otherwise unimpaired growth after entrance into log phase, with equal numbers of viable wild-type and mutant bacteria (data not shown). As such, it appears that RpoE may have evolved in Gram-positive bacteria, and the *Firmicutes* specifically, to facilitate a rapid switching of gene expression profiles that is geared toward the natural expression level of genes within a cell. The reason for this could be that by centralizing such a response within a key protein within the transcription complex, specific and global alteration in gene expression can be rapidly achieved upon environmental change, leading to expedient and efficient adaption and survival. Given that *S. aureus* is a highly human-adapted pathogen, much of its gene expression is dedicated to survival within the human host. Consequently, we contend that the δ subunit of RNAP has evolved in *S. aureus* along these virulence-adapted lines, since a large number of the most highly expressed genes in this organism are virulence specific. Therefore, RpoE provides *S. aureus* with a survival advantage upon entering the human host, which results in a survival defect upon the abrogation of its activity.

With that said, the manner by which RpoE mediates its function cannot be explained solely by mere promoter strength, since there are highly and low-level-expressed loci in *S. aureus* that are not influenced by its activity. When one compares RpoE protein sequences between various Gram-positive bacteria, there does appear to be a large variation in similarity other than in regions of the collected N termini. The N-terminal regions are characterized by the presence of several α-helices and β-sheets that were previously identified in the *B. subtilis* protein (28, 31) and are predicted to be present in the *S. aureus* counterpart (www.predictprotein.org). Such conservation is perhaps to be expected, since the N terminus is thought to be the component that mediates interaction with core RNA polymerase (9), which is itself a highly conserved protein complex. Unusually, this conserved region of α-helices and β-sheets is predicted to form a winged HTH domain (HARE-HTH). Although they have a wide range of functions in nature, including transcriptional regulation, DNA degradation, and restriction modification (32), all functions studied thus far for HARE-HTH domains involve some form of DNA binding. Thus,

even though no direct interaction with nucleic acids has yet been shown for the δ subunit of RNAP, a function in recognizing transcriptionally relevant DNA features would appear to be possible, if not probable, via this HARE-HTH domain. Such a situation would thus explain the selectivity of RpoE that extends beyond mere promoter strength.

By comparison, the C terminus of the protein shows little conservation between different species and is in fact the part of the protein responsible for significant interspecies variation and alterations in protein length. Due to its highly acidic nature and strongly disordered structure, thus far the function of the C terminus has remained obscure. Herein we show that deletion of this C terminus in *S. aureus* seemingly results in decreased stability of the RpoE protein, suggesting its importance for proper folding and function. In addition, it has been suggested that the C terminus may mediate interaction with nucleic acids and have a role in RpoE-dependent displacement of RNA and DNA from the RNAP complex (9). As such, it too may influence promoter selection in a manner hitherto not demonstrated.

Another remarkable observation from our RNA-seq analysis was the simultaneous upregulation of mobile genetic elements within the *rpoE* mutant. Specifically, we observed that most genes on the *S. aureus* pathogenicity island 5 (SaPI5) and prophage ϕSA3usa displayed strong increases in expression. In the context of the phage element, it is possible that these effects are mediated by alterations in transcription of a common regulator encoded within the prophage region (33). The shift between lytic and lysogenic pathways is commonly mediated by an altered abundance of competing regulators, which seek to swing the balance from one lifestyle to the other (reviewed in references 34 and 35). While the specific molecular details of the lytic/lysogenic decision are not known for ϕSA3usa, we do observe alterations in regulatory elements on this prophage in the *rpoE* mutant. Specifically, the phage transcriptional regulator SAUSA300_1968 is strongly altered in expression (+12-fold). This finding, together with the upregulation of most of the adjacent phage genes, leads to the postulation that SAUSA300_1968 may be a positive and global regulator of ϕSA3usa gene transcription. In terms of SaPI5, there is limited understanding of regulatory events that modulate expression of the genes it encodes. However, it is possible that an event similar to that suggested for ϕSA3usa occurs, since these elements are believed to have evolved from phages themselves. In line with this, SaPI5 in USA300 contains at least 2 uncharacterized regulatory elements (33), one of which (SAUSA300_0804) displays elevated gene expression upon *rpoE* deletion. As such, it is possible that upregulation of this element could account for changes in the expression of the pathogenicity island, as described for ϕSA3usa.

Alternatively, it is possible that RpoE has a wider role in the *S. aureus* cell in providing cellular immunity against phage infection. Although quite different, immunity systems in bacteria that seek to abrogate phage infections, such as clustered, regularly interspaced short palindromic repeat (CRISPR) elements, do exist (36). In such a scenario, RpoE may be involved in silencing foreign elements and therefore protecting the core genome.

In the context of virulence, the various defects observed for the *rpoE* mutant are readily explained. Numerous elements that are known to play integral roles in *S. aureus* disease causation, including Panton-Valentine leukocidin (PVL) (37–40) (reviewed in reference 41), alpha-hemolysin (42–44), and different proteases (21, 25), were shown to be decreased in the *rpoE* mutant. To explore how these changes are mediated, we first ensured (by Sanger sequencing and analysis of mRNA transcript fidelity from the RNA-seq reads) that unintended mutations were not present in key global regulators. We next assessed expression of key virulence regulators and found the *agr* locus to be unaffected by Δ*rpoE* deletion. We did observe downregulation of *saeR* (−2.8-fold) in our RNA-seq experiment, which may explain some of the effects observed (45). Indeed, Nygaard et al. (46) show that deletion of *saeRS* results in a downregulation of various virulence factors, many of which were also affected in the *rpoE* mutant. However, the limited alteration in *saeR* expression in the *rpoE* mutant is unlikely to be the sole reason for the effects observed. Specifically, the changes observed by Nygaard et al. result from complete loss of the *saeRS* locus (~120- and 178-fold downregulation). In our results, we see similar effects on virulence factor synthesis, with much smaller changes in *sae* system expression (e.g., for *splE*, Δ*saeRS* led to a −1.16-fold change in expression; Δ*rpoE* led to −2.89-fold change). Furthermore, *rpoE* deletion causes changes in gene expression that are not seen in the *saeRS* mutant. For example, aureolysin and several other virulence factors (e.g., V8 protease and PSMs) are either positively affected or unaffected in the *saeRS* mutant, while being negatively affected in the *rpoE* mutant. Accordingly, the effects observed in the *rpoE* mutant appear to be direct and gene specific rather than being caused solely by the differential expression of major virulence regulators.

In summary, we herein demonstrate the importance of the RNAP δ subunit for gene regulation and virulence in *S. aureus*. We reveal that *rpoE* loss results in an uncoupling of well-ordered regulatory circuits, resulting in viable yet less-fit *S. aureus* cells that are unable to rapidly adapt to stress, such as that encountered during infection. We describe a new layer of transcriptional regulation in *S. aureus* that, as in other bacteria, functions in a promoter-specific manner to deploy its global effects and, in this highly human-adapted pathogenic organism, uniquely targets those regions of the genome that are associated with virulence.

ACKNOWLEDGMENTS

We thank Karoly Szekeres for his technical help during FACs analysis.

Strain NE646 was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program, supported by NIAID, NIH contract no. HHSN272200700055C. This study was supported in part by grant AI080626 (to L.N.S.) from the National Institute of Allergies and Infectious Diseases.

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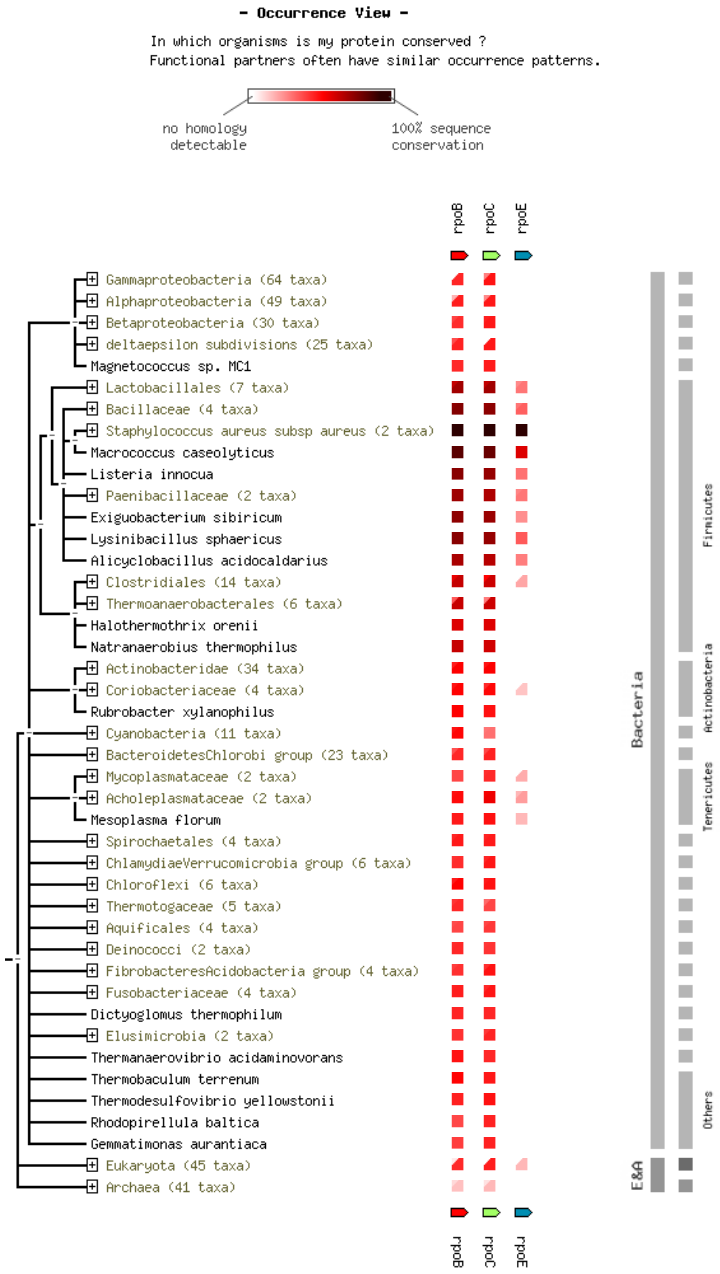
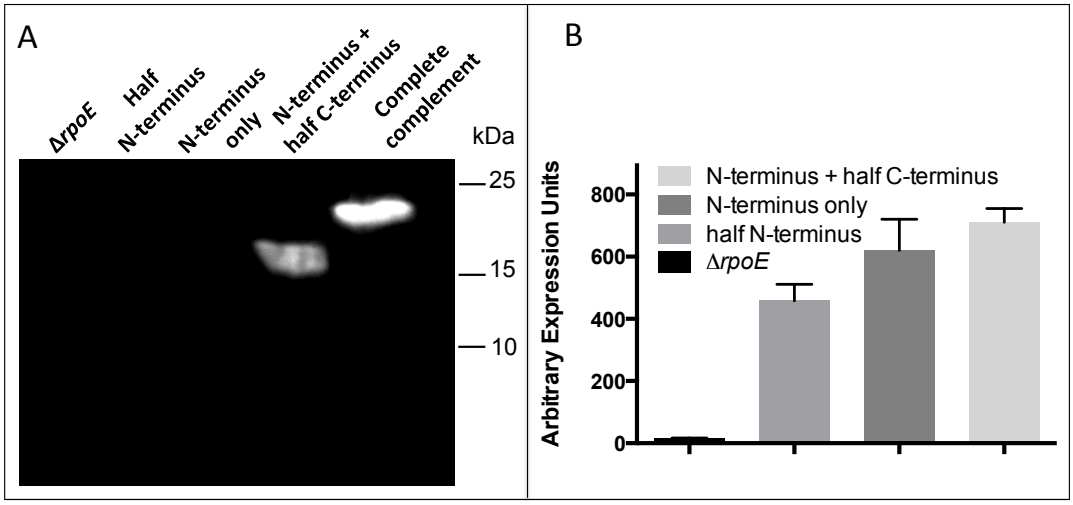
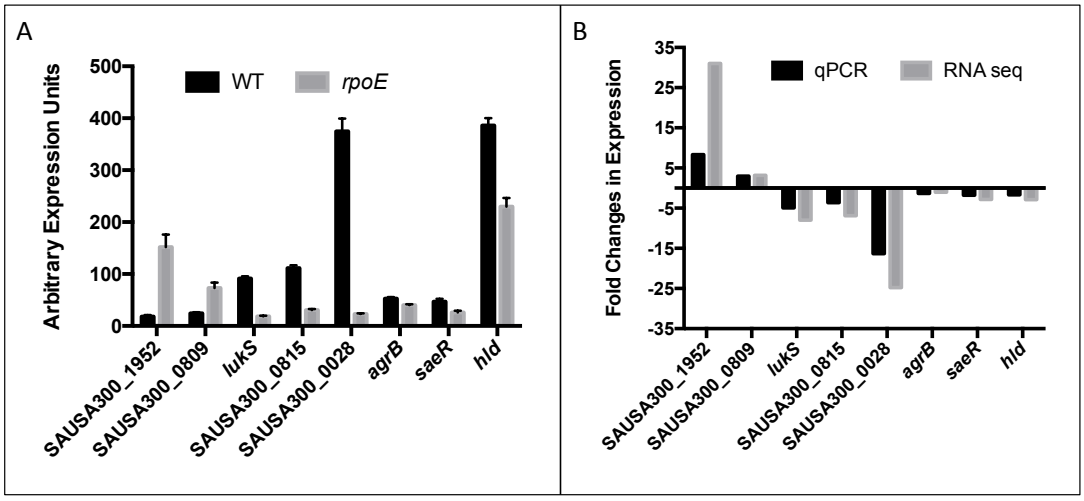


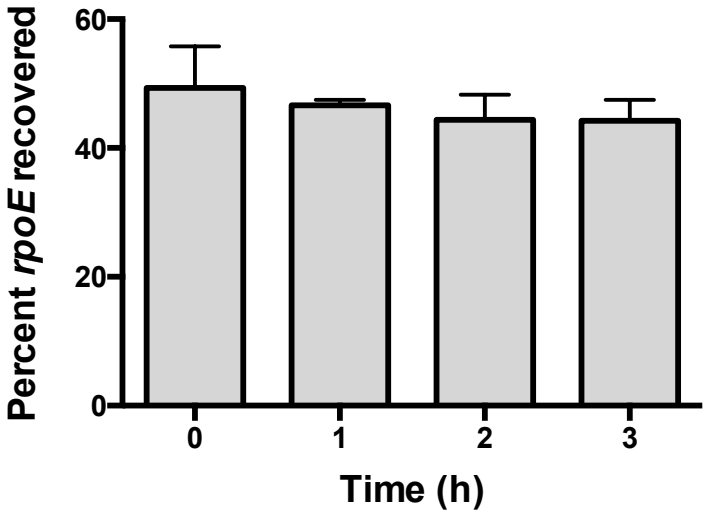
Supplemental Figure S1: The Distribution of RNAP δ subunits in nature. RpoE is found primarily in Gram-positive bacteria, and particularly the Firmicutes. It appears to be completely absent in Gram-negative species, although low-level homology hits are detected for a small number of uncharacterized eukaryotic proteins. For comparison, a similar analysis is presented for the highly conserved β (RpoB) and β' (RpoC) subunits. Images were generated using http://string-db.org/.



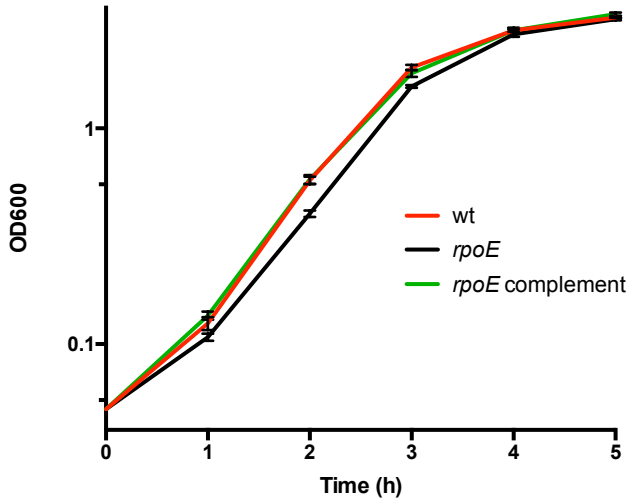
Supplemental Figure S2: Analysis of RpoE truncates. Cultures of the *rpoE* mutant bearing various plasmid based His_6 complementation constructs were grown for 3 hours, before cytoplasmic proteins and RNA were isolated. **A –** Western blot analysis using anti-His tag antibodies and the strains indicated. Detected are full length RpoE (21.7 kDa) and an RpoE variant containing the full N-terminus, and half the C-terminus (16.4 kDa). The amount of protein loaded was standardized using a Bradford assay. **B –** Quantitative real time PCR analysis was used to measure expression of the different *rpoE* versions. Each value represents data derived from 3 independent cultures. Error bars are shown ± SEM.



Supplemental Figure S3: Validation of RNAseq derived changes using qPCR analysis. A - RNAseq data was confirmed using qPCR analysis for representative genes. Error bars are shown ±SEM. **B -** qPCR fold-changes between mutant and wild-type were compared with those generated from RNAseq experiments.



Supplemental Figure S4: RNAP δ-subunit mutants are not outcompeted by the parental strain during growth under standard conditions. Both the WT and mutant strain were inoculated into TSB at a 1:1 ratio, and incubated for 3h at 37 °C. Aliquots were withdrawn at the times specified, and the CFU/mL determined by plating on either TSA, or TSA containing erythromycin (to select for the mutant strain). Data is generated from three independent replicates; error bars are shown ± SEM.



Supplemental Figure S5: *rpoE* deletion results in delayed growth upon exit from stationary phase. Strains were grown in TSB until stationary phase (18h) before being seeded into fresh media at an OD_{600} = 0.05. Strains were allowed to grow over time, and their optical density recorded. Data presented is the average from three individual experiments, with error bars shown ±SEM.

Supplemental Table S1: Changes in gene expression upon *rpoE* disruption. Listed are genes that show differential expression in the *rpoE* mutant from RNAseq analysis. Annotations were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). For hypothetical proteins, bioinformatic analyses were performed and used to complement NCBI annotations.

Gene product	Accession Number	Fold Change (M^a/WT^b)
Virulence Factors		
immunoglobulin G binding protein A precursor, Spa	SAUSA300_0113	12.67
complement inhibitory protein, Ehb	SAUSA300_1052	-2.00
V8 Protease, SspA	SAUSA300_0951	-2.05
cysteine protease precursor, SspB	SAUSA300_0950	-2.05
zinc metalloproteinase aureolysin, Aur	SAUSA300_2572	-2.07
anti protein, PSMβ2	SAUSA300_1068	-2.15
alpha-hemolysin, Hla	SAUSA300_1058	-2.20
serine protease, SplC	SAUSA300_1756	-2.34
thermonuclease, Nuc	SAUSA300_0776	-2.37
IgG-binding protein, SBI	SAUSA300_2364	-2.41
serine protease, SplA	SAUSA300_1758	-2.44
anti protein, PSMβ1	SAUSA300_1067	-2.52
serine protease, SplD	SAUSA300_1755	-2.55
gamma hemolysin, HlgC	SAUSA300_2366	-2.57
serine protease, SplF	SAUSA300_1753	-2.87
delta-hemolysin, Hld	SAUSA300_1988	-2.89
serine protease, SplE	SAUSA300_1754	-2.89
cysteine protease precursor, SspC	SAUSA300_0949	-2.95
serine protease, SplB	SAUSA300_1757	-3.22
leukotoxin, LukD	SAUSA300_1768	-3.31
Scin-b	SAUSA300_1056	-3.42
MAP/EAP domain protein	SAUSA300_2164	-3.60
Staphylococcal complement inhibitory protein A	SAUSA300_1919	-3.68
leukotoxin, LukE	SAUSA300_1769	-4.01
truncated beta-hemolysin, Hlb	SAUSA300_1918	-4.17
fibrinogen-binding protein, EfbB	SAUSA300_1055	-4.64
secretory antigen precursor, SsaA	SAUSA300_2249	-5.37
Panton-Valentine leukocidin, LukF-PV	SAUSA300_1381	-6.55
Ear Protein	SAUSA300_0815	-6.80
Panton-Valentine leukocidin, LukS-PV	SAUSA300_1382	-8.02
Hypothetical Proteins		
hypothetical protein	SAUSA300_2158	3.25
hypothetical protein	SAUSA300_1215	2.98
hypothetical protein	SAUSA300_0793	2.37

hypothetical protein	SAUSA300_1746	2.35
hypothetical protein	SAUSA300_0575	2.30
hypothetical protein	SAUSA300_0668	2.22
hypothetical protein	SAUSA300_1532	2.19
hypothetical protein	SAUSA300_1335	2.10
hypothetical protein	SAUSA300_2543	2.08
hypothetical protein	SAUSA300_0779	2.02
hypothetical protein	SAUSA300_0300	-2.25
hypothetical protein	SAUSA300_2447	-2.46
hypothetical protein	SAUSA300_0471	-2.50
hypothetical protein	SAUSA300_0302	-2.64
hypothetical protein	SAUSA300_2522	-3.05
hypothetical protein	SAUSA300_2524	-3.72
hypothetical protein	SAUSA300_0409	-4.67
hypothetical protein	SAUSA300_2482	-5.05
hypothetical protein	SAUSA300_0048	-5.31
hypothetical protein	SAUSA300_1380	-10.83
Transporters		
Na+/H+ antiporter, MnhA component	SAUSA300_0856	2.22
ABC transporter, ATP-binding protein	SAUSA300_2399	2.07
maltose ABC transporter, permease protein	SAUSA300_0211	2.05
osmoprotectant transporter, BCCT family	SAUSA300_2549	2.02
glycerol uptake facilitator protein, GlpF	SAUSA300_1191	-2.03
ABC transporter, substrate-binding protein	SAUSA300_0618	-2.10
ABC transporter, ATP-binding protein	SAUSA300_0271	-2.13
truncated calmodulin exporting ATPase	SAUSA300_0068	-2.34
peptide ABC transporter, ATP-binding protein	SAUSA300_2407	-2.37
ABC transporter, ATP-binding protein	SAUSA300_0620	-2.50
peptide ABC transporter, permease protein	SAUSA300_0202	-2.80
peptide ABC transporter, permease protein	SAUSA300_0201	-2.91
xanthine permease, PbuX	SAUSA300_0387	-3.34
copper-translocating P-type ATPase, CopA	SAUSA300_0078	-3.73
Regulators		
staphylococcal accessory regulator S, SarS	SAUSA300_0114	2.03
sigma factor, SigS	SAUSA300_1722	-2.07
regulatory protein, GntR family	SAUSA300_1914	-2.30
sensor histidine kinase, SaeS	SAUSA300_0690	-2.33
transcriptional regulator, DeoR family	SAUSA300_0683	-2.35
arsenical resistance operon repressor, ArsR	SAUSA300_1717	-2.53
DNA-binding response regulator, SaeR	SAUSA300_0691	-2.82
tRNAs		
tRNA	SAUSA300_0447	-2.07
tRNA	SAUSA300_1823	-2.12

tRNA	SAUSA300_1811	-2.12
tRNA	SAUSA300_1772	-2.76
Transposases		
putative transposase	SAUSA300_2263	-2.82
IS431 transposase/integrase	SAUSA300_0069	-6.28
IS431mec, transposase	SAUSA300_0028	-24.83
Capsule Proteins		
capsular polysaccharide biosynthesis protein, Cap5G	SAUSA300_0158	-2.06
capsular polysaccharide biosynthesis protein, Cap5F	SAUSA300_0157	-2.18
Ribosomal Proteins		
30S ribosomal protein S5, RpsE	SAUSA300_2187	2.81
ribosomal protein L33, RpmG	SAUSA300_1511	2.13
ribosomal protein L33, RpmG	SAUSA300_1233	-2.27
PTS System Components		
PTS system, IIABC components, PtsG	SAUSA300_0191	4.43
hypothetical protein	SAUSA300_0331	3.87
PTS system, IIC component	SAUSA300_1809	2.01
Nitrite/ Nitrate Metabolism		
respiratory nitrate reductase, beta subunit, NarH	SAUSA300_2342	10.18
anaerobic ribonucleoside-triphosphate reductase activating protein, NarJ	SAUSA300_2341	8.27
respiratory nitrate reductase, alpha subunit	SAUSA300_2343	6.03
nitrite reductase [NAD(P)H], small subunit, NirD	SAUSA300_2345	5.42
nitrite extrusion protein, NarK	SAUSA300_2333	4.84
nitrite reductase [NAD(P)H], large subunit, nirB	SAUSA300_2346	4.67
transcriptional regulator, NirR	SAUSA300_2347	3.68
hypothetical protein	SAUSA300_0711	2.12
respiratory nitrate reductase, gamma subunit, NarI	SAUSA300_2340	2.12
SaPI5		
Putative DNA primase, pathogenicity island	SAUSA300_0809	3.19
hypothetical protein, pathogenicity island	SAUSA300_0811	3.14
hypothetical protein, pathogenicity island	SAUSA300_0810	3.10
hypothetical protein, pathogenicity island	SAUSA300_0808	2.94
hypothetical protein, pathogenicity island	SAUSA300_0812	2.83
hypothetic XRE type regulator, pathogenicity island	SAUSA300_0804	2.72
hypothetical protein, pathogenicity island	SAUSA300_0805	2.64
hypothetical protein, pathogenicity island	SAUSA300_0806	2.46
hypothetical protein, pathogenicity island	SAUSA300_0807	2.44

Membrane Proteins/ Lipoproteins		
membrane protein, putative	SAUSA300_0111	2.80
membrane protein	SAUSA300_2056	2.32
membrane protein	SAUSA300_0823	2.31
membrane protein	SAUSA300_0872	2.30
membrane protein, putative	SAUSA300_0393	2.16
membrane protein, putative	SAUSA300_2642	2.10
membrane spanning protein	SAUSA300_2454	2.07
lipoprotein, putative	SAUSA300_0203	-2.28
lipoprotein, putative	SAUSA300_0693	-2.47
putative lipoprotein	SAUSA300_0079	-3.50
membrane Protein	SAUSA300_0692	-3.53
xanthine phosphoribosyltransferase, Xpt	SAUSA300_0386	-3.92
Other		
uroporphyrinogen III methylase SirB, putative	SAUSA300_2344	5.35
transferrin receptor	SAUSA300_0721	2.83
acetyltransferase	SAUSA300_1312	2.65
UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase, MurG	SAUSA300_1311	2.56
bifunctional acetaldehyde-CoA/alcohol dehydrogenase, AdhE	SAUSA300_0151	2.48
anaerobic ribonucleotide reductase, small subunit, NrdG	SAUSA300_2550	2.45
general stress protein 13	SAUSA300_1862	2.29
S1 RNA Binding Domain Protein	SAUSA300_0486	2.29
lantibiotic epidermin biosynthesis protein, EpiA	SAUSA300_1767	2.25
cytochrome d ubiquinol oxidase, subunit I	SAUSA300_0986	2.24
hypothetical protein	SAUSA300_1310	2.21
acetyltransferase, GNAT family	SAUSA300_2468	2.20
histidine ammonia-lyase, HutH	SAUSA300_0008	2.18
acetyltransferase, GNAT family	SAUSA300_0662	2.07
anaerobic ribonucleoside-triphosphate reductase, NrdD	SAUSA300_2551	2.07
tributyrin esterase, EstA	SAUSA300_2564	2.07
glycerol kinase, GlpK	SAUSA300_1192	2.06
penicillin-binding protein 2, MecA	SAUSA300_0032	2.06
pyrimidine-nucleoside phosphorylase, Pdp	SAUSA300_2091	2.05
threonine dehydratase, IlvA	SAUSA300_1330	2.03
N-acetyl-gamma-glutamyl-phosphate reductase, ArgC	SAUSA300_0186	-2.00
oligoendopeptidase F, PepF	SAUSA300_0902	-2.04
amidophosphoribosyltransferase, PurF	SAUSA300_0972	-2.07
cystathionine gamma-synthase, MetB	SAUSA300_0434	-2.08
riboflavin biosynthesis protein, RibBA	SAUSA300_1713	-2.12
cysteine synthase/cystathionine beta-synthase family protein, CysM	SAUSA300_0433	-2.15
GMP synthase, GuaA	SAUSA300_0389	-2.16

6,7-dimethyl-8-ribityllumazine synthase, RibH	SAUSA300_1712	-2.24
choline dehydrogenase, BetA	SAUSA300_2545	-2.48
glutamate synthase, large subunit, GltB	SAUSA300_0445	-2.50
glutamate synthase, small subunit, GltD	SAUSA300_0446	-2.55
betaine aldehyde dehydrogenase, BetB	SAUSA300_2546	-3.08
methionine aminopeptidase, Map	SAUSA300_1869	-3.39
Phage Proteins		
phi083 ORF027-like protein	SAUSA300_1952	29.2
phiPVL ORF39-like protein	SAUSA300_1962	26.58
phiPVL ORF044-like protein	SAUSA300_1959	19.69
phi77 ORF031-like protein	SAUSA300_1947	18.42
phiPVL ORF41-like protein	SAUSA300_1961	16.24
single-strand binding protein	SAUSA300_1958	14.87
phiPVL ORF046-like protein	SAUSA300_1957	12.92
dUTP diphosphatase	SAUSA300_1949	12.36
putative phage transcriptional regulator	SAUSA300_1968	11.98
hypothetical phage protein	SAUSA300_1964	11.79
phi77 ORF014-like protein, phage anti-repressor protein	SAUSA300_1966	11.45
phi77 ORF069-like protein	SAUSA300_1948	10.83
phi77 ORF040-like protein	SAUSA300_1943	9.87
hypothetical phage protein	SAUSA300_1942	9.63
phiSLT ORF53-like protein	SAUSA300_1429	9.4
putative phage-related DNA recombination protein	SAUSA300_1960	9.15
phiPVL ORF051-like protein	SAUSA300_1953	8.37
phi77 ORF026-like protein, phage transcriptional activator	SAUSA300_1944	8.29
hypothetical phage protein	SAUSA300_1963	8.07
phi77 ORF004-like protein, putative phage tail component	SAUSA300_1929	7.86
phi77 ORF100-like protein	SAUSA300_1931	7.85
holin	SAUSA300_1924	7.4
phiPVL ORF050-like protein	SAUSA300_1954	7.36
phi77 ORF015-like protein, putative protease	SAUSA300_1939	7.13
phi77 ORF003-like protein, phage terminase, large subunit	SAUSA300_1941	7.13
phi77 ORF006-like protein, putative capsid protein	SAUSA300_1938	6.19
phage portal protein	SAUSA300_1940	6.09
phi77 ORF029-like protein	SAUSA300_1935	5.74
Autolysin	SAUSA300_1923	5.73
phi77 ORF001-like protein, phage tail tape measure protein	SAUSA300_1930	5.59
hypothetical phage protein	SAUSA300_1967	5.56
phi77 ORF020-like protein, phage major tail protein	SAUSA300_1934	5.45
phi77 ORF109-like protein	SAUSA300_1927	5.38
phi77 ORF045-like protein	SAUSA300_1937	4.55
hypothetical phage protein	SAUSA300_1932	4.49
hypothetical phage protein	SAUSA300_1936	4.31
phi77 ORF002-like protein, phage minor structural protein	SAUSA300_1928	4.14

phi77 ORF044-like protein	SAUSA300_1926	3.26
hypothetical phage protein	SAUSA300_1933	3.14
phiPVL ORF17-like protein	SAUSA300_1925	2.92
Phage, phiSLT ORF151-like protein, major tail protein	SAUSA300_1396	2.9
Phage, phiSLT ORF412-like protein, portal protein	SAUSA300_1403	2.8

[a] **USA300 HOU *rpoE* mutant**
[b] **USA300 HOU wild-type**

APPENDIX III

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Editor of portion(s)	N/A
Author of portion(s)	Andy Weiss, Brittney D. Moore, Miguel H.J. Tremblay, Dale Chaput, Astrid Kremer and Lindsey N. Shaw
Volume of serial or monograph.	JB.00459-16
Issue, if republishing an article from a serial	N/A
Page range of the portion	
Publication date of portion	31 October 2016 (posted online)
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The standard identifier of New Work	University of South Florida
Title of New Work	Non-classical regulators in Staphylococcus aureus
Publisher of New Work	University of South Florida
Expected publication date	May 2017
Estimated size (pages)	130
Total (may include CCC user fee)	0.00 USD
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RESEARCH ARTICLE

The ω Subunit Governs RNA Polymerase Stability and Transcriptional Specificity in *Staphylococcus aureus*

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ABSTRACT *Staphylococcus aureus* is a major human pathogen that causes infection in a wide variety of sites within the human body. Its ability to adapt to the human host and to produce a successful infection requires precise orchestration of gene expression. While DNA-dependent RNA polymerase (RNAP) is generally well characterized, the roles of several small accessory subunits within the complex have yet to be fully explored. This is particularly true for the omega (ω or RpoZ) subunit, which has been extensively studied in Gram-negative bacteria but largely neglected in Gram-positive counterparts. In *Escherichia coli*, it has been shown that ppGpp binding, and thus control of the stringent response, is facilitated by ω. Interestingly, key residues that facilitate ppGpp binding by ω are not conserved in *S. aureus*, and consequently, survival under starvation conditions is unaffected by *rpoZ* deletion. Further to this, ω-lacking strains of *S. aureus* display structural changes in the RNAP complex, which result from increased degradation and misfolding of the β′ subunit, alterations in δ and σ factor abundance, and a general dissociation of RNAP in the absence of ω. Through RNA sequencing analysis we detected a variety of transcriptional changes in the *rpoZ*-deficient strain, presumably as a response to the negative effects of ω depletion on the transcription machinery. These transcriptional changes translated to an impaired ability of the *rpoZ* mutant to resist stress and to fully form a biofilm. Collectively, our data underline, for the first time, the importance of ω for RNAP stability, function, and cellular physiology in *S. aureus*.

IMPORTANCE In order for bacteria to adjust to changing environments, such as within the host, the transcriptional process must be tightly controlled. Transcription is carried out by DNA-dependent RNA polymerase (RNAP). In addition to its major subunits ($\alpha_2\beta\beta'$) a fifth, smaller subunit, ω, is present in all forms of life. Although this small subunit is well studied in eukaryotes and Gram-negative bacteria, only limited information is available for Gram-positive and pathogenic species. In this study, we investigated the structural and functional importance of ω, revealing key roles in subunit folding/stability, complex assembly, and maintenance of transcriptional integrity. Collectively, our data underline, for the first time, the importance of ω for RNAP function and cellular harmony in *S. aureus*.

KEYWORDS RNA polymerase subunit omega, RpoZ, *Staphylococcus aureus*, gene regulation

Received 10 June 2016 **Accepted** 26 October 2016

Accepted manuscript posted online 31 October 2016

Citation Weiss A, Moore BD, Tremblay MHJ, Chaput D, Kremer A, Shaw LN. 2017. The ω subunit governs RNA polymerase stability and transcriptional specificity in *Staphylococcus aureus*. J Bacteriol 199:e00459-16. https://doi.org/10.1128/JB.00459-16.

Editor Victor J. DiRita, Michigan State University

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Transcription in all forms of life is a tightly controlled process, necessitated by the essentiality of correct temporal and spatial expression of genes for survival. All transcriptional activity within a cell is maintained by the DNA-dependent RNA polymerase (RNAP). This multiprotein complex is structurally and functionally similar in distant forms of life, displaying only minor variations in composition, e.g., the presence/absence of certain subunits (1, 2). In bacteria RNAP consists of four main subunits, i.e.,

two α (RpoA) subunits and one subunit each of β (RpoB) and β' (RpoC), forming the $\alpha_2\beta\beta'$ complex. Together they facilitate transcriptional elongation, but they require a σ factor to initiate the process. Interestingly, a large number of bacteria, and particularly the *Firmicutes*, possess several other accessory RNAP subunits (3–6). These are considerably smaller than the major subunits, ranging from 8.5 to 21.5 kDa, and include the δ (RpoE), ε (RpoY), and ω (RpoZ) subunits. Deletion of these subunits does not result in lethality for the cell, and thus their diminutive size and nonessential nature have resulted in their being classified as the "small accessory subunits" (reviewed in reference 6). Nevertheless, for the δ factor it has been shown that, in various species, deletion is accompanied by a deregulation of the transcriptional process, leading to decreased fitness and impaired virulence in pathogenic organisms (7, 8). While there is an extensive history of research for δ, ε has only recently been described as an RNAP subunit, with only a single study thus far performed in *Bacillus subtilis* (5), which suggested a role in phage immunity. While the presence of these two subunits is largely confined to the *Firmicutes*, homologs of ω, the smallest of the three subunits, can be found not only in bacteria but also in eukaryotes (RPB6) and archaea (RpoK) (6, 9). Although this conservation might suggest a vital role and perhaps similar function across widely different species, there are in fact marked differences in how this subunit influences cells across the various kingdoms (6, 10). Most strikingly is the observation that while it is accessory in bacteria, the ω subunit is essential in eukaryotic organisms (11).

The majority of studies on ω have been carried out in Gram-negative bacteria, with a focus on the model organism *Escherichia coli* (10). In this organism it has been shown that ω influences the transcriptional machinery, and thus the transcriptional process, in a variety of ways. Most notably, ω is known to interact with β' to ensure correct folding of the subunit, as well as to facilitate docking to the $\alpha_2\beta$ complex (9, 12–14). Accordingly, deletion of ω leads to misfolding as well as degradation of β' (13, 15), which is also observed in *Mycobacterium smegmatis* (16). The crystal structure of ω in complex with the other RNAP subunits of both *E. coli* and *Thermus aquaticus* has been solved and confirms the binding of ω to β' (17, 18). Interestingly, these structures also reveal species-specific differences in the interaction of these two proteins, again highlighting the heterogeneous nature of ω function in different organisms. In contrast, no such crystal structure is available for ω, or RNAP at large, in Gram-positive organisms.

Quite strikingly, in terms of functional difference, is the finding that in *E. coli*, the stringent response, and thus adaption to nutrient-limiting conditions, is dependent on ω. Specifically, the stringent response-inducing molecule ppGpp is recognized by and binds to the interface of β' and ω (19–21), which in turn leads to the adjustment of transcriptional profiles to promote survival under nutrient-limiting conditions. While this role is true for *E. coli*, the ω protein in *B. subtilis* has been suggested to have no such role in the stringent response, due to an alternative mechanism of ppGpp recognition. Instead of binding to ω, the adaption to limiting conditions in *B. subtilis* is mediated by ppGpp-induced alterations of GTP concentrations within the cell (22, 23). Subsequently, these changes lead to alterations in gene expression, driven by the sensitivity of certain promoters to GTP availability as an initiating nucleotide. In line with this model, where onset of the stringent response does not require the interaction of ppGpp with the RNAP complex, is the observation that the conserved *E. coli* residues required for ppGpp binding to RNAP are largely absent in the *B. subtilis* ω and β' subunits (20).

The final major function of ω described in the literature is a putative role in facilitating σ factor biding to the RNAP complex. For *E. coli* and cyanobacteria, it has been reported that depletion of ω can lead to increased binding of alterative σ factors and in turn to increased expression of genes within alternative σ factor regulons (24, 25). Again, structural differences within the ω-depleted RNAP have been implicated in this alteration of σ factor affinity for the complex. As with many of the other ω phenotypes, no in-depth studies have been performed in Gram-positive bacteria regarding this role, further underscoring the need to characterize this diverse protein.

For Gram-positive organisms, only a limited number of studies exist, detailing a few phenotypic effects resulting from the abrogation of ω activity. Indeed, none of these studies have unraveled the molecular basis for alterations in mutant strains, meaning that the role of ω in Gram-positive species is still relatively elusive. Those effects that have been detailed for *rpoZ* mutants include alterations in cell wall morphology, cell motility, protein secretion, and biofilm formation (16, 26–29). Importantly, the role of this subunit in the virulence of pathogenic species has yet to be evaluated. Therefore, in this study we explored the role of ω in *Staphylococcus aureus*, demonstrating that it is not involved in the stringent response but instead mediates structural integrity of the RNAP complex. Deletion of this factor leads to individual RNAP subunit degradation and an induction of cellular stress responses. The latter effect was characterized by global transcriptional analyses, revealing that a significant number of the observed changes were due, at least in part, to altered σ factor abundance in the RNAP complex. Finally, we demonstrate that deletion of *rpoZ* influences the ability of *S. aureus* to form biofilms, a process that mediates persistent infections and the capacity to resist antibiotic treatment. We suggest that collectively, our data underline the importance of ω for RNAP stability, function, and cellular physiology in *S. aureus*.

RESULTS

ω is cotranscribed with *gmk* and is highly expressed throughout growth. Analysis of RNA sequencing data previously generated by our laboratory (31) reveals that the ω-encoding gene, *rpoZ*, is highly expressed during all phases of growth and is seemingly cotranscribed with its upstream gene, *gmk*, encoding a guanylate kinase (see Fig. S2A in the supplemental material). To validate the latter observation, we performed Northern blot analysis on RNA extracted from wild-type *S. aureus* cells grown to mid-exponential phase using *rpoZ*-specific probes. In so doing, a band of ~1100 nucleotides (nt) was observed, indicating that *rpoZ* and *gmk* are indeed organized in a bicistronic operon (Fig. S2B). In order to study the role of ω in *S. aureus*, we next created an unmarked deletion mutant, removing the majority of the *rpoZ*-encoding gene and leaving *gmk* intact. Validation of this arrangement and that no deleterious effects were observed on *gmk* expression was obtained by RNA sequencing analysis of the mutant strain and by Northern blotting (Fig. S2A and B). RNA sequencing data for the ω-depleted strain showed the expected deletion of the *rpoZ* gene but confirmed that there were no unintended effects on transcript abundance for *gmk* (see Table S1 in the supplemental material).

The *S. aureus* ω and β′ proteins lack conserved residues required for ppGpp binding. In *E. coli* the interaction of ppGpp with RNAP is mediated by conserved residues within the ω and β′ subunits, thus controlling the stringent response. Such residues are absent in *B. subtilis*, suggesting an alternate mechanism for stringent response regulation in Gram-positive organisms (20, 32). In corroboration of this, alignment analysis of ω and β′ subunits from *E. coli*, *B. subtilis*, and *S. aureus* (Fig. 1A) reveals that, although there is partial conservation of the β′ and ω proteins for all three organisms, the residues for ppGpp binding are almost entirely absent (a single aspartic acid is conserved in β′ region 2) at the primary sequence level and in the biochemical characteristics of each amino acid. This finding is further validated by the observation that growth of a Δ*rpoZ* strain is not impaired under standard, nutrient-limiting, or stringent response-inducing conditions. Specifically, in addition to growth in complex medium (tryptic soy broth [TSB]) (Fig. 1B), we investigated growth during amino acid limitation (Fig. 1C) as well as in amino acid-limiting medium completely depleted of valine and leucine (Fig. 1D). Importantly, the *rpoZ* mutant showed growth rates similar to those of the wild type in both TSB and amino acid-limiting medium and only a minor growth defect in medium devoid of valine and leucine. This defect, however, suggests an impaired ability to adapt to changing growth conditions rather than a stringent response defect, as stringent response-deficient mutants display a characteristic stalling of growth during the stringent response, rather than delayed growth (33). To further validate this finding, we assessed sensitivity to mupirocin of the *rpoZ* mutant alongside

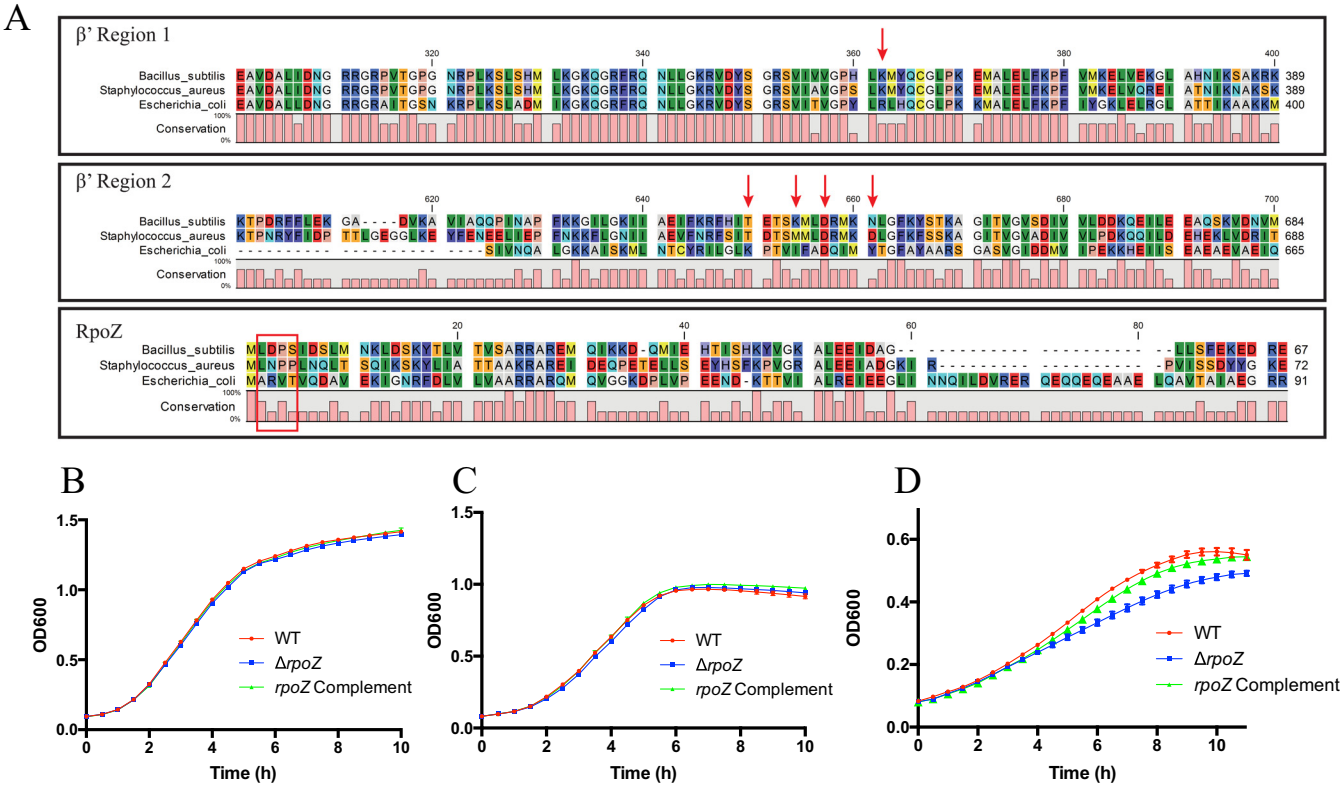

FIG 1 Influence of the *S. aureus* ω subunit on survival during nutrient-limiting conditions. (A) Alignment of select RNAP subunits from *E. coli*, *B. subtilis*, and *S. aureus*, showing conservation and divergence of amino acids sequences for RpoC (β′) and RpoZ (ω). For *E. coli*, the conserved β′ (arrows) and ω (boxed) regions important for ppGpp binding are shown. (B to D) *S. aureus* growth under standard conditions (TSB) (B), in amino acid-limiting medium (CDM) (C), and in CDM completely depleted of valine and leucine (D). Error bars show the standard error of the mean (SEM).

its parental strain and a *codY* mutant strain, as it has previously been shown that *S. aureus* mutants impaired in the stringent response display decreased resistance to this stringent response-inducing antibiotic (34). In so doing, we found that the Δ*rpoZ* strain has a mupirocin MIC (0.32 μg/ml) similar to that of the wild type, while the *codY* mutant has a 2-fold decrease in MIC (~0.16 μg/ml), comparable to that of stringent response-deficient mutants documented elsewhere (34).

Depletion of *rpoZ* leads to destabilization of the RNAP complex. We next assessed whether ω has a role in maintaining RNAP complex integrity in *S. aureus* and, more precisely, in the folding of its subunits, as described for *M. smegmatis* and *E. coli* (13, 16). We isolated cytoplasmic proteins from exponentially growing cells and determined the abundances of the intact RNAP complex in the wild-type and mutant strains. This was achieved by concentrating fractions using a 100-kDa-cutoff filter, ensuring that the resulting retentate harbored only proteins of >100 kDa in size. As β and β′ are the only RNAP subunits larger than 100 kDa, any subunit found in the concentrated fraction must originate from protein in complex with other RNAP subunits. Consequently, β and β′ are expected to be present independently and in complex with RNAP and thus should demonstrate no alteration within the mutant even if complex instability is observed. As expected, we consistently observed equimolar amounts of β for both the wild type and the *rpoZ* mutant in tested samples (Fig. 2A), which was confirmed by Western blotting using a β-specific antibody (Fig. 2B). Conversely, we found a marked decrease in β′ abundance in the mutant compared to the parent in the same samples. Given the large size of β′, these results point not only toward a release of the subunit from the complex but also to its likely degradation in the absence of ω, as observed in other organisms. Similar to β′, but to a lesser extent, the α subunit, the housekeeping σ factor, σ^A, and the small subunit δ were also decreased in abundance in the mutant strain. In order to investigate the fate of each subunit, we performed additional

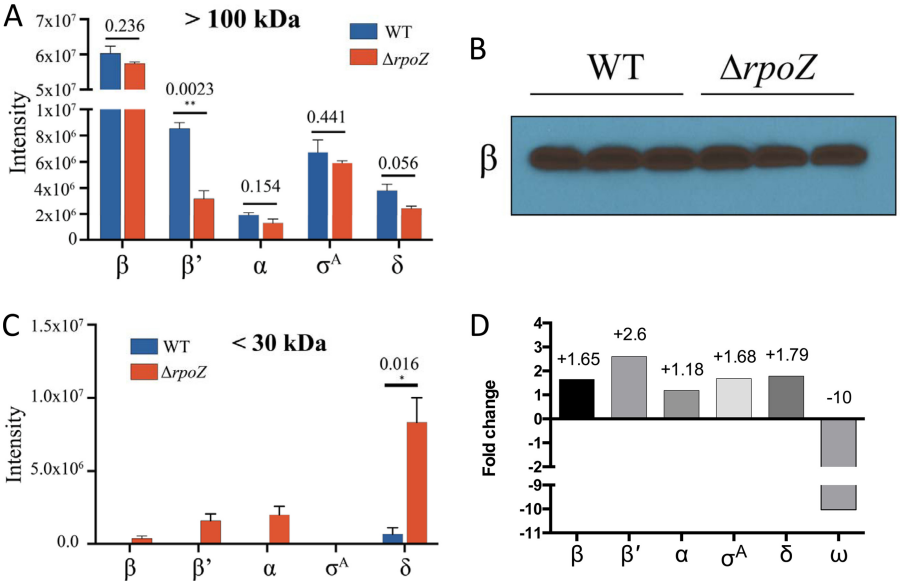

FIG 2 Cells lacking the ω factor have altered RNAP composition and individual subunit stability. Free or complex-bound subunits were separated according to their molecular masses via size selection before analysis using mass spectrometry. (A) In the >100-kDa fraction only large RNAP subunits (β and β′) and subunits within the RNAP complex should be found. (B) Data were validated by Western blotting using a β subunit antibody. (C) Protein degradation in the Δ*rpoZ* strain is highlighted by the presence of larger subunits within the <30-kDa fraction. (D) Transcriptional changes as a cause of alterations in RNAP composition were excluded by analyzing expression of RNAP genes from RNA sequencing data sets. Shown are the fold changes for each RNAP gene in the mutant strain compared to the wild type. Where relevant, error bars show SEM. Statistical significance was determined using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

concentration experiments using a 30-kDa-cutoff filter and again isolated proteins from the wild-type and *rpoZ* mutant strains (Fig. 2C). Interestingly, while only trace amounts of β were detected in these fractions (as expected), we observed significant levels of β′ in the *rpoZ* mutant strain, again suggesting β′ instability upon ω deletion. With regard to δ, which should always be found in the <30-kDa fraction, the amount of this protein was significantly higher in the Δ*rpoZ* strain than in the wild type. This would tend to indicate disassembly and release of this subunit from the RNAP complex upon ω depletion.

To exclude the possibility that alterations in subunit abundance were caused by differential expression patterns for RNAP-encoding genes in the *rpoZ*-depleted strain, we analyzed RNA sequencing data sets of the USA300 parent and its corresponding *rpoZ* mutant (Fig. 2D). The *rpoZ* gene was included as an internal control for our data set, as a portion of the locus is still present in the mutant strain (Fig. S2A), and therefore an expression value is generated from the RNA sequencing experiment. Of note, all of the subunits were shown to have a moderate increase in expression in the mutant strain. Importantly, β′, which displayed the strongest decrease in protein abundance, showed the strongest upregulation, with a 2.6-fold increase in transcription. This perhaps suggests a possible attempt by the cell to counteract β′ degradation by enhancing transcriptional activity of the *rpoC* gene within the *rpoZ* mutant strain.

The δ and ω factors of RNAP have distinct and contrasting influences on *S. aureus* gene expression. When further exploring the RNA sequencing comparison of the wild-type and *rpoZ* mutant strains, we observed 232 genes with altered expression at ≥2-fold and 63 genes at ≥3-fold (Fig. 3; Table S1). In order to validate these alterations, we performed quantitative reverse transcription-PCR (RT-qPCR) for a subset of differentially expressed genes (Fig. S3A and B), revealing similar changes in this and the RNA sequencing data sets. Since our RNAP composition studies showed that *rpoZ* deletion also results in δ depletion from RNAP, we next assessed whether removal of δ from the transcription complex was the driving force behind the observed changes. This is particularly important, as our previous studies have shown that δ is a key factor

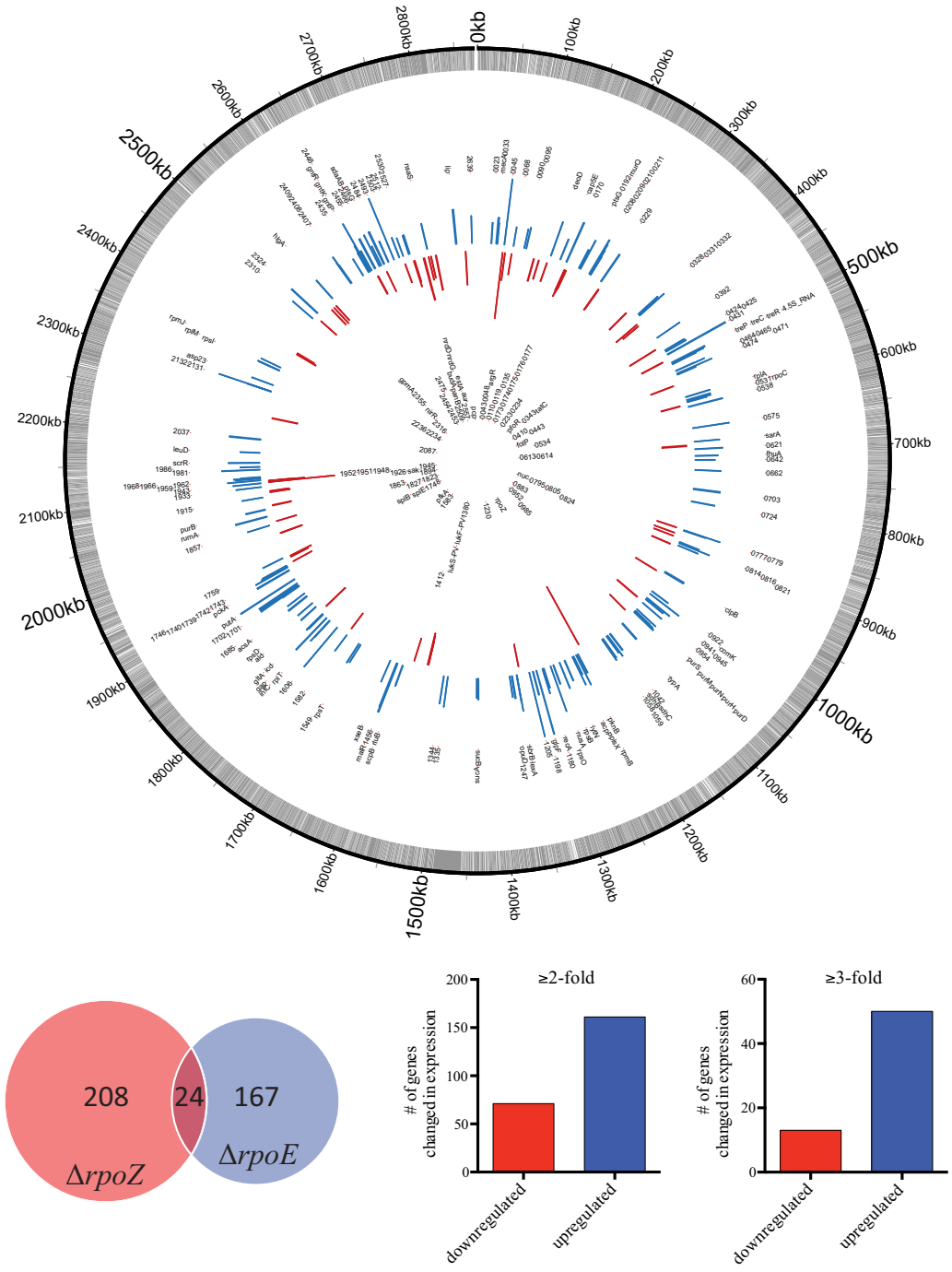

FIG 3 The δ and ω factors of RNAP have distinct and contrasting influences on *S. aureus* gene expression. (Top) Genomic map of altered transcription in the *rpoZ* mutant strain. The outermost circle (gray) represents annotations for the *S. aureus* USA300 genome. Blue (upregulation) and red (downregulation) bars show genes with differential expression in the *rpoZ* mutant (a maximum change of ±7-fold is displayed). Labels represent either gene names or USA300 gene numbers. (Bottom right) Number of genes changed in expression upon *rpoZ* depletion at ≥2-fold (232) or ≥3-fold (63). (Bottom left) Comparison of RNA sequencing data sets for Δ*rpoE* (δ) and Δ*rpoZ* (ω) strains. Shown are the number of genes within each regulon and the overlap between the two data sets (only ~10% was detected).

for maintaining transcriptional specificity within the *S. aureus* cell (8). Upon comparing RNA sequencing-derived transcriptional changes of the *rpoE* and *rpoZ* mutants, we found an overlapping regulon of only 24 genes (Fig. 3). Comparing these 24 genes to the overall number of genes changed in both mutants, only ~10.5% (Δ*rpoZ*) or 12.5% (Δ*rpoE*) of each data set is identical. This low level of regulon overlap indicates that the

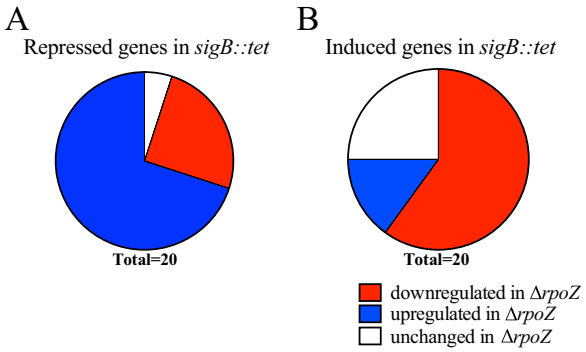

FIG 4 ω affects σ factor recruitment to the *S. aureus* RNAP complex. A comparison of RNA sequencing data sets from *sigB*::*tet* (σ^B) and Δ*rpoZ* strains is depicted. Shown are the most highly repressed (A) or highly induced (B) genes in the *sigB*::*tet* strain and their concomitant change in expression upon *rpoZ* deletion. Genes with a change of less than 10% between the *rpoZ* mutant and its parental strain were considered unchanged.

changes seen in an *rpoZ* mutant are not the result of an inability of δ to exert its influence on RNAP.

ω influences σ factor recruitment to the *S. aureus* RNAP complex. In *E. coli* and the cyanobacterium *Synechocystis*, it is known that depletion of ω causes alterations in σ factor abundance within the RNAP complex. Therefore, we next investigated if the multifaceted transcriptional changes of the *rpoZ* mutant were influenced by alterations in σ factor binding to the ω-less RNAP. Since σ^B is the major alternative σ factor in *S. aureus*, we compared transcriptome changes upon *rpoZ* deletion to those observed in a *sigB* mutant. This was facilitated using wild-type and *sigB* mutant RNA sequencing data sets previously produced in our lab in the SH1000 background. SH1000 was chosen for this study as it is known to have a σ^B overexpression phenotype. Subsequent comparison of these data sets detected an inverse relationship between the regulons of ω and σ^B. Specifically, for the 20 most strongly downregulated genes in the *sigB* mutant, the majority (70%) demonstrated increased expression upon *rpoZ* disruption (Fig. 4; Tables S2 and S3). Conversely, for the 20 most strongly upregulated genes in the *sigB* mutant, most (60%) were found to be decreased in expression in the Δ*rpoZ* strain.

It is noted that the comparison of regulons between different strains is not without drawbacks. Our rationale was that by choosing a *sigB*-overexpressing strain such as SH1000, we could obtain better resolution for the identification of σ^B-dependent genes. Nevertheless, there remains the possibility that additional genomic differences between SH1000 and USA300 drive strain-specific expression patterns and thereby influence their corresponding σ^B regulons. However, the strongly correlated inverse relationship observed suggests that there are indeed higher levels of σ^B within the ω-lacking RNAP complex, which corroborates effects seen in other bacterial species. Accordingly, it appears that ω not only is required to maintain RNAP integrity but also plays a role in association of the complex with available σ factors, thereby influencing transcriptional stringency in *S. aureus*.

Depletion of *rpoZ* leads to decreased transcriptional specificity that influences multiple cellular processes within the *S. aureus* cell. We next used functional clustering to investigate alterations in gene expression upon *rpoZ* disruption (Fig. 5). While a variety of cellular processes were shown to be affected by *rpoZ* deletion, we noted major changes in genes related to central dogma. Specifically, genes related to transcription and translation, as well as cellular processes connected to DNA, RNA, or protein synthesis, were strongly affected by the loss of ω. These include changes within purine and pyrimidine metabolism, enzymes for DNA replication and repair, and amino acid biosynthesis. Additionally, genes in more general ontological categories, such as genes for sugar uptake systems (phosphotransferase system [PTS]), the general stress

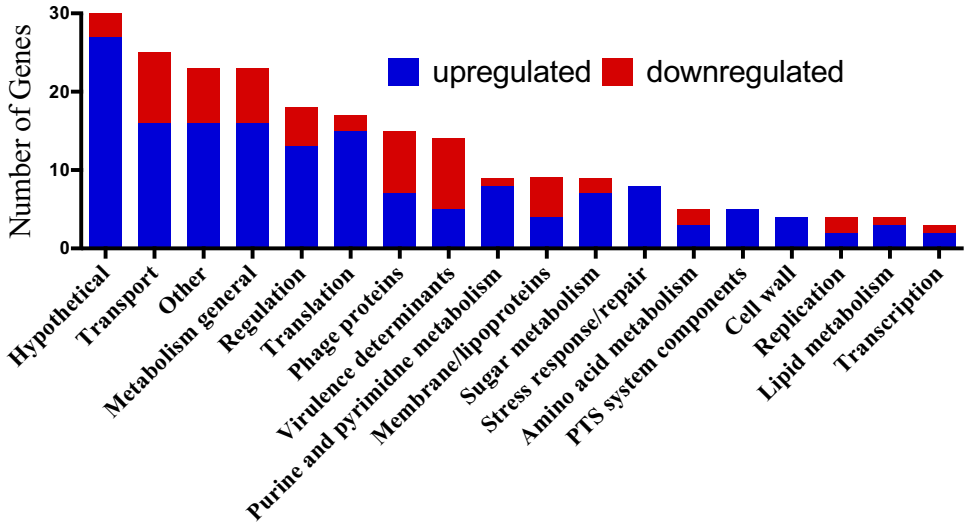

FIG 5 Ontological grouping for genes altered in expression upon *rpoZ* disruption. The transcriptional changes from Fig. 3 were analyzed and organized into ontological groups.

response, regulators, transporters, and metabolism, displayed altered expression. Interestingly, the changes also negatively affect several well-characterized virulence determinants of *S. aureus*. These include aureolysin (−3.7-fold) and the Panton-Valentine leukocidin-encoding operon (LukSF, −3.4-fold and −3.1-fold, respectively). Lastly, a significant number of phage genes were altered in their expression. This point is particularly interesting as we have previously described this phenomenon to be one of the hallmarks of *rpoE* deletion (8); thus, these effects could result from its depletion from the RNAP complex. Therefore, although overlap of the δ and ω regulons is limited (as described above) and unlikely to be causative for the majority of transcriptional changes observed in the Δ*rpoZ* strain, it cannot be entirely excluded that a number of effects seen in the *rpoZ* mutant are driven, at least in part, by δ depletion from RNAP.

Disruption of the ω subunit results in an impaired ability to circumvent multiple forms of environmental stress. To explore the physiological outcome of these findings, we sought to determine if the mutant was impaired in resisting the impact of environmental stressors. In so doing, we observed that the mutant shows an augmented sensitivity toward various antibacterial compounds with unrelated mechanisms of action. These include increased sensitivity to triclosan (2-fold; a fatty acid biosynthesis inhibitor), erythromycin and pyrogallol (both ~4-fold; a translation inhibitor and a reducing agent, respectively), and diamide (8-fold; creates disulfide stress). As these compounds all affect different cellular targets, it would appear that depletion of ω leads to widespread fitness defects, highlighting its global importance within the *S. aureus* cell.

The ω subunit of RNAP is required for biofilm formation in *S. aureus*. We next set out to investigate whether the observed changes in gene expression influence pathophysiologically relevant processes. Thus, we determined whether the formation of a bacterial biofilm, a key hallmark of *S. aureus* infection (35), was influenced by *rpoZ* depletion *in vitro*. Importantly, upon analysis we observed that loss of ω does indeed lead to an approximately 3-fold reduction in biofilm formation that could be restored via complementation in *trans* (Fig. 6). As biofilm formation is strongly influenced by extracellular proteins in *S. aureus* (36), we analyzed the secretomes of the wild-type and *rpoZ* mutant strains. Although the vast majority of proteins appeared to be unchanged in abundance, we noted a marked decrease in a major protein band at around 75 kDa in the mutant strain (see Fig. S4 in the supplemental material). In order to identify this protein, we excised the corresponding band from gels and performed mass spectrometric (MS) analysis. It was identified as two separate tributyrin lipases (the products of

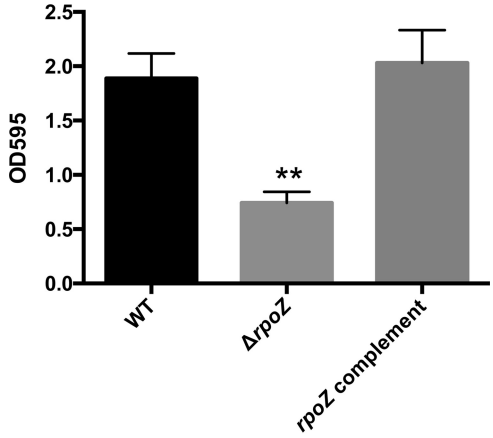

FIG 6 The ω subunit of RNAP is required for biofilm formation in *S. aureus*. Biofilm formation by the wild-type, *rpoZ* mutant, and complemented strains was measured using a 24-well plate biofilm assay. Error bars show SEM, with statistical significance measured using Student's *t* test (**, $P < 0.01$).

SAUSA300_0320 and SAUSA300_2603), with molecular masses of 76 and 77 kDa, respectively. Interestingly, a recent report on the role of lipase activity in *S. aureus* demonstrated that deleting these enzymes results in drastically impaired biofilm formation (37). This may provide some explanation for these findings, although given the complex process of biofilm formation in *S. aureus*, it is likely that other factors are also involved.

DISCUSSION

The ω subunit of RNAP is a widely distributed protein that is found in all branches of life. While it is well studied in Gram-negative bacteria (10) and eukaryotes (9), a recent review by our group highlighted the need for ω to be examined in a wider range of species, and particularly Gram-positive bacteria (6). This is especially true since (i) the subunit has a variety of possible roles that have received only limited attention in Gram-positive species, including RNAP subunit folding (9, 12, 13, 15) and stability (16), complex assembly/stabilization (10, 13, 14), maintenance of σ factor specificity (24, 25), and ppGpp binding (19–21), and (ii) Gram-positive species, and particularly the *Firmicutes*, include several epidemiologically relevant human pathogens. To the latter point, an understanding of cellular factors that are required for transcription and to maintain transcriptional stringency is crucial in order to better comprehend the physiology of pathogens and thus potentially aid in counteracting and preventing the spread of disease. Here we describe the role of ω within *S. aureus* for the first time. Our study explores previously documented functions from other bacterial species, assessing its impact on the global transcriptome as well as its key role in fitness and, most importantly, virulence, which to our knowledge has not yet been examined.

It has previously been suggested that the stringent response in *Firmicutes* is independent of ppGpp recognition by ω (32). Here we demonstrate that under amino acid-limiting conditions, as well as during exposure to stringent response-inducing compounds, the Δ*rpoZ* strain does not exhibit typical stringent response deficiency phenotypes. This is consistent with findings in *B. subtilis*, where ppGpp, instead of directly binding to ω as in Gram-negative species, exerts its function indirectly. Instead, the generation of ppGpp from GTP and ATP (38) in *B. subtilis* leads to diminished GTP pools within the cell (39, 40), which in turn causes decreased transcription of GTP-sensitive promoters (i.e., those with GTP at position +1 [22, 41, 42]) and interferes with the role of GTP as a corepressor for CodY (43). These findings are mirrored in *S. aureus*, where CodY- and GTP-regulated genes are similarly influenced by the nutritional status of the cell in a ppGpp-dependent manner (33, 44). Therefore, we hypothesize that rather than resulting from a dysfunctional stringent response, the diminished ability of a Δ*rpoZ* strain to survive challenging conditions is a consequence of basal stress levels

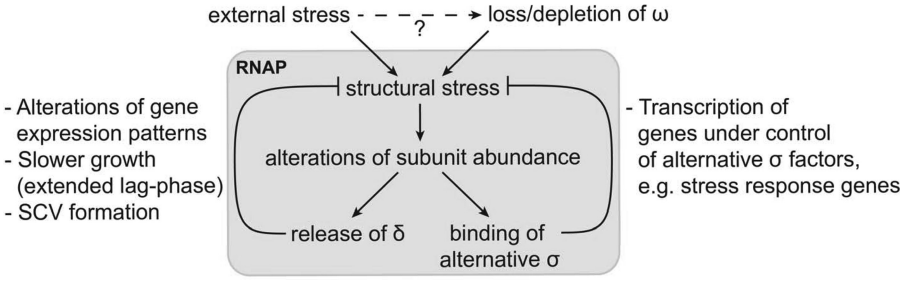

FIG 7 Model for the role of ω in the Gram-positive RNA polymerase.

experienced by the cell upon loss of the subunit. This is supported by our findings that *S. aureus* lacking the *rpoZ* gene displays a reduced-growth phenotype not only when exposed to nutrient-limiting and stringent response-inducing conditions but also during growth at elevated temperatures (data not shown).

To investigate how a lack of ω affects the transcriptional process and integrity of RNAP, we performed complex stability analysis and determined that *rpoZ* deletion causes decreased levels of intact RNAP, perhaps as a result of reduced β' within the complex. This is consistent with findings in other organisms, which describe a chaperone-like function for ω, assisting in the folding of β' and its subsequent docking to RNAP (9, 10, 12–15). Indeed, the likely misfolding of β' leads not only to degradation of the subunit but also to impaired assembly and/or dissolution of RNAP and the proteolysis of several other subunits. Importantly, in addition to this apparent failure of β' to fully associate with RNAP, ω deletion leads to a jettisoning of δ from the transcription complex and an increase in alternative σ factor binding. Indeed, the finding that δ and σ^A are preferentially released from ω-less, and presumably structurally unstable, RNAP suggests that ω-deficient RNAP can provide a unique insight into the effects of stress on the transcription complex and, more broadly, the role of the accessory subunits. Based on the current literature, as well as our findings here, we propose a model whereby stress within the cell leads to RNAP instability, resulting in an alteration of subunits present within the transcription complex that specifically facilitates survival during unfavorable conditions (Fig. 7). In support of this, those sigma factors that have increased binding to RNAP upon ω deletion (σ^S in *E. coli* [24], σ^B/σ^F in cyanobacteria [25], and σ^B in *S. aureus*) are all major components of the general stress response in their respective organism (45–49). Furthermore, as with σ factors, the release of δ from RNAP has the potential for profound alterations in gene expression, as a recent publication demonstrates the ability of δ to selectively affect individual promoters (7, 8) via binding to specific promoter elements (50). As the disruption of *rpoE* results in slower bacterial growth in a variety of organisms (e.g., an extended lag phase [8, 51, 52]), removal of this subunit from RNAP may actually present a survival advantage during stress, redirecting resources away from division and toward repair and cellular maintenance. In agreement with this is a recent study with *Streptococcus pneumoniae* biofilms, where there appears to be selective pressure for *rpoE* mutations leading to small-colony variant (SCV) phenotypes (53). SCV formation is a common mechanism used by many different bacteria which would initially appear to present a defect in viability but in actuality results in enhanced survival (54) and antibiotic resistance and the development of persister cells (55). Interestingly, our group has observed similar effects in *S. aureus*, where δ-deficient mutants display variable, and decreased, colony size after recovery from murine models of infection (unpublished observation). Thus, it would appear that the significant compositional rearrangement within ω-less RNAP is far from random. Rather, these changes in binding of accessory factors are seemingly directed to specifically adjust promoter selectivity and preference to facilitate survival. Furthermore, these findings regarding subunit swapping within the transcription complex appear to confirm long-made contentions regarding a level of cooperativity for δ and σ factor binding within RNAP (56–58).

In spite of these changes in subunit abundance, which we suggest are utilized by the cell as a survival strategy, *rpoZ* deletion itself results in wide-reaching cellular stress that is associated with an impaired transcriptional process. This contention is supported by our RNA sequencing data, which demonstrate the upregulation of various genes coding for transcriptional and translational factors, putatively in an attempt to overcome diminished or inefficient RNAP function. Furthermore, we identified increased expression of genes involved in the general stress response, sugar uptake pathways, and energy metabolism, all of which may be designed to overcome unfavorable conditions (59). The latter group emphasizes that intracellular stress resulting from ω deletion, and the subsequent countermeasures it elicits (e.g., production of chaperones, RNAP subunits, and ribosomal subunits), consumes energy and therefore has the potential to deplete cellular energy pools. This in turn leads to the upregulation of sugar uptake and energy metabolism, which in our study resulted in a minor increase in ATP pools (data not shown). In accordance with this, studies investigating *S. aureus* heat stress demonstrate that following exposure to elevated temperatures, cellular energy levels are in fact not depleted, but rather a steady (60) or transient (61) increase in ATP pools is observed, presumably due to the upregulation of energy-generating processes. It is perhaps not surprising that the transcriptional and physiological changes observed from our *in vitro* experiments also translate into the impairment of virulence-related processes, as shown by the impaired biofilm formation in the *rpoZ* mutant.

We suggest that, collectively, our findings extend the existing knowledge regarding ω and its role within RNAP, provide a unique insight into structural rearrangements within the transcription complex in response to stress, and demonstrate the necessity of this important subunit for the cellular homeostasis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. Unless otherwise indicated, cultures were grown as described by Shaw et al. (62). Briefly, *S. aureus* was grown at 37°C with shaking (250 rpm) in tryptic soy broth (TSB) or chemically defined medium (CDM) (45), while *E. coli* was cultivated in lysogeny broth (LB) with agitation (250 rpm) at 37°C. Overnight cultures of *S. aureus* were seeded at a 1:100 dilution into fresh medium. After 3 h of growth, cultures were used to inoculate fresh TSB to an optical density at 600 nm (OD_{600}) of 0.05 and grown to the desired density in a Synergy2 plate reader (BioTek) in 96-well plates.

Mutant construction. In order to investigate the role of the ω subunit, a strain carrying a markerless deletion of the *rpoZ* gene was created, using a method based on the plasmid pJB38 as outlined by Bose and coworkers (63). Briefly, the majority of the *rpoZ* gene was removed via allelic replacement, as initially described elsewhere (64). To do this, both the 5′ and 3′ ends of the *rpoZ* gene, together with ~1 kb of up- and downstream DNA, were PCR amplified using primers OL2889 to OL2892 and fused together using MluI sites contained in the amplification primers. This was then cloned into pJB38 using SacI and KpnI sites in primers OL2889 and OL2892. Following several selection and counterselection steps in *S. aureus* as described previously (63), *rpoZ* deletion was confirmed using internal (OL2991 and OL2992) and external (OL3008 and OL3009) primer pairs relative to the deleted region within the gene.

Complement construction. In order to exclude that secondary mutations were causative for phenotypic changes observed in the Δ*rpoZ* strain, a plasmid was constructed to complement the *rpoZ* gene *in trans*. Here, the complete *rpoZ* operon, including its native promoter, was PCR amplified using primers OL3753 and OL3807. After BamHI digestion of the amplicon and the pMK4 shuttle vector, the PCR fragment and plasmid were ligated together and transformed into chemically competent *E. coli* DH5α. The plasmid isolated from ampicillin-resistant clones was confirmed via Sanger sequencing (using standard M13 primers). Correct plasmids were transformed into electrocompetent *S. aureus* RN4220 (65) and finally transduced into the *S. aureus* USA300 *rpoZ* mutant using ϕ11 (66).

RNA isolation and Northern blots. The detection of RNA transcripts was performed using a Northern blotting protocol outlined by Caswell et al. (67). Briefly, RNA from cultures grown for 3 h was isolated using an RNeasy kit (Qiagen), with DNA removed using a Turbo DNA-free kit (Ambion). RNA concentrations were determined using an Agilent 2100 Bioanalyzer and an RNA 6000 Nano kit (Agilent). RNA (15 μg) was separated by gel electrophoresis (10% polyacrylamide containing 7 M urea and 1× Tris-borate-EDTA [TBE]) before being transferred to an Amersham Hybond N+ membrane (GE Healthcare) by electroblotting. In order to cross-link samples, membranes were exposed to UV radiation. Following this, membranes were prehybridized (1 h, 43°C) in ULTRAhyb-Oligo buffer (Ambion) and incubated (16 h, 43°C) with a [γ-^{32}]ATP-end-labeled oligonucleotide (OL3377) specific to the *rpoZ* target sequence. Labeling was carried out using T4 polynucleotide kinase (Thermo Scientific) according to the manufacturer's protocol. After overnight incubation, each membrane was washed (30 min, 43°C) with decreasing (2×, 1×, and 0.5×) concentrations of SSC buffer (300 mM sodium chloride, 30 mM sodium

TABLE 1 Strains, plasmids, and primers

Strain, plasmid, or primer	Description or sequence[a]	Reference or source
Species and strains		
E. coli DH5α	Cloning strain	79
S. aureus		
RN4220	Restriction-deficient transformation recipient	Lab Stocks
USA300 HOU	MRSA isolate cured of pUSA300-HOU-MRSA	72
HKM850	USA300 HOU *codY* mutant; *codY*::*tet*	75
SH1000	8325-4 derivate with functional *rsbU*; *rsbU*$^+$	80
MJH502	SH1000 *sigB* mutant; *sigB*::*tet*	80
BM2393	USA300 HOU Δ*rpoZ* mutant	This study
AW2394	USA300 HOU Δ*rpoZ*/pMK4::*rpoZ rpoZ*$^+$	This study
Plasmids		
pMK4	Gram-positive shuttle vector	81
pJB38	Counterselectable plasmid to create silent mutations in *S. aureus*	63
Primers		
OL2889	ATGGAGCTCGCCATAATTTATCTTCCACCTTC	This study
OL2890	ATGACGCGTGCAGTTGTTGCAATTAAATAC	This study
OL2891	ATGACGCGTGTTTCGACCATTAAAAATATGTG	This study
OL2892	ATGGGTACCCATTTCTTCAGCACTTTGAAC	This study
OL2991	GCGTGAAATTGATGAACAAC	This study
OL2992	CAGCAATTTCTTCTAACGCTC	This study
OL3008	AGAAGATTAGCTTAGAGAGGTC	This study
OL3009	GAAATGCTAATGGTGTCACA	This study
OL3377	CGTCAGCAATTTCTTCTAACGCTCTACCAAC	This study
OL3753	ATGGGATCCATCGCCAATTTCATCTTT	This study
OL3807	ATGGGATCCATCTCCCTTAAATATCACTATG	This study
OL2393	TCGTATGTTGTGTGGAATTG	This study
OL2394	GTGCTGCAAGGCGATTAAG	This study
OL1297	GTGGTGGCGTTTGTGC	This study
OL1298	CCGACAGCAAACACACCCAT	This study
OL2129	TAACATTCTCTGATGAAGTTG	This study
OL2130	TTAAGTCTACAGCAGCTTG	This study
OL3136	TCAATATTATCGGTGGATTTA	This study
OL3137	GCAAATTATGATGTTGAAATAG	This study
OL3747	CGTAGATGCAAATTATTACG	This study
OL3748	CGTTAATGAAACAATTGGAC	This study
OL4099	GGTTCACGTTCCTTTATC	This study
OL4100	CAGGTTTACCATCTTTAGG	This study
OL4109	GTTAAATGGTTTAATGCAGAA	This study
OL4110	GTATCCATCTTCAGCGA	This study

[a]MRSA, methicillin-resistant *S. aureus*. Underlining denotes restriction sites.

citrate). Finally, membranes were exposed to X-ray film in order to detect the size and abundance of the *rpoZ* transcript.

RNA sequencing and data analyses. RNA sequencing was performed for *rpoZ* and *sigB* mutants and their respective parental strains as previously outlined by our group (8, 68–70). Briefly, RNA was isolated and the quality determined as described for Northern blot experiments. Following this, RNA from three biological replicates was pooled in equimolar amounts and rRNA removed by consecutive treatment with the MICROBExpress (Ambion) and RiboZero (Epicentre) kits. Removal of rRNA was confirmed using an Agilent 2100 Bioanalyzer (RNA 6000 Nano kit; Agilent). The rRNA-depleted RNA samples were prepared for sequencing with an Ion Personal Genome Machine (PGM) system as described previously (68). First, cDNA libraries were constructed with an Ion Total RNA-seq kit, v2 (Ion Torrent). The prepared libraries were then used to generate template-positive ion sphere particles (ISPs) using an Ion PGM Template OT2 200 kit (Ion Torrent) in combination with an Ion OneTouch 2 system (Ion Torrent). The template-positive ISPs were loaded onto Ion 318 v2 chips (Ion Torrent) and sequencing runs performed with an Ion PGM Sequencing 200 kit, v2 (Ion Torrent). Raw data files in fastq format were exported and analyzed using the CLC Genomics Workbench software (Qiagen) and the USA300_FPR3757 and NCTC 8325 reference genomes (GenBank accession numbers CP000255 and NC_007795, respectively). Expression values for each gene were calculated as reads per kilobase per million mapped reads (RPKM), and a quantile normalization approach was applied (71) with a lower limit of 10 RPKM. Genes that displayed fold changes of $\geq$2 when comparing expression in the mutant to that in the wild-type strain were included in further analyses.

RT-qPCR. In order to confirm expression values that were generated by RNA sequencing, a subset of genes were chosen to confirm transcriptional changes via quantitative reverse transcription-PCR (RT-qPCR), as described by us previously (8, 69, 72, 73). PCR amplification was performed using primers for each gene (Table 1) (*glpF*, OL1297/OL1298; *asp23*, OL2129/OL2130; SAUSA300_0174, OL3136/OL3137; *aur*, OL3747/OL3748; *rpoC*, OL4099/OL4100; and SAUSA300_0777, OL4109/OL4110), alongside 16S rRNA-specific primers as standard controls (OL1184/OL1185) (74).

Investigation of RNA polymerase composition. In order to determine the effects of ω loss on the stability and composition of RNAP, we adopted an approach first outlined by Gunnelius et al. (an overview of this procedure can be found in Fig. S1 in the supplemental material) (25). Wild-type and Δ*rpoZ* strains were grown to exponential phase, and their cytoplasmic protein fractions were isolated as outlined previously (75). In order to differentiate between RNAP subunits unbound or within the RNAP complex, we performed two size selection steps. To do so, we first added 10 ml of phosphate-buffered saline (PBS) to the isolated protein fraction and loaded the complete mixture onto an Amicon Ultra-15 centrifugal filter unit (EMD Millipore) with a 100-kDa cutoff. After centrifugation (4,000 × *g*, 45 min), the approximately 300 μl of remaining protein fraction was washed with 10 ml of PBS and the centrifugation step repeated. The fraction on top of the filter was then recovered and stored (−80°C) for further analysis. The complete flowthrough of the first filter step was then applied to a second Amicon Ultra-15 centrifugal filter unit (EMD Millipore) with a 30-kDa cutoff. The flowthrough was collected and trichloroacetic acid (TCA) precipitated (overnight, 4°C). The pelleted proteins were washed three times in ice-cold absolute ethanol and subsequently resuspended in PBS. The different fractions were then subjected to either mass spectrometry or Western blot analysis.

Characterization of protein content using mass spectrometry. Protein extracts isolated via the size selection protocol outlined above were processed by filter-aided sample preparation (FASP), as described by us previously (73, 75). Proteins were reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and digested with trypsin–Lys-C (Promega) overnight at 37°C. Peptides were collected by centrifugation, desalted using Pierce SPE C_{18} columns with a Supelco vacuum manifold, and dried in a vacuum concentrator (Labconco). Peptides were resuspended in H_2O–0.1% formic acid and separated using a 75-μm by 10-cm C_{18} reversed-phase high-pressure liquid chromatography (HPLC) column (New Objective) on a NanoLC Ultra (Eksigent) with a 120-min gradient (4 to 40% acetonitrile [ACN] with 0.1% formic acid). Fractions were analyzed on a linear ion trap Orbitrap instrument (Orbitrap XL; Thermo Fisher Scientific), with full MS survey scans acquired at 60,000 resolution. The top 10 most abundant ions were selected for tandem MS (MS/MS) analysis in the linear ion trap. Raw data files were processed in MaxQuant (www.maxquant.org) and searched against the UniProtKB *S. aureus* USA300 protein sequence database. Search parameters included constant modification of cysteine by carbamidomethylation and the variable modification methionine oxidation. Proteins were identified using filtering criteria of 1% protein and peptide false-discovery rates. Protein levels were normalized to the overall protein content in each of the investigated fractions.

Western blotting. The assessment of β subunit abundance was performed as described by us previously (66). Size-selected protein fractions containing proteins and protein complexes of >100 kDa were prepared as for experiments determining RNAP composition. Samples were separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described by us previously (76). Separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and detected using a monoclonal mouse RNAP β antibody (8RB13; Santa Cruz Biotechnology) and a horseradish peroxidase (HRP)-conjugated secondary antibody. HRP activity was assessed using the SuperSignal West Pico substrate (Thermo Fisher Scientific) and visualized on X-ray film.

MIC assessment assay. To determine whether transcriptional changes observed within the mutant strain resulted in detectable physiological sensitivities, we assessed MICs for various antibacterial agents, as described by our group previously (77). Briefly, overnight cultures were diluted 1:1,000 into fresh medium in 96-well plates, and antibiotics at various concentrations were added at amounts no greater than 2% of the final volume. Plates were incubated for 12 h (static, 37°C), and turbidity, as a sign of bacterial growth, was manually assessed. MICs were defined as the minimum concentration of a given agent to result in no detectable turbidity in individual wells.

Secretome analysis. To evaluate alterations in secreted proteins, bacteria were grown for 24 h before being centrifuged (6,000 × *g*, 10 min). Supernatants were removed and subjected to 12% SDS-PAGE as described by us previously (76). Gels were assessed either by silver staining using a Pierce silver stain kit (Thermo Fisher Scientific) according to the manufacturer's instructions or by Coomassie brilliant blue staining, in-gel trypsin digestion, and mass spectrometric analysis, as described by us previously (76). Briefly, gel pieces were minced and destained before reduction and alkylation with dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Proteins were digested with trypsin–Lys-C overnight at 37°C, and peptides were extracted using 50:50 ACN and H_2O–0.1% formic acid and dried in a vacuum concentrator (Labconco). Peptides were resuspended in H_2O–0.1% formic acid for LC-MS/MS analysis, which was performed as described above.

Biofilm assay. The ability of strains to form biofilms was assessed as described by us previously (78). The wells of non-tissue-culture treated 12-well polystyrene plates were incubated for 48 h (static, 4°C) with 1 ml of human serum in order to facilitate attachment of cells. Concurrently, bacteria were cultured overnight in biofilm medium (BFM) (TSB supplemented with 0.5% [wt/vol] dextrose and 3% [wt/vol] NaCl) and used to seed fresh BFM to an OD_{600} of 0.05. After removal of the human serum from the wells of plates, 1 ml of these fresh cultures was transferred into each well and incubated for 48 h (static, 37°C). After incubation, supernatants were gently removed from each well and the biofilm washed twice with PBS before fixation with absolute ethanol. Biofilms were stained with crystal violet solution (2% [wt/vol])

for 10 min, followed by two more rounds of washing with PBS. Residual supernatants were aspirated, and plates were dried overnight. After this time, 300 μl of absolute ethanol was added to the wells and incubated for 10 min before being removed, and the absorbance was measured at 570 nm using a Synergy2 plate reader (BioTek).

Accession number(s). All data sets have been deposited to the NCBI Gene Expression Omnibus (GEO) (accession numbers GSE87033 and GSE87036).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00459-16.

TEXT S1, PDF file, 1.3 MB.

TEXT S2, PDF file, 0.1 MB.

ACKNOWLEDGMENT

This study was supported in part by grant AI080626 (to L.N.S.) from the National Institute of Allergy and Infectious Diseases.

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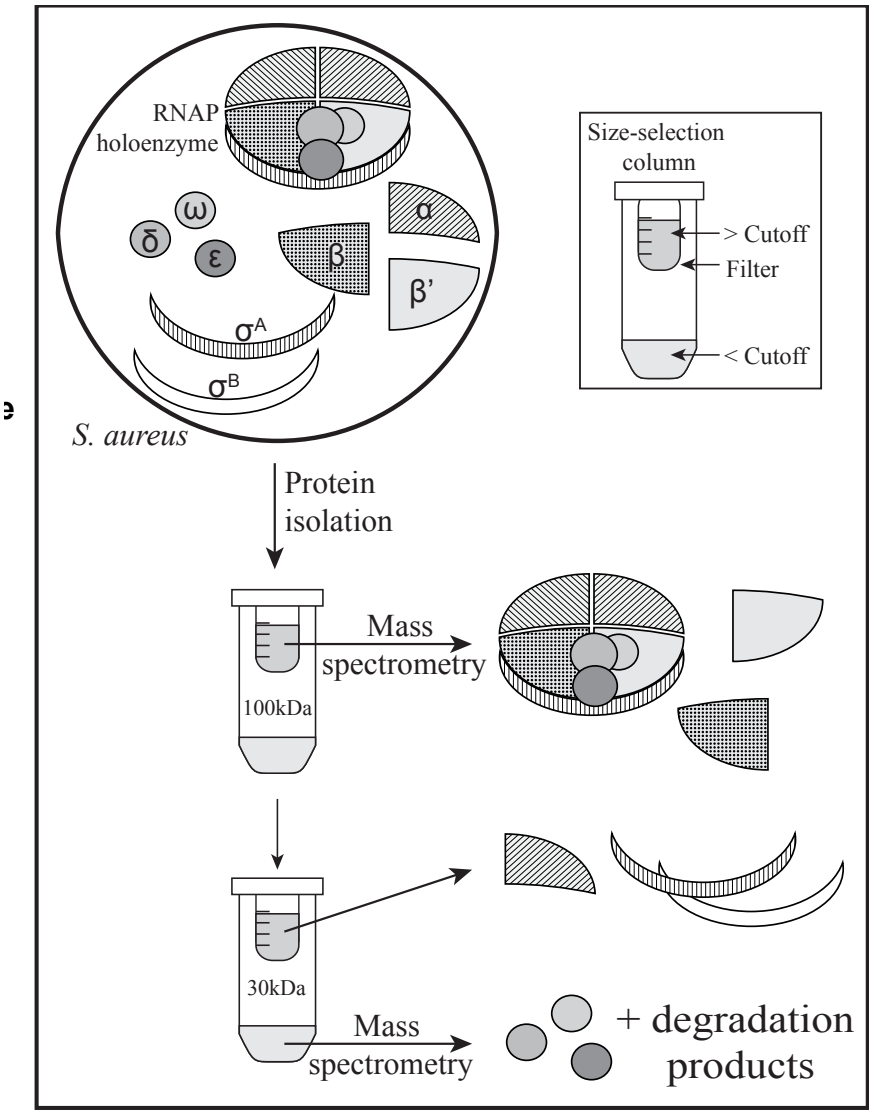

Figure S1: Workflow employed to explore RNAP composition.

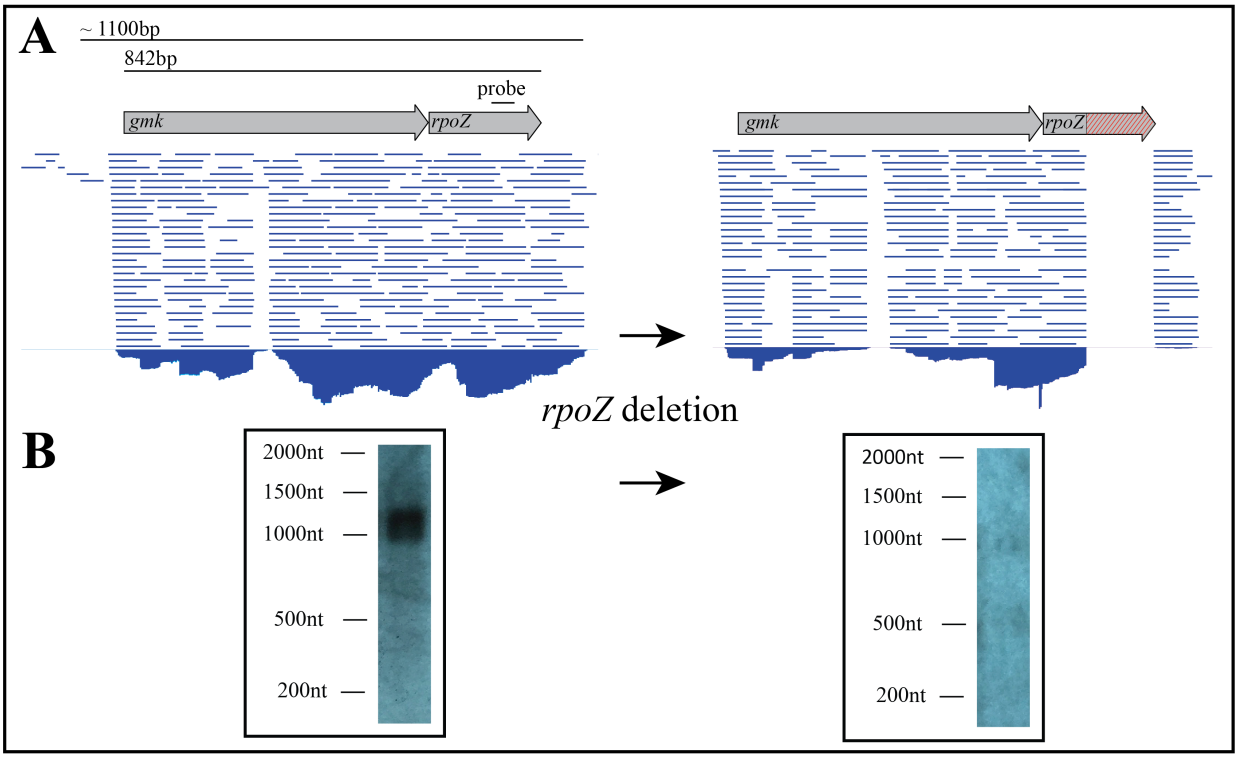

Figure S2: Exploration of the *gmk-rpoZ* operon architecture in *S. aureus*. A) RNA-sequencing analysis, or **B)** Northern blots, of the wild-type and *rpoZ* deletion mutant. The probe used in B is demarcated on the *rpoZ* gene in A. Lines denoting the lengths of 842 bp and ~1100 bp refer to the predicted size of the *gmk-rpoZ* transcript, and the actual length of this transcriptional unit detected by Northern blot, respectively.

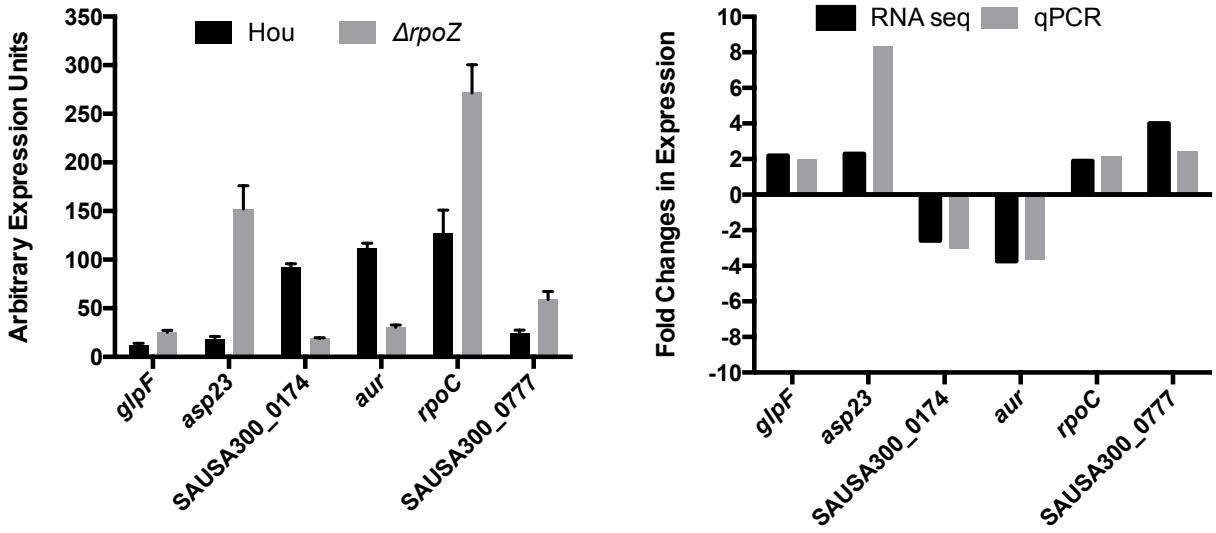

Figure S3: Validation of transcriptional changes from RNA-sequencing experiments using RT-qPCR. A) RNA-sequencing data was confirmed using RT-qPCR analysis for representative genes. Error bars are shown ±SEM. **B)** Fold-changes for RT-qPCR and RNA-sequencing were subsequently compared.

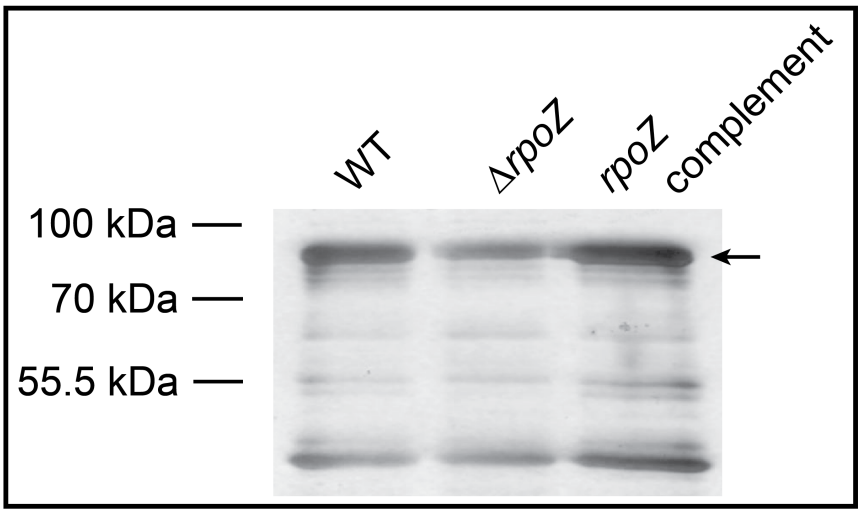

Figure S4: ω affects the abundance S. aureus lipases. Secretomes of the *S. aureus* wild-type, Δ*rpoZ* mutant and complemented strains after 24 h of growth were assessed via SDS-PAGE. A black arrow denotes the protein band found to have decreased abundance, which was identified as two different lipases by mass-spectrometric analysis.

Table S1: Differentially expressed genes in the Δ*rpoZ* mutant strain

Gene name/designation	Fold Change in Δ*rpoZ*	Function
Hypothetical proteins		
SAUSA300_0431	+∞	Hypothetical protein
SAUSA300_1205	7.54	Hypothetical protein
SAUSA300_2493	7.32	Hypothetical protein
SAUSA300_2132	5.81	Hypothetical protein
SAUSA300_1606	5.42	Hypothetical protein
SAUSA300_0575	3.88	Hypothetical protein
SAUSA300_1739	3.83	Hypothetical protein
SAUSA300_0465	3.29	Hypothetical protein
SAUSA300_1335	3.22	Hypothetical protein
SAUSA300_1742	3.11	Hypothetical protein
SAUSA300_1746	2.92	Hypothetical protein
SAUSA300_0392	2.88	Hypothetical protein
SAUSA300_1740	2.84	Hypothetical protein
SAUSA300_1180	2.73	Hypothetical protein
SAUSA300_2131	2.58	Hypothetical protein
SAUSA300_1857	2.41	Hypothetical protein
SAUSA300_0779	2.4	Hypothetical protein
SAUSA300_1743	2.34	Hypothetical protein
SAUSA300_1759	2.31	Hypothetical protein
SAUSA300_1247	2.23	Hypothetical protein
SAUSA300_1915	2.21	Hypothetical protein
SAUSA300_0814	2.2	Hypothetical protein
SAUSA300_1701	2.12	Hypothetical protein
SAUSA300_0642	2.08	Hypothetical protein
SAUSA300_1582	2.07	Hypothetical protein
SAUSA300_2527	2.06	Hypothetical protein
SAUSA300_2236	-2.04	Hypothetical protein
SAUSA300_0824	-2.08	Hypothetical protein
SAUSA300_0177	-2.09	Hypothetical protein
SAUSA300_1863	-2.18	Hypothetical protein
SAUSA300_1748	-2.21	Transposase, frameshift
SAUSA300_1230	-2.39	Hypothetical protein
SAUSA300_1380	-2.62	Hypothetical protein
SAUSA300_0048	-2.9	Hypothetical protein
SAUSA300_0174	-3.02	Hypothetical protein
SAUSA300_0192	2.19	Hypothetical protein
SAUSA300_0805	-2.19	Hypothetical protein

Transport		
glpF	8.74	Glycerol uptake facilitator
SAUSA300_0208	4.17	Putative maltose ABC transporter, ATP-binding protein
ptsG	3.76	Glucose transporter subunit IIABC
SAUSA300_2466	3.67	ABC transporter permease protein
SAUSA300_0209	3.65	Putative maltose ABC transporter, maltose-binding protein
SAUSA300_2408	3.37	Oligopeptide permease, ATP-binding protein
SAUSA300_0941	3.07	Putative ferrichrome ABC transporter
SAUSA300_0210	2.98	Maltose ABC transporter, permease protein
4.5S RNA	2.74	Signal recognition particle
SAUSA300_2407	2.6	Peptide ABC transporter, ATP-binding protein
fhuA	2.4	Ferrichrome transport ATP-binding protein
opuD	2.39	Glycine betaine transporter
gntP	2.35	Gluconate permease
SAUSA300_2409	2.25	Oligopeptide permease
SAUSA300_0211	2.21	Maltose ABC transporter, permease protein
SAUSA300_0068	2.02	Cadmium-exporting ATPase, truncation
SAUSA300_2454	-2.05	ABC Transporter
tatC	-2.07	Sec-independent protein translocase
SAUSA300_2453	-2.1	ABC Transporter
SAUSA300_0176	-2.27	ABC transporter, permease protein
SAUSA300_0173	-2.33	Dipeptide/oligopeptide/nickel ABC transporter permease
SAUSA300_2557	-2.49	ABC transporter protein
SAUSA300_0613	-2.57	Putative Na^+/H^+ antiporter, MnhD component
SAUSA300_0175	-2.58	Putative lipoprotein/ transporter
SAUSA300_0614	-2.6	Putative Na^+/H^+ antiporter, MnhE component

Other		
SAUSA300_0045	7.99	HNH nucleases
SAUSA300_0471	4	Veg protein
sbrB	3.87	Small peptide encoding gene
comK	3.83	Competence transcription factor
SAUSA300_0538	3.7	Uncharacterized epimerase/dehydratase
SAUSA300_0328	2.74	Lipoate-protein ligase
SAUSA300_1198	2.53	Putative GTP-binding protein
cap5E	2.29	Capsular polysaccharide biosynthesis protein
SAUSA300_0821	2.23	SUF system FeS assembly protein
SAUSA300_2503	2.23	Secretory antigen SsaA
pknB	2.19	Serine/threonine kinase
typA	2.19	GTP-binding protein
SAUSA300_0474	2.1	Putative endoribonuclease, L-PSP
SAUSA300_0464	2.06	Predicted O-methyltransferase
scpB	2.04	Chromosome segregation and condensation protein B

SAUSA300_2512	2.01	Glyoxalase family protein
SAUSA300_0135	-2.04	Superoxide dismutase
SAUSA300_0534	-2.04	Putative amidohydrolases
SAUSA300_0795	-2.25	Thioredoxin like protein
SAUSA300_0985	-2.26	Hypothetical protein
SAUSA300_0234	-2.51	Putative flavohemoprotein
pcp	-3.52	CDD-2,6-dichloro-p-hydroquinone 1,2-dioxygenase
SAUSA300_0043	-9.73	Metallo-beta-lactamase/Zn-dependent hydrolase
Metabolism general		
gap	4.65	Glyceraldehyde-3-phosphate dehydrogenase 2
SAUSA300_0170	4.56	Aldehyde dehydrogenase
SAUSA300_0229	3.45	Acyl-CoA transferase
SAUSA300_0662	2.73	Acetyltransferase, GNAT family
sdhC	2.67	Succinate dehydrogenase, cytochrome b-558 subunit
acsA	2.45	Acetyl-coenzyme A synthetase
SAUSA300_0425	2.44	NADH dehydrogenase I, F subunit
SAUSA300_2484	2.38	3-hydroxy-3-methylglutaryl CoA synthase
gltA	2.37	Citrate synthase II
SAUSA300_0945	2.34	Isochorismate synthase family protein
SAUSA300_0424	2.32	Putative Cobalamin protein
sucB	2.21	Dihydrolipoyllysine-residue succinyltransferase
icd	2.14	Isocitrate dehydrogenase
sdhB	2.12	Succinate dehydrogenase, iron-sulfur subunit
SAUSA300_1986	2.05	Nitroreductase family protein
sucA	2.03	2-oxoglutarate dehydrogenase, E1 component
SAUSA300_1894	-2	Nicotinate phosphoribosyltransferase
SAUSA300_2316	-2.15	Acetyltransferase
SAUSA300_2475	-2.27	Acyl-CoA thioester hydrolase
budA 2736735..2737440)	-2.32	Alpha-acetolactate decarboxylase
folP	-2.53	Dihydropteroate synthase
panB	-2.83	3-methyl-2-oxobutanoate hydroxymethyltransferase
SAUSA300_0343	-2.84	Acetyltransferase
Regulation		
gntR	5.78	Transcriptional regulator, GntR family
malR	4.94	Transcriptional regulator, maltose operon repressor
scrR	3.3	Sucrose operon repressor
SAUSA300_0621	2.92	MntR Mn Repressor
treR	2.78	Trehalose operon Repressor
SAUSA300_2310	2.77	Putative transcriptional regulator
nsaS	2.74	NsaS Histidine Kinase
sarA	2.66	Staphylococcal accessory regulator A

SAUSA300_0954	2.61	Transcriptional regulator, MarR family
SAUSA300_0095	2.26	LysR Regulator, LysR family
SAUSA300_0023	2.2	YycI protein
SAUSA300_2530	2.08	Transcriptional regulator, TetR family
SAUSA300_0090	2.05	Hypothetical protein
SAUSA300_1583	-2	Cystine metabolism regulator
SAUSA300_2509	-2.07	Transcriptional regulatory, TetR family
nirR	-2.12	Nitrite reductase transcriptional regulator
argR	-2.19	Arginine repressor homolog
SAUSA300_0110	-2.41	Transcriptional Regulator, GntR family
Translation		
rpsO	5.08	30S ribosomal protein S15
rpmB	3.5	50S ribosomal protein L28
rumA	3.41	RNA methyltransferase
SAUSA300_2037	3.31	ATP-dependent RNA helicase
rpsB	2.91	30S ribosomal protein S2
infC	2.91	Translation initiation factor IF-3
rplA	2.67	Ribosomal protein L1
rplM	2.48	50S ribosomal protein L13
rpsI	2.37	30S ribosomal protein S9
rluB	2.3	Pseudouridine synthase B
rplT	2.28	50S ribosomal protein L20
rpmJ	2.27	50S ribosomal protein L36
rpsT	2.19	30S ribosomal protein S20
SAUSA300_0531	2.13	30S ribosomal protein S7
rpsD	2	30S ribosomal protein S4
SAUSA300_1823	-2.38	tRNA-Ser
SAUSA300_1827	-2.42	tRNA-Met
Phage proteins		
SAUSA300_1981	4.03	Phage terminase family protein
SAUSA300_1959	3.48	PhiPVL ORF044-like protein
SAUSA300_1943	2.94	Phi77 ORF040-like protein
SAUSA300_1966	2.82	Phi77 ORF014-like protein, phage anti-repressor protein
SAUSA300_1933	2.67	Hypothetical phage protein
SAUSA300_1962	2.63	PhiPVL ORF39-like protein
SAUSA300_1968	2.05	Putative phage transcriptional regulator
SAUSA300_1926	-2.02	Phi77 ORF044-like protein
SAUSA300_1954	-2.13	PhiPVL ORF050-like protein
SAUSA300_1948	-2.21	Phi77 ORF069-like protein
SAUSA300_1412	-2.49	PhiSLT ORF 50-like protein
SAUSA300_1953	-2.68	PhiPVL ORF051-like protein

SAUSA300_1951	-3.06	PhiPVL ORF052-like protein
SAUSA300_1952	-3.81	Phi083 ORF027-like protein
SAUSA300_1945	-7.94	Phi77 ORF071-like protein
Virulence determinats		
mecA	2.78	Penicillin-binding protein 2a
SAUSA300_1059	2.64	Staphylococcal superantigen like protein 12
hlgA	2.53	Gamma-hemolysin, component A
SAUSA300_1058	2.42	Alpha-hemolysin
SAUSA300_0033	2.21	Methicillin resistance regulator protein
splE	-2.02	Serine protease
sak	-2.11	Staphylokinase
nuc	-2.13	Nuclease
splB	-2.44	Serine protease
SAUSA300_0883	-2.54	Eap/Map protein
SAUSA300_2087	-2.73	Putative peptidase
lukF-PV	-3.13	Panton-Valentine leukocidin, LukF-PV
lukS-PV	-3.4	Panton-Valentine leukocidin, LukS-PV
aur	-3.74	Zinc metalloproteinase aureolysin
Purine and pyrimidne metabolism		
deoD	3.79	Purine nucleoside phosphorylase
purD	2.76	Phosphoribosylamine--glycine ligase
purS	2.67	Phosphoribosylformylglycinamidine synthase
purN	2.55	Phosphoribosylglycinamide formyltransferase
purH	2.5	Bifunctional purine biosynthesis protein
xseB	2.44	Exodeoxyribonuclease VII, small subunit
purM	2.11	Phosphoribosylformylglycinamidine cyclo-ligase
purB	2.08	Adenylosuccinate lyase
SAUSA300_2234	-2.32	Purine nucleosidase
Membrane/lipoproteins		
SAUSA300_0724	2.49	Putative lipoprotein
SAUSA300_2448	2.35	Putative membrane protein
SAUSA300_1685	2.23	Putativ membrane protein
SAUSA300_0922	2.1	Membrane protein
SAUSA300_2355	-2.23	Putative lipoprotein
pfoR	-2.47	Perfringolysin O regulator protein
SAUSA300_0443	-2.49	Putative Membrane protein
SAUSA300_0233	-2.64	Putative Membrane protein
SAUSA300_0410	-3.18	Staphylococcal tandem lipoprotein

Sugar metabolism		
pfkA	-2.79	6-phosphofructokinase
gpmA	-2.04	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase
sdaAB	2.24	L-serine dehydratase, beta subunit
gntK	3.14	Gluconate kinase
murQ	3.4	N-acetylmuramic acid-6-phosphate etherase
SAUSA300_2455	3.54	Fructose-1,6-bisphosphatase
treC	3.69	Alpha-phosphotrehalase
pckA	3.87	Phosphoenolpyruvate carboxykinase
SAUSA300_1456	5.52	Alpha-amylase
Stress response/repair		
SAUSA300_0777	4.01	Cold shock protein C
SAUSA300_0816	3.44	CsbD-like protein
lexA	2.89	LexA repressor
SAUSA300_2639	2.88	Cold shock protein D
asp23	2.39	Alkaline shock protein 23
SAUSA300_1549	2.37	ComE operon protein I
clpB	2.31	Chaperone
recA	2.14	Recombinase A
Amino acid metabolism		
putA	6.78	Proline dehydrogenase
ald	2.08	Alanine dehydrogenase
leuD	2.06	3-isopropylmalate dehydratase, small subunit
SAUSA300_0119	-2.05	Ornithine cyclodeaminase
SAUSA300_0952	-2.75	Aminotransferase, class I
PTS system components		
treP	3.89	PTS system, trehalose-specific IIBC component
SAUSA300_2324	3.72	PTS system, sucrose-specific IIBC component
SAUSA300_0332	3.45	PTS system, IIA component
SAUSA300_0331	2.74	PTS IIB - ascorbate, lactose or cellobiose
ptsG	2.17	PTS system, glucose-specific IIABC component
Cell wall		
SAUSA300_1702	4.86	Cell wall surface anchor family protein
SAUSA300_0703	2.84	Lipoteichoic acid synthase
lytN	2.31	Cell wall hydrolase
SAUSA300_2435	2.01	Cell wall surface anchor family protein

Replication		
SAUSA300_1344	2.95	Putative DNA-replication protein, DnaD
SAUSA300_1042	2.15	DNA polymerase IV (family X)
nrdG	-3.48	Anaerobic ribonucleotide reductase, small subunit
nrdD	-4.59	Anaerobic ribonucleotide reductase, large subunit
Lipid Metabolism		
lip	3.67	Triacyglycerol lipase
acpP	3.25	Acyl carrier protein
plsX	2.11	Phosphate acyltrasferase
estA	-2.33	Tributyrin esterase
Transcription		
rpoC	2.6	RNAP Beta' subunit
nusA	2.11	Transcription elongation factor
rpoZ	-10.03	RNAP omega subunit

Table S2: List comparing the 20 most downregulated genes in a *S. aureus* SH1000 σ^B mutant strain (which have homologues in USA300) to the alterations in transcript abundance of the homologue genes in a USA300 Δ*rpoZ* strain.

Feature ID SH1000	Fold-Change (*sigB::tet*)	Feature ID USA300	Gene name	Fold Change (Δ*rpoZ*)
SAOUHSC_00624	-∞	SAUSA300_0609		-1.61
SAOUHSC_02442	-∞	SAUSA300_2143		-1.10
SAOUHSC_02882	-∞	SAUSA300_2502		1.41
SAOUHSC_02443	-15,213.97	SAUSA300_2144		1.31
SAOUHSC_00845	-4,178.80	SAUSA300_0816		3.44
SAOUHSC_02441	-3,188.61	SAUSA300_2142	*asp23*	2.39
SAOUHSC_02444	-1,540.56	SAUSA300_2145		-1.16
SAOUHSC_00356	-667.21	SAUSA300_0372		1.11
SAOUHSC_01729	-546.86	SAUSA300_1581		1.45
SAOUHSC_00358	-321.83	SAUSA300_0374		1.86
SAOUHSC_02466	-142.55	SAUSA300_2164		1.52
SAOUHSC_02387	-139.11	SAUSA300_2097		-1.39
SAOUHSC_03032	-97.75	SAUSA300_2629		-1.13
SAOUHSC_02774	-95.56	SAUSA300_2418		-1.08
SAOUHSC_01730	-89.95	SAUSA300_1582		2.07
SAOUHSC_02772	-77.38	SAUSA300_2416		1.13
SAOUHSC_00831	-75.12	SAUSA300_0786		1.19
SAOUHSC_02880	-70.09	SAUSA300_2500		1.64
SAOUHSC_02881	-62.08	SAUSA300_2501		1.44
SAOUHSC_02812	-54.8	SAUSA300_2447		1.90

Table S3: List comparing the 20 most upregulated genes in a *S. aureus* SH1000 σ^B mutant strain (which have homologues in USA300) to the alterations in transcript abundance of the homologue genes in a USA300 Δ*rpoZ* strain.

Feature ID SH1000	Fold-Change (*sigB::tet*)	Feature ID USA300	Gene name	Fold Change (Δ*rpoZ*)
SAOUHSC_00260	∞	SAUSA300_0281		-1.45
SAOUHSC_01315	∞	SAUSA300_1221		1.20
SAOUHSC_00069	80.09	SAUSA300_0113	*spa*	-1.54
SAOUHSC_02821	75.93	SAUSA300_2454		-2.05
SAOUHSC_00818	29.22	SAUSA300_0776	*nuc*	-2.13
SAOUHSC_03002	21.68	SAUSA300_2600	*icaA*	-1.06
SAOUHSC_02820	16.26	SAUSA300_2453		-2.10
SAOUHSC_00674	13.42	SAUSA300_0654	*sarX*	-1.50
SAOUHSC_00259	11.98	SAUSA300_0280		1.01
SAOUHSC_00913	11.1	SAUSA300_0878		-1.68
SAOUHSC_00072	10.88	SAUSA300_0116	*sirB*	1.19
SAOUHSC_02461	10.74	SAUSA300_2160		1.19
SAOUHSC_00244	9.97	SAUSA300_0266		-1.77
SAOUHSC_03004	9.64	SAUSA300_2601	*icaB*	-1.41
SAOUHSC_00492	9.19	SAUSA300_0495		1.01
SAOUHSC_02639	7.73	SAUSA300_2305		-1.21
SAOUHSC_00250	7.03	SAUSA300_0272		1.07
SAOUHSC_03036	6.24	SAUSA300_2633		1.00
SAOUHSC_01488	6.23	SAUSA300_1361		-1.12
SAOUHSC_01941	6.02	SAUSA300_1757	*splB*	-2.44

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RESEARCH ARTICLE

Genome-wide Annotation, Identification, and Global Transcriptomic Analysis of Regulatory or Small RNA Gene Expression in *Staphylococcus aureus*

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ABSTRACT **In *Staphylococcus aureus*, hundreds of small regulatory or small RNAs (sRNAs) have been identified, yet this class of molecule remains poorly understood and severely understudied. sRNA genes are typically absent from genome annotation files, and as a consequence, their existence is often overlooked, particularly in global transcriptomic studies. To facilitate improved detection and analysis of sRNAs in *S. aureus*, we generated updated GenBank files for three commonly used *S. aureus* strains (MRSA252, NCTC 8325, and USA300), in which we added annotations for >260 previously identified sRNAs. These files, the first to include genome-wide annotation of sRNAs in *S. aureus*, were then used as a foundation to identify novel sRNAs in the community-associated methicillin-resistant strain USA300. This analysis led to the discovery of 39 previously unidentified sRNAs. Investigating the genomic loci of the newly identified sRNAs revealed a surprising degree of inconsistency in genome annotation in *S. aureus*, which may be hindering the analysis and functional exploration of these elements. Finally, using our newly created annotation files as a reference, we perform a global analysis of sRNA gene expression in *S. aureus* and demonstrate that the newly identified *tsr25* is the most highly upregulated sRNA in human serum. This study provides an invaluable resource to the *S. aureus* research community in the form of our newly generated annotation files, while at the same time presenting the first examination of differential sRNA expression in pathophysiologically relevant conditions.**

IMPORTANCE **Despite a large number of studies identifying regulatory or small RNA (sRNA) genes in *Staphylococcus aureus*, their annotation is notably lacking in available genome files. In addition to this, there has been a considerable lack of cross-referencing in the wealth of studies identifying these elements, often leading to the same sRNA being identified multiple times and bearing multiple names. In this work, we have consolidated and curated known sRNA genes from the literature and mapped them to their position on the *S. aureus* genome, creating new genome annotation files. These files can now be used by the scientific community at large in experiments to search for previously undiscovered sRNA genes and to monitor sRNA gene expression by transcriptome sequencing (RNA-seq). We demonstrate this application, identifying 39 new sRNAs and studying their expression during *S. aureus* growth in human serum.**

Received 13 November 2015 **Accepted** 22 December 2015 **Published** 9 February 2016

Citation Carroll RK, Weiss A, Broach WH, Wiemels RE, Mogen AB, Rice KC, Shaw LN. 2016. Genome-wide annotation, identification, and global transcriptomic analysis of regulatory or small RNA gene expression in *Staphylococcus aureus*. mBio 7(1):e01990-15. doi:10.1128/mBio.01990-15.

Invited Editor Paul Dunman, University of Rochester **Editor** Gerald B. Pier, Harvard Medical School

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In recent years, a number of studies have been carried out, employing both experimental and computational methods to identify regulatory or small RNAs (sRNAs) in *Staphylococcus aureus* (1–12). Hundreds of sRNAs have been identified, and many, in addition to the *agr* effector RNAIII, have been shown to play a role in gene regulation (while these molecules go by a variety of names such as regulatory RNAs or noncoding RNAs, we will use sRNAs to refer to them all, as previously recommended [13]). Despite advancements in sRNA identification, the roles of most of these molecules remain unknown, because in many cases, limited functional information can be gathered from analysis of their sequence alone.

One additional factor that has hampered the study of sRNAs in *S. aureus* has been the lack of a clear nomenclature and annotation system. This absence of a systematic identification and annotation process has led to the repeated discovery of the same sRNAs on multiple occasions, the reidentification of already known sRNAs (e.g., RNAIII), and even to important protein-coding genes being ascribed as sRNAs (e.g., the α-PSM transcript, which is not annotated in most *S. aureus* genome files). Recent work by Sassi et al. (14) established an online database for staphylococcal sRNAs; however, most sRNAs, including the well-studied RNAIII, are still not included in annotated *S. aureus* GenBank genome files. This is a marked oversight, as annotated genome files serve as the reference for global genomic and transcriptomic studies; thus, the absence of sRNAs from these files severely impedes their study and prevents us from gaining an overarching picture of regulatory circuits.

In *S. aureus*, most sRNA identification studies have been performed in a single background, the hospital-acquired methicillin-resistant *S. aureus* (MRSA) strain N315. The existence of many of these sRNAs has been demonstrated experimentally in strain N315 (1, 3, 4, 6, 7); however, very few of them have been investigated in other *S. aureus* strains, including the epidemic community-associated MRSA (CA-MRSA) strain USA300 (15). As such, their existence, location, and copy number in most *S. aureus* isolates is unknown, preventing us from gaining a sense of their role in the physiological and pathogenic differences between strains.

To better understand the sRNA content of multiple *S. aureus* strains, we explored the genomes of three well-studied *S. aureus* strains (USA300, MRSA252, and NCTC 8325). We identified the location(s) of previously discovered sRNAs and created new GenBank genome annotation files for each strain, inserting ~260 sRNAs. These newly annotated files serve as a valuable resource allowing us to do the following: (i) search the genome of each strain for as yet unidentified sRNAs without mistakenly reidentifying known species, and (ii) calculate expression values for these genes using transcriptome sequencing (RNA-seq) data.

To demonstrate the application of these new files, we performed RNA-seq on CA-MRSA strain USA300 growing in laboratory media and human serum and aligned the data to our newly created sRNA annotated genome files. Examining the data, we identified 39 novel putative sRNAs that had not been previously reported. These novel sRNAs were annotated, cross-referenced in the genomes of strains NCTC 8325 and MRSA252, and added to the newly created genome files as well. During the cross-referencing process, we observed numerous examples of inconsistent genome annotation in different *S. aureus* strains. We highlight examples clearly demonstrating genome misannotation and demonstrate how this phenomenon is adversely affecting the identification and characterization of sRNAs. Finally, we calculate expression values and examine the global sRNA expression profile of strain USA300, uncovering a wealth of molecules that display differential expression in human serum. The latter point is of significant importance, as it gives us a unique look into the sRNA transcriptome, not only during growth of *S. aureus* but also in a pathophysiologically relevant growth environment. The new genome annotation files described in this work have been deposited in the figshare depository and are freely available for download. We suggest that these newly reannotated genomes will be a valuable resource to the *S. aureus* research community for sRNA identification and analysis hereafter, as they can be incrementally added to as new sRNAs are discovered.

RESULTS

Annotation of sRNAs on the *S. aureus* genome. Previous studies in our lab have utilized RNA-seq to determine the global transcriptomic profile of *S. aureus* in the community-associated MRSA strain USA300 (16, 17). Analysis of these data sets revealed a large number of *S. aureus* transcripts that map to intergenic regions where no protein-coding genes have been annotated. We hypothesized that many of these transcripts represent sRNAs because of the following. (i) Most *S. aureus* genome annotation files do not contain annotations for sRNA genes. (ii) Recent studies carried out in the *S. aureus* N315 background have demonstrated that there are several hundred sRNAs encoded in the *S. aureus* genome (12, 14). To facilitate improved global transcriptomic analysis of *S. aureus* by RNA-seq, we created new GenBank genome annotation files for three commonly used *S. aureus* strains, NCTC 8325, MRSA252, and USA300. To do this, we elected to expand the sRNA annotation and nomenclature system already present for 25 sRNAs in strain MRSA252 (see Table S1 in the supplemental material) and apply a similar annotation/nomenclature system to strains USA300 and NCTC 8325 (for details, see supplemental material). To include annotations for known sRNAs, we performed a literature search to identify studies in which sRNAs in *S. aureus* were reported. A total of 12 papers that employed a variety of methods to identify sRNAs, including computational approaches, microarray studies, cDNA cloning, and high-throughput sequencing, were investigated (1–12). Using the information provided in these publications, a list of 928 potential sRNAs was assembled (Table S2) (1–12). In order to ensure accurate annotation of each sRNA from this list, RNA-seq experiments were performed for each of the three strains growing under standard laboratory conditions. Reads generated from these works were aligned to the respective genomes and used as a guide to identify the specific location of each sRNA for each of the three strains (see Fig. S1 in the supplemental material).

This work condensed the 928 putative sRNAs to a total of 248 annotations for strain MRSA252, 254 annotations for strain NCTC 8325, and 264 annotations for strain USA300 (for a more-comprehensive explanation of how sRNAs were identified and annotated, see Text S1 in the supplemental material). Such a dramatic reduction in the number of sRNAs points to the scale of overlapping identification, reidentification, and duplicate naming that was extant in the literature for these elements and the poor state of sRNA curation in *S. aureus* genomes. Our newly generated GenBank files for all three strains represent the first comprehensive list of sRNAs annotated directly in the *S. aureus* genome, which will serve as a valuable reference point for future sRNA discovery, and more broadly, global transcriptomic analyses of *S. aureus* by RNA-seq.

Detection of novel sRNAs in strain USA300. The creation of GenBank files containing annotations for previously identified sRNAs provided us with a unique opportunity to examine RNA-seq data for novel forms of these elements, without mistakenly reidentifying those that are already known. Accordingly, we set out to perform such an exploration using the community-associated MRSA isolate USA300, an undeniably relevant clinical strain for which no sRNA identification studies have yet been performed. To maximize the probability of identifying novel sRNAs, we performed RNA-seq with USA300 grown under both laboratory conditions (tryptic soy broth [TSB]), and in media that was more pathophysiologically relevant to its infectious lifestyle (human serum) (18).

RNA-seq reads from bacteria grown under these conditions were mapped to the newly created USA300 GenBank file (containing 264 sRNA annotations), followed by a thorough examination for the presence of novel sRNA transcripts. A total of 39 potential sRNAs were identified from both conditions, which we named *tsr1* to *tsr39* for Tampa small RNA (Table 1) (for details regarding the criteria used for our determination of novel sRNAs, see Text S1 in the supplemental material). Although the majority of the *tsr* genes were located in intergenic regions, a number were identified as being antisense to annotated genes or as partially overlapping annotated coding DNA sequence (CDS) genes. The novel sRNAs were also added to our newly created USA300 genome file, using

TABLE 1 Novel sRNAs identified in strain USA300

sRNA designation	sRNA gene	Strand[a]	Location	Size (nt)	Chromosomal location of *tsr* gene in strain:		
					USA300[b]	MRSA252[b]	NCTC 8325[b]
SAUSA300s265	*tsr1*	>	52438−53094	656	IG	−	−
SAUSA300s266	*tsr2*	<	57712−57804	92	IG	−	−
SAUSA300s267	*tsr3*	<	61388−61550	162	IG	−	−
SAUSA300s268	*tsr4*	>	73511−74139	628	AS	AS	AS
SAUSA300s269	*tsr5*	<	79346−79425	79	AS	−	−
SAUSA300s270	*tsr6*	>	120785−120897	112	IG	IG	IG
SAUSA300s271	*tsr7*	>	169903−170079	176	IG	−	IG
SAUSA300s272	*tsr8*	<	170013−170214	201	IG	−	IG
SAUSA300s273	*tsr9*	<	228412−228796	384	IG	CDS	CDS
SAUSA300s274	*tsr10*	>	349895−350058	163	IG	OL	OL
SAUSA300s275	*tsr11*	<	356689−356782	93	OL	OL	OL
SAUSA300s276	*tsr12*	>	457272−457333	61	IG	OL	AS
SAUSA300s277	*tsr13*	>	484942−485025	83	OL	OL	OL
SAUSA300s278	*tsr14*	>	834340−834885	545	IG	IG	AS
SAUSA300s279	*tsr15*	>	896563−897379	816	AS	AS	AS
SAUSA300s280	*tsr16*	<	911246−911364	118	AS	OL	OL
SAUSA300s281	*tsr17*	<	973559−973971	412	IG	CDS	CDS
SAUSA300s282	*tsr18*	<	1074292−1074484	192	IG	CDS	CDS
SAUSA300s283	*tsr19*	<	1080302−1080394	92	IG	IG	IG
SAUSA300s284	*tsr20*	<	1154300−1154753	453	IG	−	IG
SAUSA300s285	*tsr21*	<	1154827−1156001	1174	CDS	CDS_3	CDS_3
SAUSA300s286	*tsr22*	>	1165484−1165865	381	IG	CDS	CDS
SAUSA300s287	*tsr23*	>	1256521−1256545	24	IG	IG	IG
SAUSA300s288	*tsr24*	>	1429517−1429754	237	IG	−	IG
SAUSA300s289	*tsr25*	>	1442862−1443042	180	IG	AS	AS
SAUSA300s290	*tsr26*	>	1641611−1641732	121	IG	IG	−
SAUSA300s291	*tsr27*	<	1642820−1642923	103	IG	IG	−
SAUSA300s292	*tsr28*	<	1715900−1715975	75	OL	OL	OL
SAUSA300s293	*tsr29*	<	1954961−1955091	130	IG	−	IG
SAUSA300s294	*tsr30*	>	2126434−2126545	111	IG	−	−
SAUSA300s295	*tsr31*	<	2244964−2245035	71	IG	IG	IG
SAUSA300s296	*tsr32*	>	2337922−2338072	150	IG	IG	CDS
SAUSA300s297	*tsr33*	<	2410564−2410648	84	IG	IG	IG
SAUSA300s298	*tsr34*	>	2591032−2591131	99	IG	IG	IG
SAUSA300s299	*tsr35*	>	2608047−2608594	547	IG	CDS	CDS
SAUSA300s300	*tsr36*	<	2608120−2608645	525	IG	AS	AS
SAUSA300s301	*tsr37*	>	2620285−2620621	336	IG	CDS	CDS
SAUSA300s302	*tsr38*	<	2664856−2664945	89	IG	IG	IG
SAUSA300s303	*tsr39*	<	2811278−2811330	52	IG	IG	IG

[a] >, forward strand; <, reverse strand.

[b] Characteristics of the chromosomal location of the *tsr* gene. IG, located in the intergenic region; −, absent, deleted, or no homologue; AS, antisense to the annotated gene; CDS, located within an existing annotated CDS; OL, partially overlaps CDS gene; CDS_3, the corresponding locus contains three annotated CDSs.

the nomenclature system described in Text S1, resulting in a GenBank file with a total of 303 sRNAs in the USA300 genome. This brings the total number of genes annotated on the USA300 genome from 2,850 to 3,153, representing an approximately 10% increase in annotated genes.

To investigate the conservation of *tsr* genes in *S. aureus*, we analyzed their corresponding chromosomal loci on the *S. aureus* NCTC 8325 and MRSA252 genomes by performing a BLAST search (Table 1). Of the 39 *tsr* genes, 29 were found in strain MRSA252, while 32 were found in strain NCTC 8325. Annotations were again added to the MRSA252 and NCTC 8325 genome files for each of the *tsr* genes identified using the nomenclature outlined (see Text S1 in the supplemental material). Importantly, five of the *tsr* genes appear to be unique to USA300 (*tsr1*, *tsr2*, *tsr3*, *tsr5*, and *tsr30*), with no homologues found in either MRSA252 or NCTC 8325. In addition, five *tsr* genes were present in USA300 and NCTC 8325 but absent in MRSA252 (*tsr7*, *tsr8*, *tsr20*, *tsr24*, and *tsr29*), while two were present in USA300 and MRSA252 but absent in NCTC 8325 (*tsr26* and *tsr27*) (see Fig. S2 in the supplemental material).

Northern blot analysis of *tsr* transcripts. To validate our RNA-seq-based approach for sRNA discovery, five putative *tsr* transcripts were examined by Northern blot analysis. The transcripts investigated had different expression patterns in TSB compared to human serum. Three of the transcripts investigated (*tsr8*, *tsr26*, and *tsr31*) showed no alteration in expression between TSB and human serum, one (*tsr25*) demonstrated an increase in expression, while another (*tsr33*) showed a decrease (see Table S3 in the supplemental material). Northern blot analysis confirmed the predicted size and orientation of *tsr25*, *tsr26*, and *tsr31* (Fig. 1). In the case of *tsr8* and *tsr33*, bands were identified; however, they were considerably larger than those predicted by RNA-seq analysis (Fig. 1). For example, the predicted size of *tsr33* was 85 nucleotides (nt); however, the size observed by Northern blotting was

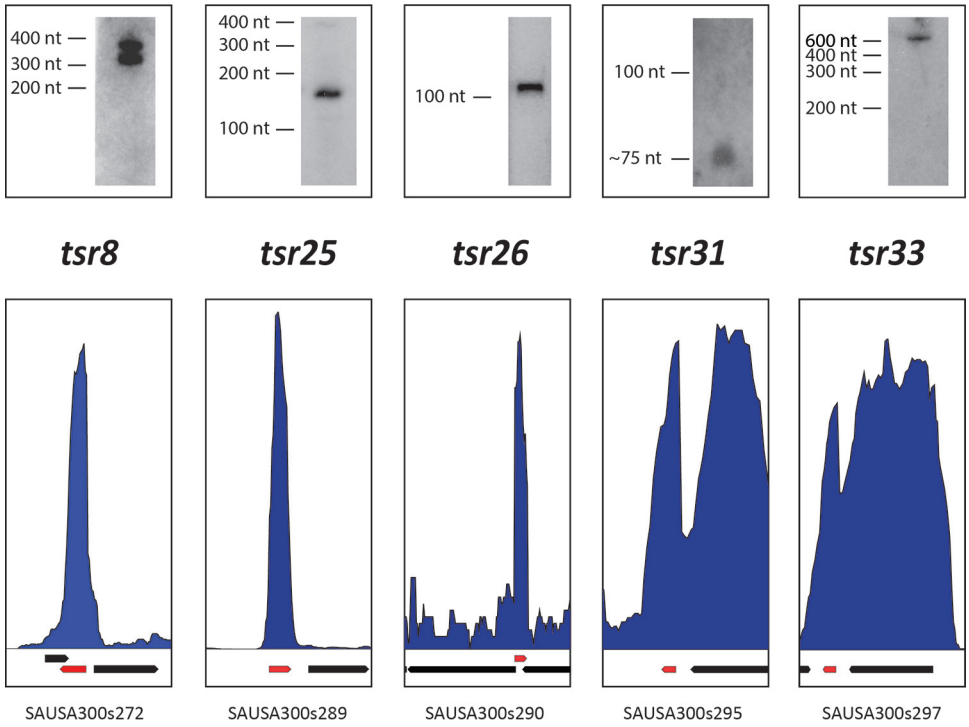

FIG 1 Northern blot analysis of *tsr* transcripts. Northern blotting was performed using oligonucleotide probes specific for the *tsr* transcripts (*tsr8*, *tsr25*, *tsr26*, *tsr31*, and *tsr33*). RNA-seq read alignments for each corresponding chromosomal location are shown, as are CDS genes (black arrows), and sRNAs (red arrows). The depth of read coverage on the genome is shown by the blue histograms.

approximately 600 nt. When looking at the *tsr33* transcript, one observes that it is located at the 3′ end of the *sarR* gene and is transcribed in the same orientation. The combined size of *sarR* and *tsr33* would be around 600 nt, therefore suggesting that *tsr33* represents a large 3′ untranslated region (UTR) for the *sarR* gene. However, RNA-seq alignment from strain USA300 growing in human serum demonstrates a much greater depth of coverage (and hence abundance) of *tsr33* than of *sarR*, suggesting that a *tsr33*-specific RNA may exist under these conditions (see Fig. S3 in the supplemental material). On the basis of the above information, we predict that *tsr33* is cotranscribed as a 3′ UTR of *sarR*, but under certain conditions (e.g., growth in human serum), it is possible that a *tsr33* RNA may exist independently of *sarR*.

The second *tsr* demonstrating a size difference was *tsr8*, which was predicted by RNA-seq to be 201 nt; however, two bands were observed by Northern blotting with approximate sizes between 300 and 400 nucleotides. A possible explanation for this may come from the fact that *tsr8* and *tsr7* are convergently transcribed, that the two transcripts are complementary at the 3′ ends, and that these regions overlap. The density of reads, in both directions, mapping to this region of complementarity make it difficult to predict the precise location of each transcript based on RNA-seq data, resulting in an underestimation of *tsr8* size. Given our experimental findings, the size of *tsr8* was amended to ~350 nt in the GenBank files. It is interesting to note that this type of genetic organization (convergent transcripts overlapping at the 3′ end) is common among toxin-antitoxin (TA) systems in *S. aureus* (19, 20), hence *tsr7* and *tsr8* could potentially represent a novel serum-induced TA system.

For some of the *tsr* elements expressed at low levels, Northern blot detection proved unsuccessful (data not shown); therefore, we employed a reverse transcriptase PCR (RT-PCR)-based approach, which is inherently more sensitive. Using this methodology, we were able to validate the presence of an additional six transcripts, *tsr1*, *tsr2*, *tsr18*, *tsr24*, *tsr29*, and *tsr32* (see Fig. S4 in the supplemental material), suggesting that our RNA-seq-based identification approach is effective at identifying legitimate sRNA molecules.

Inconsistent genome annotation in strains USA300, MRSA252, and NCTC 8325. Twenty-seven *tsr* genes were found in the genomes of all three strains (USA300, MRSA252, and NCTC 8325). For 14 of these genes, the corresponding genomic loci were similarly annotated in all three strains, e.g., *tsr6* is located in an intergenic region in all three strains, while *tsr15* is located antisense to an annotated CDS. Interestingly, for 13 *tsr* genes, the genomic loci in the three strains studied are differentially annotated (Table 1). In many cases, the NCTC 8325 and MRSA252 genomes contain annotations for CDS genes, while the USA300 genome specifies these loci as being intergenic (e.g., *tsr9*, *tsr17*, *tsr18*, etc.). An open reading frame (ORF) search reveals that 11 *tsr* genes have the potential to encode proteins (of 30 amino acids or larger in size). Seven of these genes are annotated as CDS in strains MRSA252 and NCTC 8325. This raises the possibility that some *tsr* genes may in fact be protein-coding genes that were omitted from the USA300 genome annotation. Conversely, it is also possible that the NCTC 8325 and MRSA252 genomes may be incorrect and that these annotated genes do not encode proteins. Upon close examination, our data clearly demonstrate that incorrect genome annotation accounts for at least some of the discrepancies observed. For example, the *tsr12* locus is annotated differently in all three

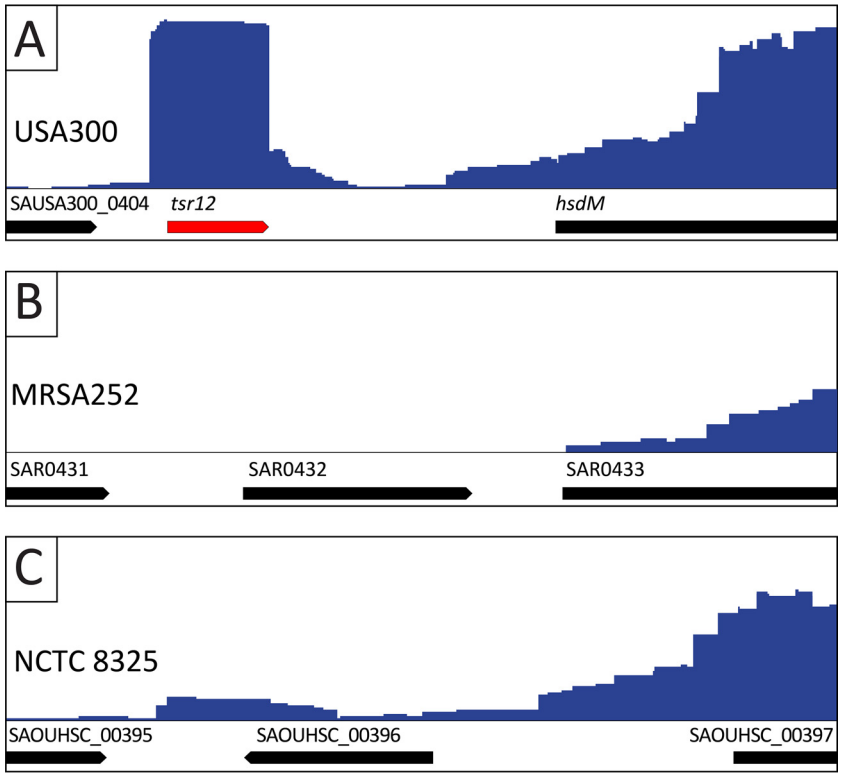

FIG 2 Variation in genome annotation of the *tsr12* locus. RNA-seq read alignment data are shown for strains USA300 (A), MRSA252 (B), and NCTC 8325 (C). Annotations for CDS genes are shown by black arrows, and the depth of coverage is shown by the blue histograms. The location of *tsr12* is shown by a red arrow. (A) There is no CDS annotation at the *tsr12* locus in strain USA300. (B) In strain MRSA252, a gene (SAR0432) is annotated in the forward direction at the *tsr12* locus. (C) In strain NCTC 8325, a gene (SAOUHSC_00396) is annotated in the reverse direction at the *tsr12* locus.

strains (Fig. 2). In strain USA300, *tsr12* is located in an intergenic region (between SAUSA300_0404 and *hsdM*). In strain MRSA252, *tsr12* incompletely overlaps with a CDS gene annotated in the same orientation, while in strain NCTC 8325, it partially overlaps with a CDS gene annotated in the opposite direction. Bioinformatic analysis of these CDS genes reveals that they both specify very small proteins (43 amino acids in MRSA252 and 33 amino acids in NCTC 8325) that possess no known structural motifs and have no homology to any protein in the database beyond counterparts in a handful of other *S. aureus* strains. Furthermore, in USA300 and NCTC 8325, the *tsr12* locus is 100% identical at the nucleotide level, making large-scale differences in coding sequences (e.g., inverse open reading frames) unlikely. Collectively, this demonstrates that there is likely misannotation in the genomes of MRSA252 and NCTC 8325 and that our suggested annotation of *tsr12* as an sRNA is likely the correct one.

tsr12 is an example where incorrect genome annotation is rather clear and raises important questions regarding how commonplace this type of genome misannotation may be. To investigate the issue of inconsistent genome annotation in *S. aureus* further, we selected another sRNA gene for additional study. Teg23 (SAUSA300s148, SARs145, SAOUHSCs144) was originally identified by Beaume et al. (7) as a potential 5′ UTR of the SAS083 gene in *S. aureus* strain N315. In strains NCTC 8325 and MRSA252, a gene is annotated in the position corresponding to SAS083 (SAOUHSC_02572 and SAR2384, respectively), but no gene is annotated in strain USA300 (Fig. 3A). Upon analysis, SAS083, SAOUHSC_02572, and SAR2384 once again encode small, hypothetical proteins with no known structural features, functional domains, or apparent homologues and are therefore likely misannotated genes (as noted above for the *tsr12* locus).

Northern blot analysis using *S. aureus* USA300 grown in human serum (where Teg23 is strongly upregulated) detected a band of around 310 nt (as predicted by RNA-seq), alongside an additional band of approximately 215 nt (Fig. 3B). The smaller band (which we designated Teg23.1) was more abundant than the larger band (designated Teg23.2) and was repeatedly detected in multiple Northern blots (data not shown), suggesting that two forms of the transcript may exist within the cell. The CDS annotated in the NCTC 8325 genome at the Teg23 position (SAOUHSC_02572) potentially encodes a protein of 80 amino acids (Fig. 3C, red), while the corresponding locus in strain MRSA252 (SAR2384) potentially encodes a protein of 64 amino acids (Fig. 3C, blue). The difference is due to a 4-bp insertion in the MRSA252 genome that results in the creation of a stop codon (Fig. 3C). The nucleotide sequence of the 432-bp intergenic region from strain USA300 is almost identical with the corresponding locus in strain NCTC 8325 (431/432 identical), and it does not contain the 4-bp insertion found in MRSA252 (Fig. 3C). Therefore, an 80-amino-acid protein could potentially be generated from a transcript originating from this locus in USA300 (Fig. 3C, green), similar to NCTC 8325 (for simplicity, this potential protein will be referred to as Teg23P for Teg23 protein).

To test whether Teg23P is produced in strain USA300, we cloned a His_6-tagged variant of its putative coding sequence, along with its native promoter, into an *S. aureus* shuttle vector. Western

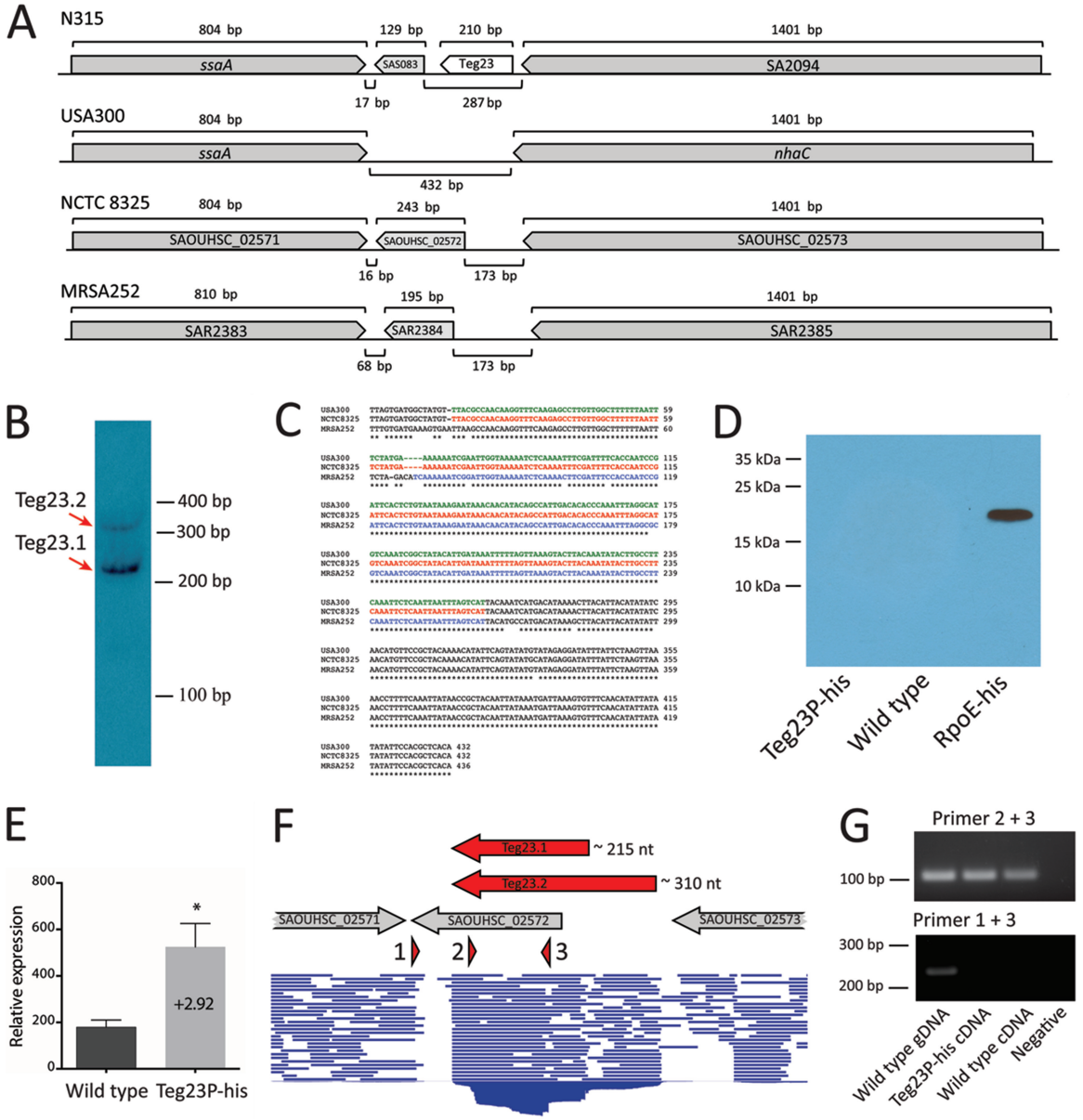

FIG 3 Analysis of the Teg23 locus. (A) Teg23 locus in *S. aureus* strains N315, USA300, NCTC 8325, and MRSA252. Annotated CDS genes are shown in gray. The previously reported location of Teg23 is shown in white. (B) Northern blot analysis of Teg23. The Teg23 transcript was detected by Northern blot analysis with a strand-specific oligonucleotide probe. Two bands were detected and were designated Teg23.1 (approximately 215 nt) and Teg23.2 (approximately 310 nt). (C) Alignment of Teg23 DNA sequence in strains USA300, NCTC 8325, and MRSA252. Sequence corresponding to the SAOUHSC_02572 and SAR2384 CDS genes is highlighted in red and blue, respectively. Sequence potentially encoding an 80-amino-acid protein (Teg23P) in strain USA300 is highlighted in green. (D) Western blot to detect histidine-tagged Teg23P. No Teg23P protein was detected in *S. aureus* expressing the Teg23P-his plasmid (lane 1) or in a wild-type *S. aureus* strain without the plasmid (lane 2). Histidine-tagged RpoE was detected as a positive control (lane 3). (E) Quantitative RT-PCR analysis of Teg23 expression. Expression of Teg23 was analyzed in the wild-type *S. aureus* strain with or without the Teg23P-his plasmid. Approximately 3-fold-higher expression of Teg23 was detected in the strain expressing the Teg23P-his plasmid. Statistical significance was determined using Student's *t*-test. *, $P < 0.05$. (F) RNA-seq read alignment at the Teg23 locus in strain NCTC 8325. RNA-seq reads aligned to the NCTC 8325 genome are shown in blue. The locations of annotated genes are shown in gray. The locations of primers used for RT-PCR are shown in red. The predicted size and location of the Teg23.1 and Teg23.2 transcripts are shown by red arrows. (G) RT-PCR analysis of Teg23 transcript. PCR was performed using the primer pairs indicated and *S. aureus* genomic DNA (gDNA) (lane 1), cDNA from *S. aureus* containing the Teg23P-his plasmid (lane 2), cDNA from wild-type *S. aureus* (lane 3), or no DNA (lane 4). No PCR product was detected using primers 1 and 3 with *S. aureus* cDNA as the template.

blots of USA300 strain bearing this construct revealed no detectable band using an antihistidine antibody, while the positive control (histidine-tagged RpoE) was detected (Fig. 3D). Quantitative RT-PCR (qRT-PCR) to confirm that the construct was being expressed demonstrated a 2.92-fold-higher expression of Teg23 in *S. aureus* containing the Teg23P-his$_6$ plasmid (Fig. 3E). These data confirm that the plasmid gene-encoded Teg23 is expressed; however, no protein corresponding to Teg23P-his$_6$ is generated. This strongly suggests that no protein corresponding to Teg23P is produced in strain USA300.

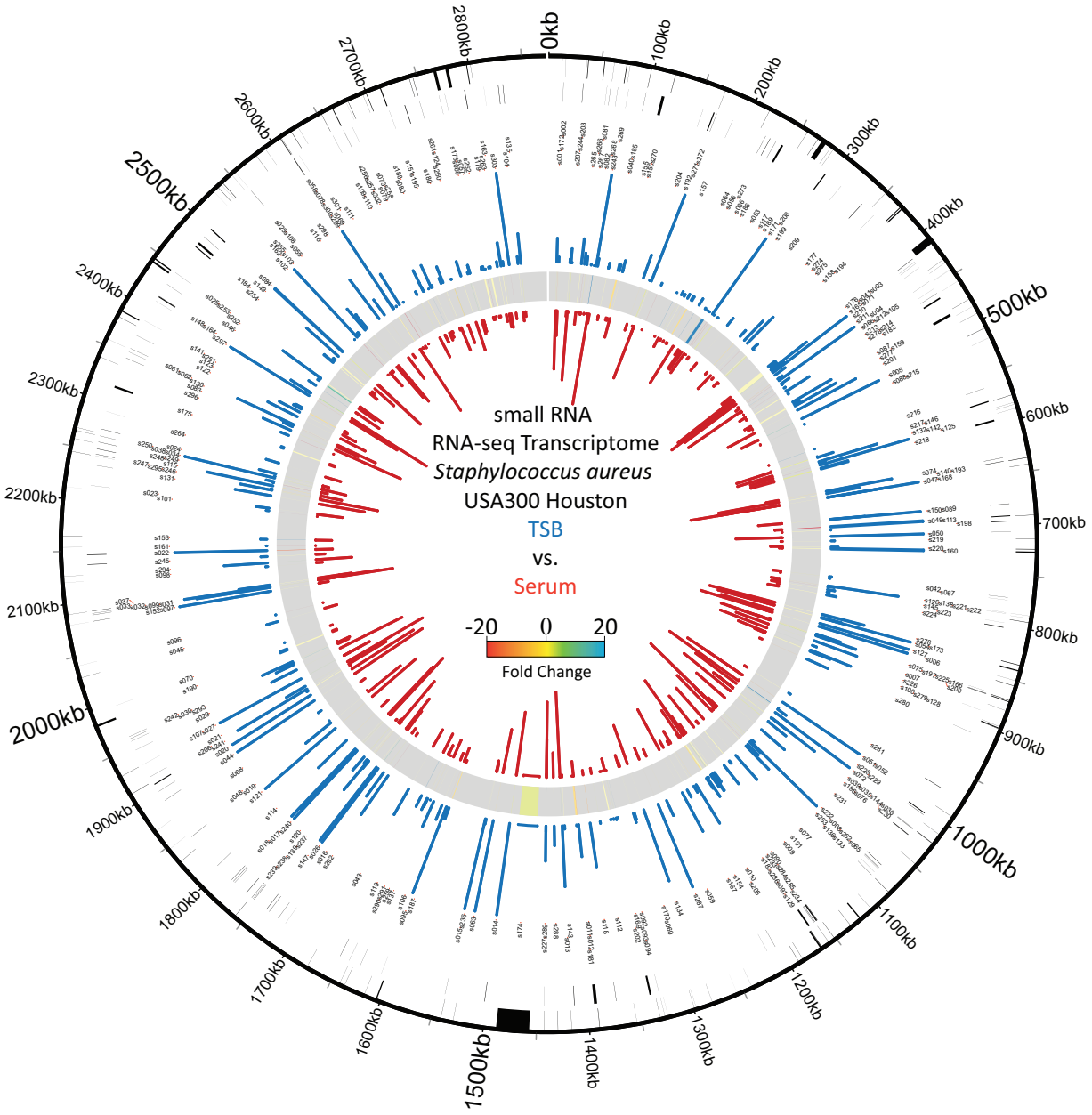

FIG 4 Location and comparative expression analysis of the 303 *S. aureus* USA300 sRNAs. The black outer ring shows the chromosomal locations of 303 sRNAs in strain USA300 (the outer circle shows the forward strand, while the inner circle shows the reverse strand). The inner rings show sRNA gene expression profile in TSB (outside, blue), human serum (inside, red), and heat map (middle) showing differences in expression. The heights of the blue or red bars are proportional to the expression values (the maximum displayed expression value was 1,000).

The RNA-seq read alignment data demonstrate that >99% of the Teg23 reads in strain NCTC 8325 terminate 88 nucleotides upstream of the annotated 3′ end of the gene (Fig. 3F). Consequently, transcript generated at this locus terminates before the end of the annotated gene, and therefore, an 80-amino-acid protein corresponding to the SAOUHSC_02572 gene is unlikely to be made. A similar pattern of transcript termination is observed in the *S. aureus* USA300 RNA-seq data set, further suggesting that the Teg23P protein could not be produced in this strain (data not shown). To test this further, we performed PCR using cDNA generated from total RNA from strain USA300. PCR performed using primers that bind within the Teg23 sequence (primers 2 and 3 [Fig. 4F]) generated products using template cDNA from USA300 and USA300 containing the Teg23P-his$_6$ plasmid (Fig. 3G). In contrast, no product was generated in PCRs using one primer within the Teg23 sequence and a second primer located at the 3′ end of the Teg23P sequence (primers 1 and 3 [Fig. 3F]). These data show that the Teg23 RNA is being generated but that it terminates prior to the 3′ end of Teg23P, confirming the results from the Western blot analysis that Teg23 does not encode an 80-amino-acid protein.

While it is impossible to completely rule out the existence of a

TABLE 2 42 sRNAs that are upregulated in human serum versus TSB

sRNA designation	sRNA gene name or feature	TSB expression value (RPKM)[b]	Serum expression value (RPKM)	Fold change
SAUSA300s289	*tsr25*	60.67	35,374.06	583.02
SAUSA300s046	*rsaOG*	90.99	34,267.77	376.61
SAUSA300s053	RsaG	80.85	3,463.09	42.83
SAUSA300s119	*ssr24*	130.78	3,217.86	24.6
SAUSA300s153	Teg16	71.05	1,174.01	16.52
SAUSA300s066	Sau-63	104.99	1,690.95	16.11
SAUSA300s030	*sprC*	149.58	2,144.32	14.34
SAUSA300s026	*ssrS*	2,991.16	37,212.81	12.44
SAUSA300s005	*ffs*	929.74	11,188.00	12.03
SAUSA300s148	Teg23	256.6	2,950.59	11.5
SAUSA300s050	RsaD	2,737.91	30,473.59	11.13
SAUSA300s226	JKD6008sRNA173	50.05	539.79	10.78
SAUSA300s013	Lysine riboswitch	659.93	6,917.84	10.48
SAUSA300s260	JKD6008sRNA396	16.13	150.26	9.31
SAUSA300s294	*tsr30*	470.64	4,383.00	9.31
SAUSA300s027	*sprA1*	1,097.34	9,306.35	8.48
SAUSA300s233	JKD6008sRNA205	96.96	717.53	7.4
SAUSA300s003	T-box riboswitch	158.5	1,156.13	7.29
SAUSA300s094	Teg108	19.39	132.54	6.83
SAUSA300s099	Teg124	37.1	245.19	6.61
SAUSA300s110	*sprA3*	36.05	233.31	6.47
SAUSA300s282	*tsr18*	105.4	653.5	6.2
SAUSA300s024	GlmS ribozyme	185.53	1,064.95	5.74
SAUSA300s117	*ssr8*	12.13	67.9	5.6
SAUSA300s038	*sprG3*	191.04	1,049.33	5.49
SAUSA300s120	*ssr28*	188.03	1,012.53	5.39
SAUSA300s028	*sprA2*	99.08	504.62	5.09
SAUSA300s210	JKD6008sRNA071	136.39	691.13	5.07
SAUSA300s100	Teg124	469.39	2,313.98	4.93
SAUSA300s002	SAM[a] riboswitch	56.06	268.4	4.79
SAUSA300s031	*sprD*	804.11	3,810.91	4.74
SAUSA300s021	SAM riboswitch	239.09	941.21	3.94
SAUSA300s075	*rsaOL*	273.5	1,010.42	3.69
SAUSA300s091	Teg60	95.9	350.09	3.65
SAUSA300s034	*sprF3*	1,227.97	4,261.30	3.47
SAUSA300s016	T-box riboswitch	316.76	1,059.28	3.34
SAUSA300s084	Teg27	15,788.22	52,571.03	3.33
SAUSA300s042	*rsaOC*	26.75	88.89	3.32
SAUSA300s135	*ssr100*	322.65	1,050.22	3.26
SAUSA300s163	Teg28as	46.74	149.53	3.2
SAUSA300s079	*rsaOU*	0	85.01	∞
SAUSA300s151	Teg134	0	204.13	∞

[a] SAM, *S*-adenosylmethionine.
[b] RPKM, reads per kilobase of transcript per million mapped reads.

protein corresponding to Teg23P, these results strongly suggest that the CDS annotations for Teg23P in strains N315, MRSA252, and NCTC 8325 (i.e., SAS083, SAR2384, and SAOUHSC_02572, respectively) are incorrect. Teg23 was originally identified as a 5′ UTR for the SAS083 gene (i.e., the gene encoding Teg23P in N315) (7); however, the data presented herein demonstrates that this is incorrect and Teg23 likely represents a nontranslated sRNA. This comprehensively highlights how overannotation of genomes with CDS genes may be masking the identification of transcripts that encode sRNAs.

Global analysis of sRNA gene expression in strain USA300. The inclusion of 303 sRNA gene annotations in the GenBank file of strain USA300 allowed us to calculate global gene expression values for sRNAs using our RNA-seq data sets. To examine variation in sRNA gene expression, we calculated and compared expression values for *S. aureus* USA300 growing in TSB and human serum, two conditions known to result in widespread changes in gene expression (18). The location, relative expression, and fold change for each of the 303 sRNA genes in strain USA300 are shown in Fig. 4 and Table S3 in the supplemental material. To identify sRNA genes with meaningful differences in expression, we applied cutoffs to eliminate genes expressed at low levels and those displaying fold changes that are less than 3-fold (see Text S1 in the supplemental material for details). This resulted in a total of 83 sRNAs displaying alterations in gene expression under the two conditions tested. Forty-two were upregulated in human serum (Table 2), while 41 were downregulated (Table 3). Of the newly identified *tsr* genes, 19 displayed differential regulation, with 16 downregulated in serum, while 3 (*tsr18*, *tsr25*, and *tsr30*) were upregulated. Interestingly, the newly identified *tsr25* sRNA demonstrated the largest upregulation in serum of any sRNA (583-fold). To validate these findings, we performed Northern

TABLE 3 41 sRNAs that are downregulated in human serum versus TSB

sRNA designation	sRNA gene name or feature	TSB expression value (RPKM)[a]	Serum expression value (RPKM)	Fold change
SAUSA300s277	*tsr13*	509.65	0.43	−1,187.67
SAUSA300s113	*sbrC*	1,834.12	6.3	−291.07
SAUSA300s266	*tsr2*	122.44	0.42	−290.6
SAUSA300s296	*tsr32*	92.33	0.44	−210.63
SAUSA300s171	Sau-6569	41,077.46	250.83	−163.77
SAUSA300s049	RsaC	8,079.72	77.42	−104.37
SAUSA300s004	Purine riboswitch	2,277.90	45.36	−50.22
SAUSA300s303	*tsr39*	3,132.51	64.48	−48.58
SAUSA300s162	Teg25as	6,668.30	191.51	−34.82
SAUSA300s125	*ssr47*	6,272.84	256.03	−24.5
SAUSA300s052	RsaF	35,374.06	1,713.79	−20.64
SAUSA300s275	*tsr11*	398.93	20.01	−19.94
SAUSA300s301	*tsr37*	390.39	23.36	−16.72
SAUSA300s292	*tsr28*	513.06	31.91	−16.08
SAUSA300s297	*tsr33*	8,987.32	592.2	−15.18
SAUSA300s062	Sau-31	141.32	9.7	−14.57
SAUSA300s022	RNAIII	66,968.70	5,178.15	−12.93
SAUSA300s283	*tsr19*	162.04	13.93	−11.63
SAUSA300s118	*ssr16*	478.47	49.19	−9.73
SAUSA300s302	*tsr38*	251.17	27.55	−9.12
SAUSA300s280	*tsr16*	254.42	30.88	−8.24
SAUSA300s095	Teg116	1,149.05	155.21	−7.4
SAUSA300s211	JKD6008sRNA073	59.06	8	−7.38
SAUSA300s287	*tsr23*	970.48	133.71	−7.26
SAUSA300s054	RsaH	4,110.36	629.99	−6.52
SAUSA300s087	Teg41	481.01	74.55	−6.45
SAUSA300s078	*rsaOT*	17,890.11	2,790.22	−6.41
SAUSA300s127	*ssr54*	3,245.19	584.15	−5.56
SAUSA300s051	RsaE	2,929.20	534.76	−5.48
SAUSA300s074	*rsaOI*	721.93	135.52	−5.33
SAUSA300s237	JKD6008sRNA258	100.89	20.87	−4.83
SAUSA300s298	*tsr34*	200.17	41.54	−4.82
SAUSA300s267	*tsr3*	85.01	19.21	−4.43
SAUSA300s114	*sbrE*	424.58	102.21	−4.15
SAUSA300s073	Sau-6072	265.82	64.27	−4.14
SAUSA300s276	*tsr12*	295.29	73.2	−4.03
SAUSA300s291	*tsr27*	135.22	34.48	−3.92
SAUSA300s204	RsaX04	265.58	70.09	−3.79
SAUSA300s086	Teg39	50.39	13.67	−3.69
SAUSA300s141	*ssr153*	74.69	21.06	−3.54
SAUSA300s010	T-box riboswitch	487.82	149.37	−3.27

[a] RPKM, reads per kilobase of transcript per million mapped reads.

blot analysis of *tsr25* and *rsaOG* (a previously identified sRNA which showed the second highest degree of upregulation in serum, 376-fold), confirming the predicted size and upregulation of both sRNAs in human serum (Fig. 5A). RNAIII was downregulated 13-fold in the RNA-seq data analysis, and we also confirmed this by Northern blotting (Fig. 5B). These results corroborate previously published data (18) and provide validation for the experimental techniques described herein.

The data presented above represent the first global transcriptomic analysis of sRNA gene expression in the CA-MRSA strain USA300 and demonstrate the utility of the sRNA annotation files constructed in this study. The biological functions of most sRNAs are unknown, making it difficult to ascertain the impact on the bacterial cell of their differential regulation. Studying the variation in sRNA expression in response to changing environmental conditions may provide insight into which sRNAs play a role in the adaptive nature of *S. aureus*. *tsr25* and *rsaOG*, for example, are upregulated >300-fold in human serum, suggesting a role for these molecules under these conditions. In addition, a number of conserved, well-studied sRNAs were among those differentially regulated in human serum. 4.5S RNA (SAUSA300s005) and *ssrS* (SAUSA300s026) demonstrated increased expression in human serum (12-fold increase for both). The increased expression of these RNA species may reflect physiological changes to the bacterial cell in this environmental niche (see Discussion).

DISCUSSION

In 1995, the release of the first fully sequenced bacterial genome heralded a new era of bacterial genomic research (21). Over the past 20 years, the number of sequenced bacterial genomes has risen exponentially, and new research strategies, techniques, and applications have emerged to exploit the opportunities that these resources provide. While raw genomic sequence data are valuable, the availability of fully annotated genome sequences, outlining the positions of known genes and genomic features, dramatically increases their utility. Global expression analysis techniques such as

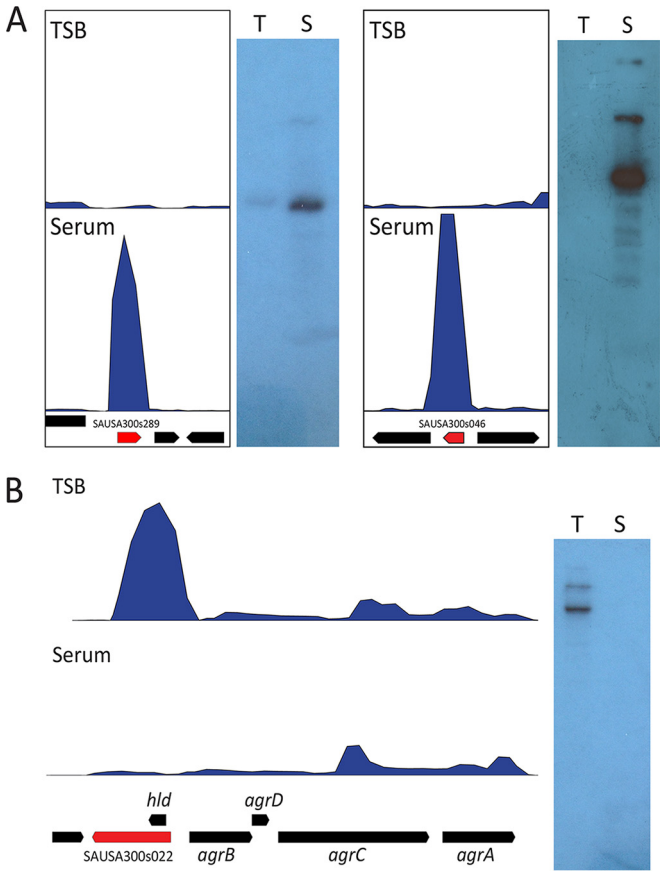

FIG 5 Northern blot analysis of serum-regulated sRNAs in strain USA300. (A) Analysis of sRNAs demonstrating upregulation in human serum. RNA-seq read alignment data and Northern blot analysis are shown for SAUSA300s289 (*tsr25*) and SAUSA300s046 (*rsaOG*) during growth in TSB (T) or human serum (S). (B) Analysis of RNAIII expression in human serum and TSB. RNA-seq read alignment data are shown for the entire *agr* locus. Northern blot analysis was performed using an oligonucleotide probe specific for the RNAIII transcript. Annotations for CDS genes are shown by black arrows, annotations for sRNAs are shown by red arrows, and depth of read coverage on the genome is shown by the blue histograms.

microarrays and RNA-seq depend heavily on annotated genome sequences as a reference source for genes in the bacterial cell. These techniques have proved extremely useful; however, recently, certain limitations to their application are becoming apparent. A major concern in this regard is that they do not provide expression data for genes that are not included in genome annotation files. Bacterial sRNAs represent a class of genes that are frequently absent from genome annotation files; consequently, their expression is rarely studied on a global level. In this work, we added annotations for 303 sRNA genes to the *S. aureus* USA300 genome, increasing the number of annotated genes from 2,850 to 3,153 (a 10% increase). Including sRNA gene annotations in *S. aureus* GenBank files facilitates global expression analysis of these understudied molecules. The 303 newly added annotations undoubtedly do not represent an exhaustive list of the complete *S. aureus* sRNA repertoire, as it is likely that subsequent studies, using different techniques and environmental conditions, will continue to add to this number. It is also likely that among the 303 annotations, there may be false positives as only 92 of the 303 sRNAs reported and annotated here (30%) have been confirmed by independent experimental methods other than high-throughput sequencing and microarray hybridization. Therefore, while the sRNA annotated GenBank files generated herein may not be definitive, they nonetheless represent a significant step forward and pave the way for future studies that can validate the existence, elucidate the role, and demonstrate the biological impact of these molecules on *S. aureus* physiology and virulence.

The 303 sRNA genes annotated in this study (representing 10% of all known genes on the *S. aureus* genome) were identified in *S. aureus* growing under a limited number of environmental conditions. How many sRNAs remain unidentified is unknown; however, on the basis of the data presented in this study and by others (14), it seems likely that sRNA genes may account for >15 to 20% of all transcripts in the *S. aureus* cell. Importantly, a benefit of the nomenclature system used in this study to annotate sRNAs (in addition to keeping original names intact) is that it can be expanded upon to accommodate new sRNAs as they are identified.

An unexpected observation arising from this study is the lack of consistency in genome annotation across multiple strains of *S. aureus*. The examples highlighted in this study (specifically *tsr12* and Teg23) demonstrate that this phenomenon occurs even at genomic loci with a high degree of homology (100% identical in the case of *tsr12* in strains USA300 and NCTC 8325). Genome annotation is typically performed bioinformatically and is rarely validated and curated (22); therefore, this variation in annotation likely arises because different annotation pipelines have been used to annotate different genomes. This raises the important question of whether certain genomes have been under- and/or overannotated. It is likely that examples of both situations occur; however, on the basis of our data, it appears that the overannotation of genomes (i.e., the inclusion of CDS annotations that are not legitimate mRNA transcripts) is common and could have deleterious consequences for sRNA identification and study. sRNA transcripts that map to genomic regions containing CDS annotations will mistakenly be assumed to encode proteins. Highlighting this point is the data presented in Fig. 3, which strongly suggest that Teg23 is an sRNA and not a protein. Nonetheless, we acknowledge that this type of data, by its very nature, cannot exclude the possibility that such a gene/protein exists, and hence, once a CDS has been annotated, it is difficult or impossible to conclusively prove that it does not exist. Careful attention must be paid to the location of transcript start/stop sites, the existence of ribosome binding sites and the predicted function of annotated genes. We observed many instances of inconsistent genome annotation where a given gene encoded a protein of unknown function with no homologues and no functional domains. This lack of homology may indicate that while sequence analysis alone may suggest that a gene is possible at this locus, it is not certain to exist. The results presented herein are a timely reminder that, although genome annotation files are valuable resources that are increasingly relied upon by next-generation DNA sequencing technology, these annotations should be treated with a reasonable degree of caution and not seen as an infallible reference. Confirmation of the existence of a gene/transcript/protein by traditional biochemical methods (such as 5′ and 3′ rapid amplification of cDNA ends [RACE], primer extension, and *in vivo* translation) remains essential.

Including sRNA annotations in GenBank files allowed us to perform global sRNA expression analysis by RNA-seq for the first time in the CA-MRSA isolate USA300. The data generated dem-

onstrated that 83 sRNAs are differentially expressed in TSB versus human serum (Tables 2 and 3). This represents 27% of the known sRNAs in strain USA300. It is not easy to interpret how their differential regulation affects the bacterial cell (because the biological functions of most of them are unknown); however, certain inferences can be made. The newly identified *tsr25* sRNA demonstrated a 582-fold increase in expression in human serum. It is tempting to speculate that increased expression of *tsr25* in serum suggests that it plays an important role during *S. aureus* bloodstream infections. A small number of conserved, well-studied sRNAs were also among the differentially regulated sRNAs in serum. 4.5S RNA, a component of the signal recognition particle, was upregulated 12-fold in human serum, perhaps reflecting altered protein secretion and/or protein composition in the cell membrane in this environment. Another important cellular RNA that has been well explored is 6S RNA (*ssrS*), which we demonstrate also has a 12-fold increase in expression during growth in serum. In *Escherichia coli*, 6S RNA binds to the housekeeping sigma factor σ^{70} and inhibits transcription from σ^{70}-dependent promoters. It is thought that this diverts RNA polymerase to alternative sigma factors (such as the stress response sigma factor), resulting in increased expression of adaptive and stress-circumventing genes (23). The upregulation of *ssrS* in human serum suggests that a similar situation occurs in *S. aureus*, whereby σ^{A}-dependent genes are downregulated and σ^{B}-dependent genes are upregulated by the action of 6S RNA.

Examining the global transcriptome can provide valuable insights into bacterial physiology and adaptation to environmental conditions. In the past, global transcriptomic analysis has focused on protein-coding genes, but here, we conduct global transcriptomic analysis and include newly annotated sRNA genes. Like protein-coding genes, sRNA genes display differential regulation that allow bacteria to adapt to environmental changes. The annotation files presented herein, which facilitate this kind of global analysis will prove to be a valuable resource for the future study of sRNAs in *S. aureus* and will more generally broaden our understanding of regulatory circuits.

MATERIALS AND METHODS

Strains, plasmids, and primers. Bacterial strains, plasmids, and primers used in this study are listed in Table 4. *S. aureus* and *E. coli* were grown routinely at 37°C with shaking in tryptic soy broth (TSB) and lysogeny broth (LB), respectively. Pooled human serum from anonymous donors, purchased from MP Biomedicals, was used for growth of *S. aureus* strain USA300. When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}.

RNA-seq. Samples for transcriptome sequencing (RNA-seq) were prepared as follows for *S. aureus* USA300 and SH1000. Overnight cultures were diluted 1:100 in 100 ml of fresh TSB and grown at 37°C with shaking for 3 h. Exponentially growing cultures were then diluted and synchronized by inoculating fresh 100-ml flasks of TSB at an optical density at 600 nm (OD_{600}) of 0.05, or in the case of strain USA300 growing in serum, 10 ml of human serum in a 50-ml tube. Synchronized cultures were grown for 3 h at 37°C with shaking, at which time bacteria were pelleted by centrifugation and stored at −80°C prior to RNA isolation. *S. aureus* UAMS-1 (a USA200 strain and close relative of MRSA252) overnight cultures were diluted to an optical density of 0.05 in 40 ml of TSB containing no dextrose (1:12.5 volume-to-flask volume ratio). Cultures were grown at 37°C with shaking for 4 h, before cells were harvested and stored as described above. For each RNA-seq sample, three replicate cultures were grown, and three biological replicate RNA isolations were performed (using the procedure outlined in reference 24). For each sample, equimolar amounts of the three biological replicate RNA preparations were mixed prior to rRNA reduction. The subsequent RNA-seq analysis therefore represents the average of three biological replicates. RNA-seq and data analysis were carried out using the protocol previously published by our research group (24).

Bioinformatics, sRNA identification, and genome annotation. The CLC Genomics Workbench software platform (Qiagen) was used for all RNA-seq data analysis and for construction of new GenBank files. Annotated genome files for *S. aureus* N315, USA300, MRSA252, and NCTC 8325 (GenBank accession numbers NC_002745, CP000255, NC_002952, and NC_007795, respectively) were downloaded from NCBI. The location, sequence, and orientation of previously described sRNAs on the N315 genome were calculated based on the information provided in published manuscripts and supplemental information files of the relevant studies (1–12). Using the CLC Genomics Workbench built-in BLAST feature, the corresponding positions for sRNAs were identified in the genomes of strains USA300, MRSA252, and NCTC 8325. The location and orientation of each sRNA was then annotated.

Northern blots. Northern blots to identify the size and abundance of sRNAs were performed by the method of Caswell et al. (25). Briefly, 10 μg of total RNA isolated from a 3 h USA300 culture in TSB were loaded on a 10% polyacrylamide gel (7 M urea, 1× Tris-borate-EDTA [TBE]) and separated by gel electrophoresis. The samples were then transferred via electroblotting to an Amersham Hybond N+ membrane (GE Healthcare). The membrane was exposed to UV light to cross-link samples to the membrane. Subsequently, membranes were prehybridized (1 h, 45°C) in ULTRAhyb-Oligo buffer (Ambion) and then incubated (16 h, 45°C) with sRNA-specific oligonucleotide probes end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Thermo Scientific). After incubation, membranes were washed with 2×, 1×, and 0.5× SSC buffer (1× SSC is 300 mM sodium chloride and 30 mM sodium citrate) at 45°C for 30 min each to remove unspecific bound probes. Finally, X-ray film was exposed to membranes for sRNA detection.

Cloning of histidine-tagged Teg23P. The genomic region containing Teg23P (including its native promoter) was amplified using USA300 genomic DNA and primers OL3222 and OL3223. The reverse primer OL3223, in addition to the gene-specific region, carries a sequence that encodes a hexahistidine (His_6) tag, which allows the detection of a possible encoded protein via Western blotting. The amplified 761-bp product was cloned into shuttle vector pMK4, and the plasmid was transformed into chemically competent *E. coli* DH5α. The resulting colonies were screened for correct constructs employing a colony PCR approach using identical primers to those used for the amplification of the initial fragment. After identification of positive clones, the plasmid was verified via Sanger sequencing with the plasmid-specific standard primers M13Fw (Fw stands for forward) and M13Rv (Rv stands for reverse) (Eurofins MWG Operon). This construct was then transformed into *S. aureus* RN4220 and confirmed via PCR. Finally, the plasmid was transduced into *S. aureus* USA300 using a φ11 phage lysate, and after final confirmation of the construct using PCR, the strain was utilized for subsequent analysis.

qRT-PCR. Quantitative reverse transcriptase PCR (qRT-PCR) was conducted as described by our research group previously (26), employing the *teg23*-specific primers OL3281, OL3282, OL3232, and OL3282 and RNA isolated from *S. aureus* USA300 cultures grown in TSB as described above for RNA-seq experiments. As a reference, 16S rRNA was amplified using primers OL1184 and OL1185 (27). All experiments were performed in triplicate.

Western blots. The evaluation of Teg23 protein abundance was performed by Western immunoblotting as described previously (28). USA300 cells harboring the His_6-tagged version of Teg23P were grown for 3 h in TSB, before they were pelleted and their cytoplasmic proteins were isolated. Following SDS-polyacrylamide gel electrophoresis and transfer of the separated proteins to a polyvinylidene difluoride (PVDF) membrane, detection was performed using anti-His monoclonal mouse antibody (Covance) and horseradish peroxidase (HRP)-conjugated anti-

TABLE 4 Bacterial strains, plasmids, and primers used in this study

Bacterial strain, plasmid, or primer	Characteristic(s) or sequence	Reference or source	Comment[a]
S. aureus strains			
USA300 Houston	Community-associated MRSA clinical isolate	26	
SH1000	Laboratory strain; *rsbU* functional	29	
UAMS-1	Osteomyelitis clinical isolate	30	
RN4220	Restriction-deficient transformation recipient	31	
AW2192	USA300 pMK4::*teg23p*-his_6	This study	
E. coli strain			
DH5α	Routine cloning strain	Invitrogen	
Plasmids			
pMK4	Shuttle vector; Cm^r	32	
pAW105	pMK4::*teg23P*-his_6	This study	
Primers			
OL1184	5′ TCTGGACCGTGTCTCAGTTCC 3′	27	
OL1185	5′ AGCCGACCTGAGAGGGTGA 3′	27	
OL2701	5′ CCAAATTTAGGCATGTCAAATCGGC 3′		*teg23* probe
OL3201	5′ GGATTCCCAATTTCTACAGACAATGCA 3′		*tsr8* probe
OL3208	5′ ACGGGCATATAAAAGGGGAATATTTGAAAA 3′		*tsr25* probe
OU0121	5′ GTGTTAAAAAAATAACTGGGATGTG 3′		*tsr26* probe
OL3216	5′ CTCACAAATTCTGTAAGGGGAGCGTAT 3′		*tsr31* probe
OL3217	5′ TTATGTCCCAATGCTGAATAAATAACTTC 3′		*tsr33* probe
OL3222	5′ ACGCGTCGACGCGCTTGTATTCGCTGCAGG 3′		*teg23P* F
OL3223	5′ CGGGATCCTTAGTGGTGGTGGTGGTGGTGCGCCAACAAGGTTTCAAGAGC 3′		*teg23P*-his_6 R
OL3232	5′ CGCCAACAAGGTTTCAAGAGC 3′		*teg23* OL1
OL3281	5′ TAAACAACATACAGCCATTG 3′		*teg23* OL2
OL3282	5′ GAGAATTTGAAGGCAAGTAT 3′		*teg23* OL3
OL3880	5′ GCCAGGATAATGTAGTCTTAA 3′		*tsr1* F
OL3882	5′ CCATTAATTTACTCAAACCG 3′		*tsr1* R
OL3885	5′ GCTTCTGTTCGATCTC 3′		*tsr2* F
OL3886	5′ CACGTCTTCTGATTAAAC 3′		*tsr2* R
OL3916	5′ CATACCTCTTTAACAACAG 3′		*tsr18* F
OL3917	5′ GGAGGAATTAATCATGTC 3′		*tsr18* R
OL3935	5′ GAAGGGATCCAACACA 3′		*tsr24* F
OL3937	5′ GTCTCGCCATTAATACTAC 3′		*tsr24* R
OL3946	5′ GTCTTTTCACAACCAAAG 3′		*tsr29* F
OL3948	5′ GGTTTTATCTTTGGAAAAAG 3′		*tsr29* R
OL3956	5′ GATGCGGAAAATTTGG 3′		*tsr32* F
OL3957	5′ GTGCGCAATGAATATTATG 3′		*tsr32* R

[a] F, forward; R, reverse.

mouse secondary antibody. Subsequently, HRP activity was detected and visualized on X-ray film. Histidine-tagged RpoE, a protein previously described by our group, was included as a control for successful protein transfer and immunodetection (16).

Accession numbers. The GenBank files generated have been deposited in figshare (https://dx.doi.org/10.6084/m9.figshare.2061132.v1). The files are provided in .gkb format and can be viewed using a variety of genome browser software (examples of freely available genome browsers include Artemis, Genome Compiler, and CLC Sequence Viewer). The RNA-seq data files have been deposited in GEO under accession number GSE74936. Newly identified sRNAs (i.e., the *tsr* sRNAs) have been deposited in GenBank under accession numbers KU639719-KU639757 for SAUSA300s265-SAUSA300s303, KU639758-KU639789 for SAOUHSCs255-SAOUHSCs286, and KU639790-KU639818 for SARs249-SARs277.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01990-15/-/DCSupplemental.

Text S1, DOCX file, 0.2 MB.
Figure S1, TIF file, 2 MB.
Figure S2, TIF file, 0.5 MB.
Figure S3, TIF file, 0.4 MB.
Figure S4, TIF file, 0.5 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank members of the Shaw and Rice labs for useful discussions. R.K.C. thanks Padraig Deighan for many years of advice and assistance.

FUNDING INFORMATION

IFAS Early Career Scientist award provided funding to Kelly C. Rice. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Lindsey N. Shaw under grant number AI080626.

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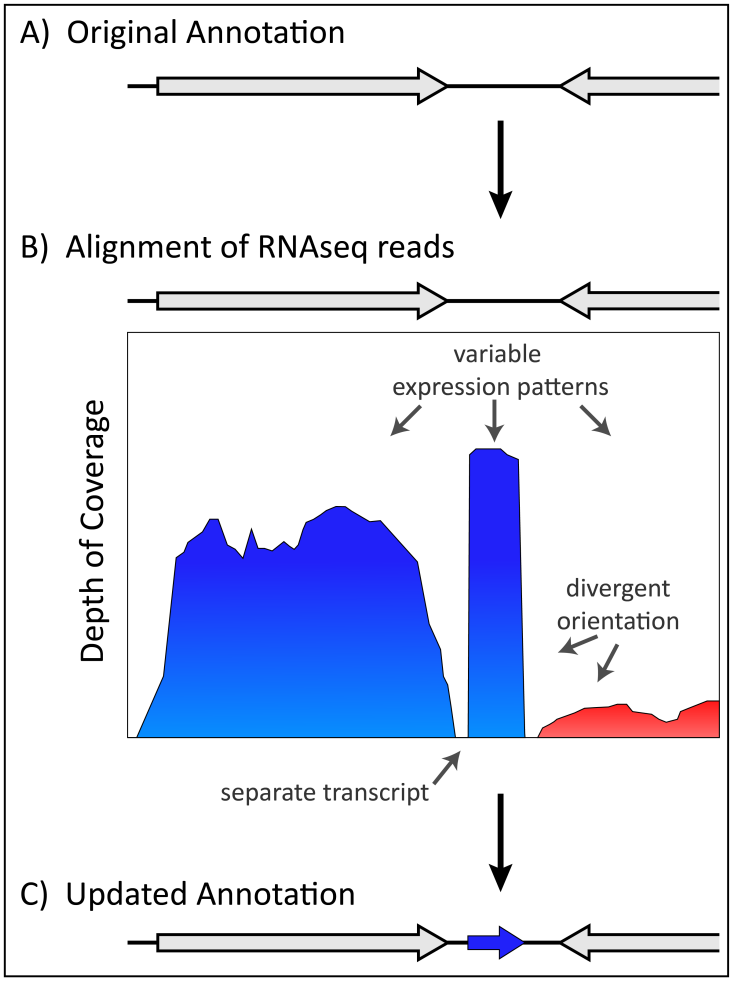

Figure S1. Schematic outlining the procedure used to locate and annotate sRNAs in *S. aureus.* A. Using information provided in previous studies the approximate location of reported sRNAs was identified. **B.** RNAseq was performed and reads were aligned to the relevant *S. aureus* genome. **C.** Location of aligned reads was used to precisely locate and annotate sRNAs (blue arrow).

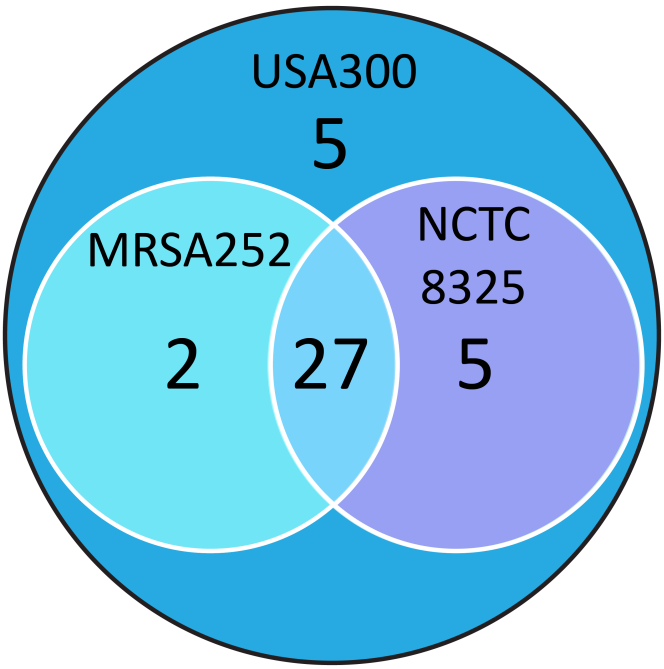

Figure S2. **Distribution of *tsr* genes in USA300, NCTC 8325 and MRSA252.** Five *tsr* genes are unique to USA300. An additional five are found in USA300 and NCTC 8325 (but not MRSA252), while two are found in USA300 and MRSA252 (but not NCTC 8325). Twenty-seven *tsr* genes are found in all three species.

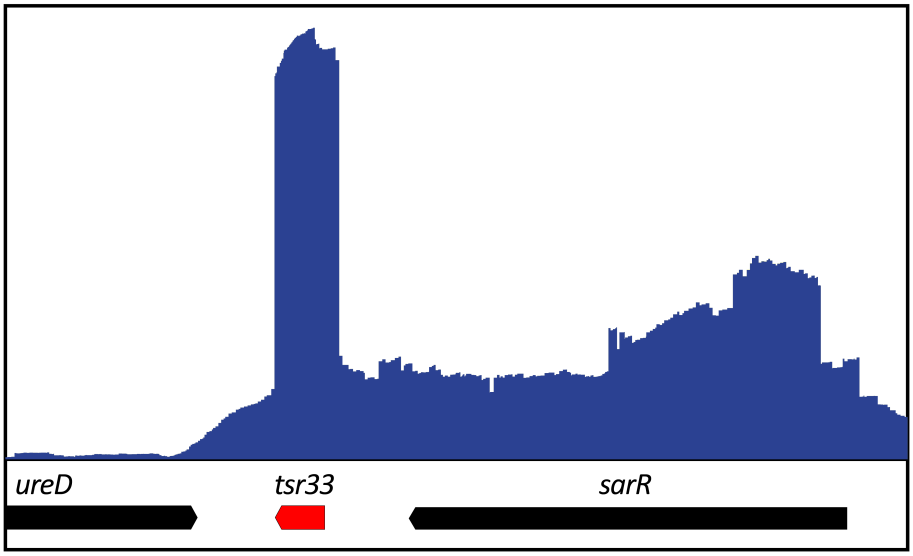

Figure S3. RNAseq read alignment data for *sarR* and *tsr33* during growth in human serum. Annotations for CDS genes are shown by black arrows, annotations for sRNA is shown by red arrow. Depth of read coverage on the genome is shown by blue histogram. Greater depth of read coverage for *tsr33* suggests that a *sarR*-independent tsr33 exists under these conditions.

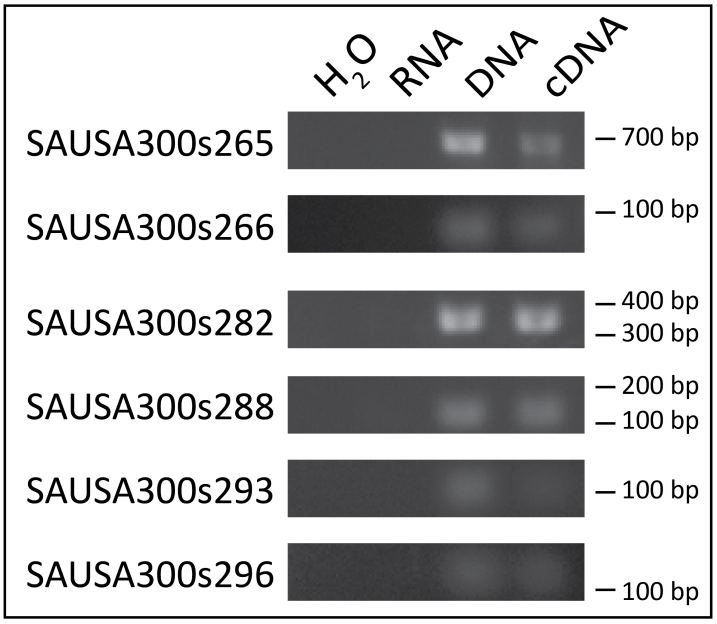

Figure S4. Reverse transcriptase-PCR based detection of newly identified sRNAs in *S. aureus*. PCR reactions were performed with primers specific to newly identified sRNA genes. DNA-depleted RNA, DNA and H_20 were employed as templates to verify the complete removal of DNA from RNA samples, the correct size of the respective amplicons and the absence of DNA contaminations. Successful, size-specific amplification of a target from cDNA suggests the presence of transcript at the position where a novel sRNA annotation was added to the genome file.

Table S1. List of 25 miscellaneous RNAs from MRSA252 genome

MRSA252 designation	Gene name	Strand	Position	Description	NCTC 8325 designation	USA300 designation
SARs001		>	12491..12722	T-box riboswitch	SAOUHSCs001	SAUSA300s001
SARs002		>	15939..16037	SAM riboswitch	SAOUHSCs002	SAUSA300s002
SARs003		<	403755..404014	T-box riboswitch	SAOUHSCs003	SAUSA300s003
SARs004		>	441050..441153	Purine riboswitch	SAOUHSCs004	SAUSA300s004
SARs005	*ffs*	>	509502..509604	Signal recognition particle	SAOUHSCs005	SAUSA300s005
SARs006	*ssrA*	>	883681..884039	tmRNA	SAOUHSCs006	SAUSA300s006
SARs007		>	908405..908508	SAM riboswitch	SAOUHSCs007	SAUSA300s007
SARs008		<	1098190..1098290	TPP riboswitch	SAOUHSCs008	SAUSA300s008
SARs009		>	1156281..1156504	T-box riboswitch	SAOUHSCs009	SAUSA300s009
SARs010		>	1212605..1212799	T-box riboswitch	SAOUHSCs010	SAUSA300s010
SARs011		>	1437490..1437684	T-box riboswitch	SAOUHSCs011	SAUSA300s011
SARs012		>	1437691..1437899	T-box riboswitch	SAOUHSCs012	SAUSA300s012
SARs013		>	1463346..1463521	Lysine riboswitch	SAOUHSCs013	SAUSA300s013
SARs014	*rnpB*	<	1549260..1549607	Ribonuclease P	SAOUHSCs014	SAUSA300s014
SARs015		<	1588874..1589008	FMN riboswitch	SAOUHSCs015	SAUSA300s015
SARs016		<	1759722..1759920	T-box riboswitch	SAOUHSCs016	SAUSA300s016
SARs017		<	1824382..1824557	Lysine riboswitch	SAOUHSCs017	SAUSA300s017
SARs018		<	1826732..1826959	T-box riboswitch	SAOUHSCs018	SAUSA300s018
SARs019		<	1882961..1883179	T-box riboswitch	SAOUHSCs019	SAUSA300s019
SARs020		<	1943687..1943822	FMN riboswitch	SAOUHSCs020	SAUSA300s020
SARs021		<	1958937..1959048	SAM riboswitch	SAOUHSCs021	SAUSA300s021
SARs022	RNAIII	<	2183350..2183839	RNAIII	SAOUHSCs022	SAUSA300s022
SARs023		<	2253957..2254059	TPP riboswitch	SAOUHSCs023	SAUSA300s023
SARs024		<	2311657..2311873	GlmS ribozyme	SAOUHSCs024	SAUSA300s024
SARs025		<	2480682..2480777	SAM riboswitch	SAOUHSCs025	SAUSA300s025

Supplemental Methods

sRNA genome annotation

The annotated Genbank (.gbk) files in the NCBI database for most strains of *S. aureus* do not contain entries for sRNAs, however, the genome file for MRSA252 (USA200) includes annotations for 25 RNAs designated as “miscellaneous RNAs” (Table S1). The nomenclature used for these miscellaneous RNA species, SARs001 to SARs025, distinguishes them from coding DNA sequence genes (which are annotated SAR0001 to SAR2800). We used these 25 RNAs as a starting point to annotate sRNAs in the genomes of two additional *S. aureus* strains i.e. NCTC 8325 and USA300. Using the sequences of SARs001 to SARs025 as a reference, the corresponding position of their homologues in the NCTC 8325 and USA300 genomes was identified by BLAST search. Matches were found for each of the 25 sequences. Genbank files for NCTC 8325 and USA300 were downloaded from NCBI and new gene annotations were added to each file for the 25 miscellaneous RNA sequences. A nomenclature system similar to that for MRSA252 was used to annotate these new genes in the NCTC 8325 and USA300 genomes. In NCTC 8325 sRNA annotations were added using the prefix SAOUHSCs (to distinguish from CDS annotations which begin with the prefix SAOUHSC) and in USA300 sRNA annotations were added with the prefix SAUSA300s (CDS annotations begin with the prefix SAUSA300). Using this system the 25 RNAs from MRSA252 (SARs001 to SARs025) were annotated as SAOUHSCs001 to SAOUHSCs025 in NCTC 8325 and SAUSA300s001 to SAUSA300s025 in USA300 (Table S1).

Having introduced annotations in the NCTC 8325 and USA300 genomes for these 25 “miscellaneous RNAs”, next we sought to add annotations for other previously identified

sRNAs in *S. aureus*. To do this a literature search was performed to identify studies in which sRNAs were described in *S. aureus*. A total of 12 studies were selected that employed a variety of methods to identify sRNAs, including computational approaches, microarray studies, cDNA cloning and high throughput sequencing (1-12). The majority of these studies have been performed in a single *S. aureus* background (i.e. strain N315). Using the information provided in these publications a list of 928 potential sRNAs was assembled (Table S2) (1-12). To identify the positions of these sRNAs in the MRSA252, NCTC 8325 and USA300 genomes we adopted a two-pronged approach. First, we assembled all information regarding sRNA sequence, genome position, and orientation in the N315 genome, from each respective publication. This information was used to identify the approximate position of each sRNA in MRSA252, NCTC 8325 and USA300. Second, to facilitate an accurate annotation of each sRNA position, RNAseq experiments were performed using UAMS-1 (a USA200 isolate, and close relative of MRSA252), SH1000 (a close relative of NCTC 8325) and USA300 growing under standard laboratory conditions. The reads generated were aligned to the respective genomes and used as a guide to identify the specific location of each sRNA on each of the three genomes (Fig. S1). Using this approach we identified and annotated an additional 223 sRNAs on the MRSA252 genome, 229 sRNAs on the NCTC 8325 genome and 239 sRNAs on the USA300 genome (Table S1). When combined with the list of 25 RNAs from the MRSA252 genome this brings the total number of sRNAs annotated to 248 in MRSA252, 254 in NCTC 8325 and 264 on the USA300 genome. Throughout the annotation process it became apparent, that many of the sRNAs reported had been identified multiple times in different studies, as has been

noted recently (14). Where multiple copies of sRNAs exist, each copy was individually annotated to distinguish them from each other. In addition, we observed that the α-phenol soluble modulins (α-PSM) transcript was mistakenly identified as a sRNA (6). The most likely reason for this is that the α-PSMs are not annotated in the *S. aureus* genome and therefore any transcript mapping to that location was thought to represent a sRNA. To avoid future confusion we have included annotations for the PSMs in the updated MRSA252, NCTC 8325 and USA300 Genbank files.

sRNA gene expression analysis by RNAseq

Expression values for each annotated gene were calculated as RPKM (reads per kilobase material per million reads) values, using the CLC Genomics Workbench software platform (Qiagen). TSB and serum data sets were normalized by quantile normalization (16). To identify sRNA genes differentially expressed in serum Vs TSB, eliminate lowly expressed genes, and reduce the impact of non-unique reads, three cut off criteria were applied to the data.

1. Percent unique reads. RPKM values are calculated based upon the number of reads mapping to a gene. These reads can be unique or non-unique. Non-unique reads map to multiple locations on the genome and consequently their mapping location cannot be precisely determined. To avoid artificially inflating RPKM values due to the incorporation of ambiguous non-unique reads we imposed a cut off whereby >80% of the reads mapping to a gene (in both data sets) must be unique.

2. Expression values. Small differences in expression values can translate into large fold-differences for lowly expressed genes. To eliminate lowly expressed genes we

imposed a cut-off whereby the RPKM expression value of a gene must be greater than or equal to 50 in at least one data set.

3. Fold-change. We imposed a cut-off of 3-fold to identify genes showing differentially expressed in serum Vs TSB.

sRNA identification in USA300

RNAseq reads generated for USA300 growing in TSB and human serum were aligned to the newly created Genbank file containing annotations for known sRNAs. These read alignment files were subsequently examined for transcripts that did not map to annotated genes. Two types of potential sRNA were identified (i) those mapping to intergenic regions and (ii) those that were antisense to, and located within annotated genes. The following criteria were applied during the selection process to avoid excessive false positive identifications. The minimum number of unique reads mapping to a potential sRNA, and minimum RPKM expression value generated must exceed 25 and 40 respectively. Using these selection criteria 39 potential sRNAs were identified which we named tsr1-39.

Reverse transcriptase polymerase chain reaction

Overnight cultures of wild-type *S. aureus* USA300 were diluted 1:100 into 5 mL of fresh TSB and incubated for 3 hours at 37°C in a rotating incubator at 250 RPM. The cultures were diluted to an OD_{600} of 0.05 in 100 mL of fresh TSB and incubated for 3 hours at 37°C in a rotating incubator at 250 RPM. Duplicate samples were immediately removed into 3 volumes of ice cold PBS and centrifuged for 10 minutes at 4150 RPM. RNA was

extracted as previously outlined (16) and contaminating DNA removed using TURBO™ DNase (ThermoFisher Scientific) per their protocol. Finally, 1 µg DNA-free RNA was used to create cDNA using the iScript™ Reverse Transcription Supermix Kit (BioRad) per their instructions.

To isolate genomic DNA the pelleted cells were re-suspended in 600 µL of TE buffer in screw cap tubes with glass beads. The cells were then beaten for a total of 3 minutes in 30-second bursts. The cells were then centrifuged at full speed for 2 minutes. Next, 500 µL of supernatant was transferred to a sterile microfuge tube with 100 µL of 1.6% n-lauroylsarcosine and 25 µg Proteinase K (US Biologicals) then incubated for 1 hour at 60°C. After incubation 500 µL of phenol:chloroform (1:1), briefly vortexed, and then centrifuged at full speed for 2 minutes. The aqueous layer was removed and the DNA was precipitated with 3 M sodium acetate and isopropanol overnight at -80°C. The DNA was centrifuged at 4°C at full speed for 20 minutes. DNA pellets were rinsed with ice cold 70% ethanol, dried at 45°C, and re-suspended in 500 µL of nuclease free water.

Green GoTaq (Promega) was used in an amplification reaction containing 1 mM primers and 100 ng nucleic acid template (DNase-treated RNA, genomic DNA, or cDNA) or water. All PCR reactions were performed in a DNA Engine Peltier Thermal Cycler (BioRad) with an annealing temperature of 45°C and an extension time of 1 minute at 72°C for 35 cycles. PCR products were evaluated for size and specificity by separation on a 2% (w/v) agarose gel with a 100 bp DNA Molecular Marker (Promega).