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The *Staphylococcus aureus* ArlRS two-component system regulates virulence factor expression through MgrA

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Summary

The Gram-positive bacterium *Staphylococcus aureus* is a versatile pathogen that can sense and adapt to a wide variety of environments within the human host, in part through its 16 two-component regulatory systems. The ArlRS two-component system has been shown to affect many cellular processes in *S. aureus*, including autolysis, biofilm formation, capsule synthesis, and virulence. Yet the molecular details of this regulation remained largely unknown. We used RNA-sequencing to identify the ArlRS regulon, and found 70% overlap with that of the global regulator MgrA. These genes included cell wall-anchored adhesins (*ebh*, *sdrD*), polysaccharide and capsule synthesis genes, cell wall remodeling genes (*lytN*, *ddh*), the urease operon, genes involved in metal transport (*feoA*, *mntH*, *sirA*), anaerobic metabolism genes (*adhE*, *pflA*, *nrdDG*), and a large number of virulence factors (*lukSF*, *lukAB*, *nuc*, *gehB*, *norB*, *chs*, *scn*, and *esxA*). We show that ArlR directly activates expression of *mgrA* and identify a probable ArlR binding site (TTTTCTCAT-N₄-TTTTAATAA). A highly similar sequence is also found in the *spx* P2 promoter, which was recently shown to be regulated by ArlRS. We also demonstrate that ArlS has kinase activity toward ArlR *in vitro*, although it has slower kinetics than other similar histidine kinases.

Abbreviated Summary

Methicillin Resistant *Staphylococcus aureus* (MRSA) is a leading cause of bloodstream infections, pneumonia, and surgical site infections. One of the hallmarks of *S. aureus* is its diverse array of virulence factors, such as secreted toxins, cell wall-anchored adhesins, and immune evasion factors. We demonstrate that the ArlRS two-component system regulates more than 200 genes, including many virulence factors, primarily through controlling expression of the global regulators MgrA and Spx.

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Keywords

Staphylococcus aureus; virulence factors; histidine kinase; RNA sequencing; binding site; regulon

Introduction

The Gram-positive pathogen *Staphylococcus aureus* is a commensal that colonizes the nostrils, throat, and skin of ~30% of the population (Mertz *et al.*, 2009, Gorwitz *et al.*, 2008, Miller & Diep, 2008). It causes a broad spectrum of invasive infections, including osteomyelitis, bacteremia, endocarditis, and pneumonia (Lowy, 1998), and it is one of the leading causes of healthcare-associated infections such as surgical site infections and central line-associated bloodstream infections (Weiner *et al.*, 2016). *S. aureus* is such a versatile pathogen in part because of its large arsenal of virulence factors that help it to establish a foothold in diverse body sites and ultimately spread to other sites. These virulence factors include secreted toxins and enzymes (Tam & Torres, 2019), cell wall-anchored adhesins (Foster *et al.*, 2014), and a variety of proteins involved in immune evasion (Thammavongsa *et al.*, 2015). Expression of many of these virulence genes is controlled by two-component systems (TCS) (Haag & Bagnoli, 2017) such as the *agr* quorum-sensing system (Thoendel *et al.*, 2011), as well as SaeRS (Liu *et al.*, 2016), SrrAB (Yarwood *et al.*, 2001), and ArlRS (Liang *et al.*, 2005). These TCSs allow *S. aureus* to sense extracellular conditions and change gene expression accordingly. A typical TCS consists of a membrane bound histidine kinase that responds to a particular stimulus, causing it to autophosphorylate. It then transfers that phosphate to a cytoplasmic response regulator protein, which can then bind DNA and affect transcription of a subset of genes (Stock *et al.*, 2000).

The ArlRS TCS was originally identified as a regulator of autolysis (autolysis-related locus) and biofilm formation (Fournier & Hooper, 2000). Mutations in *arlRS* lead to increased biofilm formation (Toledo-Arana *et al.*, 2005), but also decreased clumping in the presence of human plasma (Walker *et al.*, 2013) and decreased adhesion to host extracellular matrix proteins (Kwiecinski *et al.*, 2019). ArlRS is also important for virulence in a number of animal models of infection, including systemic infection in mice (Benton *et al.*, 2004, Radin *et al.*, 2016), infectious endocarditis in rabbits (Walker *et al.*, 2013), and a mouse catheter-associated biofilm infection (Burgui *et al.*, 2018). Despite its importance in infection, the molecular details of the ArlRS TCS and specifically what it regulates are largely unknown. An early microarray study suggested that ArlRS directly or indirectly regulated over 100 genes, including other regulators like the *agr* quorum-sensing system and the LytSR TCS (Liang *et al.*, 2005). Since then, ArlRS has been shown to activate expression of the global regulator MgrA (Luong & Lee, 2006, Crosby *et al.*, 2016). MgrA in turn regulates a variety of genes, including those for surface proteins and secreted virulence factors (Crosby *et al.*, 2016). ArlRS was shown to affect amino acid metabolism and sensitivity to the Zn-, Mn-, and Fe-binding protein calprotectin (Radin *et al.*, 2016). It was also reported that ArlRS is required for leukocidin expression, perhaps through regulation of *agr* (Harper *et al.*, 2018). Recent work showed that ArlRS also activates expression of Spx, a transcriptional regulator involved in the response to oxidative stress and antibiotic resistance (Bai *et al.*, 2019). ArlR

was shown to directly bind to the *spx* promoter region, although the exact binding site was not determined (Bai *et al.*, 2019).

ArlS is a membrane-bound histidine kinase with an extracellular domain that senses an unknown signal. Upon activation, ArlS autophosphorylates a conserved histidine residue (H242), and then transfers this phosphate to an aspartate residue within ArlR (D52) (Villanueva *et al.*, 2018, Delgado *et al.*, 1993, Stock *et al.*, 2000). ArlR is an OmpR/PhoB-type response regulator protein that binds DNA upon phosphorylation by ArlS. Many histidine kinases have both kinase and phosphatase activity toward their cognate response regulator, and the dominant activity depends on the presence of their specific signal and the balance between kinase/phosphatase activities (Casino *et al.*, 2010). The phosphatase activity serves as a way to reset signaling activity after the stimulus is gone, and also suppresses non-specific signaling from other histidine kinases and inorganic phosphate donors like acetyl-phosphate (Huynh & Stewart, 2011, Siryaporn & Goulian, 2008, Hentschel *et al.*, 2014, Wayne *et al.*, 2012). Distinct residues within the catalytic domain of histidine kinases are required for kinase and phosphatase activity (Willett & Kirby, 2012, Dutta *et al.*, 2000, Wayne *et al.*, 2012).

In this work we used RNA-seq to investigate the ArlRS regulon and compare it to the group of genes regulated by MgrA (Crosby *et al.*, 2016). The two regulons largely overlap, indicating that MgrA is one of the major targets of ArlR. We also confirm that the disulfide stress regulator Spx is regulated by ArlRS, but not by MgrA. Both *mgrA* and *spx* promoters share a potential ArlR binding site that is located 11 bp upstream of the -10 sequence, suggesting that they are directly regulated by ArlR. Lastly, we investigated the kinase and phosphatase activity of ArlS using targeted point mutations.

Results

Analysis of the ArlRS regulon

We previously determined the MgrA regulon using RNA-sequencing (Crosby *et al.*, 2016), and in this work we sought to compare this to the ArlRS regulon under similar growth conditions. *S. aureus* USA300 strain LAC and an isogenic *arlRS* deletion mutant were grown in tryptic soy broth to mid-log phase (OD600 of 1.5), and changes in gene expression were determined using RNA-sequencing. Using a 2-fold cutoff limit and false discovery rate (FDR)-corrected p-value of <0.05, 126 genes were up-regulated and 124 genes were down-regulated in the *arlRS* mutant compared to LAC (Table S1). As expected, *mgrA* expression was down ~5-fold in the *arlRS* mutant, confirming that ArlRS activates *mgrA* expression. Expression of *spx* was down 2.7-fold in the *arlRS* mutant, and no change in expression was seen for the *agrBDCA* operon or the *lytSR* TCS (Table S1).

There was substantial overlap between the ArlRS regulon and that of MgrA. Of the 250 genes regulated by ArlRS, 70% were also differentially expressed in the *mgrA* mutant, and in all cases the direction of regulation was the same (Table S2). Focusing on the genes regulated 4-fold by ArlRS, 55 out of 57 (96%) were also regulated 2-fold by MgrA (Fig. 1). Up-regulated genes included the cell surface adhesins *ebh*, *sdrD*, *fntB*, and *sasC*, cell wall hydrolase genes such as *lytN*, the urease operon genes *ureABC*, putative polysaccharide

synthesis loci *cap1ABC* and *SAUSA300_0130–0133*, and the Mn transporter *mntH*. Down-regulated genes in common between ArlRS and MgrA included the capsule biosynthetic genes *cap5A* and *cap5D*, and a variety of secreted virulence factors, such as *lukSF* (Panton Valentine Leukocidin), *lukAB*, chemotaxis inhibitor *chs* (CHIPS), complement inhibitor *scn* (SCIN), and nuclease (*nuc*). Both the capsule operon (Luong & Lee, 2006) and the leukocidins (Harper *et al.*, 2018) have been previously reported to be regulated by ArlRS. The first ten genes in the type VII secretion system gene cluster, starting with *esxA*, were down 3–6 fold in the *arlRS* mutant, consistent with a previous report that *esxA* is regulated by ArlRS (Schulthess *et al.*, 2012). Three transcriptional regulators, *sarV*, *atlR*, and *SAUSA300_1202*, were also repressed by both ArlRS and MgrA, suggesting that there are likely several levels to the ArlRS regulatory cascade, and that some genes may be indirectly regulated by MgrA. ArlRS-regulated genes that appeared to be independent of MgrA included the putative peptidoglycan hydrolase *SAUSA300_0739*, and the catechol siderophore transport genes *sstB* and *sstC* (Morrissey *et al.*, 2000, Beasley *et al.*, 2011).

To confirm a subset of the RNA-seq results, we constructed GFP transcriptional fusions for 12 of these genes, 6 predicted to be up-regulated and 6 down-regulated (Fig. 2). Construction of fusion plasmids was based on predicted promoter sequences from Prados *et al.* (Prados *et al.*, 2016) or other published promoter sequences where possible. Reporter expression at 24 h is shown in Fig. 2, and representative growth curves for a subset of these reporters are shown in Supplemental Fig. 1. As expected based on previous results (Crosby *et al.*, 2016), expression from the upstream *mgrA* promoter (*mgrA* P2) was strongly dependent on ArlRS, but was not autoregulated (Fig. 2A). Expression of nuclease (*nuc*), the leukocidins *lukA* and *lukS*, the type VII secretion gene *esxA*, and the secreted lipase *gehB* were all significantly down in the *arlRS* mutant and could be complemented (Fig. 2B–F), verifying the RNA-seq results. All five of these genes were also significantly affected by the *mgrA* deletion (Fig. 2B–F), suggesting that ArlRS may indirectly regulate their expression through MgrA. Likewise, the up-regulated genes that we investigated, *ebh*, *mntH*, *sdrD*, *lytN*, *SAUSA300_0130*, and *ureA* all showed significantly higher expression in both the *arlRS* and *mgrA* mutants, and the *arlRS* effect could be complemented (Fig. 2G–L). We have previously shown that under these growth conditions ~90% of MgrA expression is driven by the ArlRS-regulated upstream promoter *mgrA* P2 (Crosby *et al.*, 2016). In the *arlRS* mutant there is residual low-level expression of MgrA from the downstream *mgrA* P1 promoter, which likely explains the difference in target gene expression in the *arlRS* and *mgrA* mutants.

Identifying the ArlRS binding site

The RNA-seq data suggest that ArlR likely directly regulates expression of *mgrA*, although the exact binding site remains unknown. The *mgrA* gene has two promoters (Fig. 3A), and we have previously shown that expression from only the upstream promoter, *mgrA* P2, is activated by ArlR (Crosby *et al.*, 2016). Two potential transcription start sites (TSSs) for *mgrA* P2 have been identified (Ingavale *et al.*, 2003, Prados *et al.*, 2016), located 300 and 302 bp upstream of the *mgrA* start codon. Prados *et al.* (Prados *et al.*, 2016) selected the adenine 300 bp upstream as the “representative TSS” based on its lower p-value in their work, so we used that as the *mgrA* P2 TSS going forward. There is a consensus –10

sequence (TATAAT) centered 11 bp upstream of this TSS, but this promoter appears to lack an identifiable -35 sequence. Expression from this promoter requires *arlRS* (Fig. 3B), suggesting that ArlR may compensate for the poor -35 site.

Our first step in locating the ArlR binding site was to clone a series of truncated *mgrA* P2 promoter fragments in front of GFP (Fig. 3A–C). The shortest fragment with full activity in the LAC wild-type strain, pHC137, included 72 bp upstream of the TSS. Shorter promoter fragments showed decreasing activity, and the shortest reporter, pHC146 (32 bp upstream of the TSS) had virtually no activity. This information suggested that ArlR likely binds somewhere between 30 and 72 bp upstream of the TSS. Next, we took the shortest fully active reporter, pHC137, and constructed staggered 6-bp mutations across this entire region (Fig. 4A–B). When these mutated reporters were introduced into LAC, six reporters showed minor decreases in expression, and seven consecutive reporters had almost no activity (Fig. 4A). These reporters with minimal activity had mutations spanning a region located ~45–25 bp upstream of the TSS, overlapping the area where a -35 sequence would normally be (Fig. 4B). We determined that this region was likely to contain the ArlR binding site. We then made a series of 2 bp mutations spanning this region to better define the ArlR binding site (Fig. 5A–B). One of these reporters, pHC198, containing a 2 bp change 46 and 45 bp upstream of the TSS, actually had higher activity, while the remaining reporters had much lower activity than the wild-type sequence (pHC137). The reason for the higher activity of pHC198 is unclear, but perhaps introducing these mutations helps ArlR to bind productively to the *mgrA* P2 promoter by disrupting another potential binding position. Mutations in the middle of the binding site, pHC194, pHC195, and pHC196, were less detrimental than mutations on either side, suggesting a symmetrical binding site separated by a short gap. We surmised that the likely ArlR binding site is TTTTCTCAT-N₄-TTTAAATAA, located 44 to 23 bp upstream of the TSS. This near direct repeat is consistent with other OmpR-family response regulators, which often bind direct repeats separated by a 4–5 bp spacer (de Been *et al.*, 2008).

To show direct binding of ArlR to this potential binding site we used electrophoretic mobility shift assays (EMSAs). Increasing concentrations of phosphorylated ArlR (0 to 2 μ g, which corresponds to 0 to 7.2 μ M) were incubated with an IRDye labeled 50 nt DNA probe overlapping the proposed binding site, or a probe directly upstream of the first probe (Fig. 6). ArlR was able to bind to the probe containing the proposed binding site and showed minimal binding to the upstream non-specific probe. This indicates that ArlR likely directly regulates *mgrA* expression and that we have identified the correct location of the binding site.

Spx regulation

It was recently shown that ArlRS activates expression of the transcriptional regulator *spx* (Bai *et al.*, 2019). Spx interacts directly with RNA polymerase and is involved in the disulfide stress response in Firmicutes (Hillion & Antelmann, 2015). Expression of *spx* was down 2.7-fold in the *arlRS* mutant in our RNA-seq experiment (Table S1). A *spx* reporter (pHC169) was constructed containing the majority of the intergenic region upstream of *spx* fused to GFP. Expression of this reporter was significantly lower in the *arlRS* mutant and

could be complemented (Fig. 7A). However, expression in the *mgrA* mutant was similar to wild-type (Fig. 7A), suggesting that ArlRS may directly regulate *spx*. The *spx* gene has two promoters within this upstream region, a distal promoter P1 and a proximal promoter P2 (Fig. 7B) (Pamp *et al.*, 2006, Jousselin *et al.*, 2013, Donegan *et al.*, 2014, Prados *et al.*, 2016). To investigate *spx* regulation by ArlRS further, we constructed separate reporters for each promoter. Time courses using each of these three reporters in the wild-type LAC background and the *arlRS* mutant demonstrated that only *spx* P2 is regulated by ArlRS (Fig. 7C–E). In the absence of *arlRS* there was essentially no expression from this P2 reporter, similar to the *mgrA* P2 promoter, whose expression requires ArlRS. Alignment of *mgrA* P2 and *spx* P2 demonstrated that both promoters have very similar sequences in the ArlR binding region (Fig. 7F). The first repeat is identical, and in the second repeat seven out of nine nucleotides are identical. In addition, the spacing between the proposed ArlR binding site and canonical –10 sequence is identical in the two promoters (Fig. 7F), lending strength to our proposed ArlR binding site.

Search for other promoters containing a potential ArlR binding site

Alignment of the *mgrA* P2 and *spx* P2 promoters suggests a potential ArlR consensus binding site TTTTCTCAT-N₄-TTTTAATNN. A search for 90% matches to this sequence in the *S. aureus* USA300_FPR3757 genome yielded 38 hits (Table S3). Only 13 of these hits were located upstream of a nearby gene and in the same orientation. This set of 13 included *mgrA* and *spx*, as expected. The only other overlap with our RNA-seq experiment was SAUSA300_0130, a putative UDP-glucose 4-epimerase and the first gene in a possible five-gene glycan synthesis locus. Two of the 13 potential ArlR binding sites were located just upstream of SAUSA300_0130 (Supplemental Fig. 2). Although the location of the SAUSA300_0130 promoter is unknown, these binding sites are likely too close to the start codon to substitute for a –35 promoter element. In addition, expression of SAUSA300_0130 was up ~23-fold in the *arlRS* mutant, suggesting that ArlR might repress expression of SAUSA300_0130. Our RNA-seq results and SAUSA300_0130-GFP fusion (Fig. 2K) indicate that SAUSA300_0130 is repressed by MgrA. It seems unusual, but possible, that this potential glycan synthesis operon would be repressed both directly and indirectly (through MgrA) by ArlRS.

ArlS kinase/phosphatase activity

In this study, we also characterized ArlS histidine kinase enzyme kinetics that dictate its ability to regulate the phosphorylation state of its cognate response regulator ArlR. Although ArlS has already been identified as a HisKA (or Subtype 1A) family member (e.g. ScanProsite (de Castro *et al.*, 2006)), the N-terminal region has not been annotated. Homology modeling using the Phyre2 (Kelley *et al.*, 2015) server predicts that ArlS is a multi-domain protein (Fig. 8A) containing two transmembrane spanning regions (from residue 1 to 33 and 154 to 186) and a putative extracellular sensor or Cache domain (Anantharaman & Aravind, 2000) (residue 34 to 153). The second transmembrane region extends into the cytoplasmic HAMP (named for histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and Phosphatases) domain (Parkinson, 2010). The catalytic core of ArlS consists of dimerization and histidine phosphotransfer (DHp) and catalytic ATP binding (CA) domains. It has been reported that 75–85% of histidine kinases from 1500

sequenced bacterial genomes belong to the HisKA family (Trajtenberg *et al.*, 2010, Willett & Kirby, 2012). The HisKA family of histidine kinases is characterized by the presence of an H-box consensus motif (Stock *et al.*, 1989) within the DHp domain that contains the phosphorylatable histidine residue: H E X R T/N P, where X is a hydrophobic residue. Within this same motif, the conserved threonine/asparagine residue has been shown to be important for phosphatase activity (Willett & Kirby, 2012, Huynh & Stewart, 2011).

Our data show that, like other HisKA proteins, ArlS is a dual function enzyme with kinase and phosphatase activity that regulates the phosphorylation state of ArlR. We purified full-length ArlR (~26 kDa), as well as soluble ArlS constructs corresponding to just the DHp and CA domains (DHpCA, ~26 kDa), or the HAMP, DHp and CA domains (HAMP-DHpCA, ~31 kDa) (Fig. 8B). Fig. 8C shows the autophosphorylation kinetics of ArlS DHpCA and HAMP-DHpCA constructs. Although most studies performed on histidine kinases have focused on the autophosphorylation activity of the DHpCA domain, recent data indicate that the HAMP domain can influence kinase and phosphatase activity (Wang *et al.*, 2013). Fig. 8C (left and right panels) indicates that ArlS HAMP-DHpCA and ArlS DHpCA have similar autophosphorylation rates. Moreover, the HAMP-DHpCA and DHpCA domains alone have slow autophosphorylation kinetics ($t_{1/2}$ = 15–20 minutes) compared to $t_{1/2}$ ~30–60 seconds for other histidine kinases, such as WalK (Gutu *et al.*, 2010), CrdS (Willett & Kirby, 2011), and AgrC (Srivastava *et al.*, 2014) (Fig. S4A). ArlS also undergoes phosphotransfer to ArlR as shown in Fig. 8D (left and right panels). 50% of the phosphoryl group transfers to ArlR within 1 minute of the reaction when ArlR is incubated with ArlS HAMP-DHpCA. The phosphotransfer kinetics is further enhanced when ArlR is phosphorylated in the absence of HAMP. 50% phosphoryl group is transferred within 15 seconds of the reaction compared to 1 minute when the HAMP domain is present (Fig. S4B–C). In contrast to the autophosphorylation kinetics, the phosphatase kinetics is very similar to that observed in other histidine kinases (Willett & Kirby, 2012, Gutu *et al.*, 2010, Willett & Kirby, 2011, Srivastava *et al.*, 2014). Also, of note is the phosphatase activity of ArlS, which is evident by the disappearance of phosphorylated ArlR over time (shown by arrow in Fig. 8D). Similar to the phosphotransfer activity, the phosphatase activity was enhanced without the HAMP domain.

In order to further characterize the ArlRS two-component system, we investigated the importance of the key residues in ArlS involved in its kinase and phosphatase function. The signature phosphorylated histidine is clearly important for kinase activity, while the conserved threonine/asparagine residue is important for phosphatase activity (Willett & Kirby, 2012, Huynh & Stewart, 2011). Based on the amino acid sequence alignment with other histidine kinases (Fig. 9A), we hypothesized that His242 and Thr246 are important for autophosphorylation and phosphatase activity of ArlS, respectively. Additionally, we examined the role of the conserved phosphorylation site in ArlR, Asp52 (Fig. 9B). We used our chromosomally complemented LAC *arlRS* strain to construct alanine substitutions in place of His242 and Thr246 in ArlS, providing single-copy versions of these variants in a *arlRS* background. As expected, the *mgrA* P2-GFP reporter pHc68 had no activity in the *arlRS* mutant and was fully complemented with a wild-type copy of *arlRS* on the chromosome (Fig. 9C). Mutating His242 to an alanine in ArlS decreased expression of the *mgrA* reporter by ~80%, and was comparable to complementing the *arlRS* mutation with

arlR alone (Fig. 9C), indicating that His242 is required for ArlS kinase activity. Conversely, mutating Thr246 to an alanine increased *mgrA* expression two-fold compared to wild-type ArlRS, demonstrating that Thr246 is crucial for ArlS phosphatase function (Fig. 9C). A His242/Thr246 double mutant had similar activity to the His242 single mutant (Fig. 9C). We also showed that the conserved phosphorylation site in ArlR, Asp52, is important for ArlR function. Mutation of Asp52 to either an alanine or a glutamate decreased *mgrA* expression by 92–96% (Fig. 9C). For some OmpR-family response regulators, replacement of this conserved aspartate with glutamate mimics phosphorylation and results in a constitutively active state (Delaune *et al.*, 2012, Lan & Igo, 1998), but this does not appear to be true for ArlR.

We investigated protein expression levels of two genes under the control of the ArlRS/MgrA cascade, the large surface protein Ebh (Fig. 9D) and the PVL leukocidin component LukS (Fig. 9E). LukS is down-regulated in the *arlRS* mutant (Fig. 1, 2D), and its expression in the various *arlR* and *arlS* point mutations showed a similar pattern to that of the *mgrA* reporter (Fig. 9E). Ebh, on the other hand, is directly repressed by MgrA (Fig. 2G) (Crosby *et al.*, 2016), and its expression showed the opposite pattern as *mgrA* and LukS (Fig. 9D).

In vitro kinase assays showed that the autophosphorylation activity of the ArlS H242A mutant is completely abolished, while the WT control had activity (Fig. S3). Hence, H242 is critical for the ArlRS two-component system to be activated *in vitro* and *in vivo*. *In vitro* kinase and phosphotransfer experiments were also performed with the ArlS T246A mutant. The kinase activity of the mutant (Fig. 10A) was similar to ArlS wild type, but the phosphotransfer data (shown by arrow in Fig. 10B) demonstrated that the T246A mutation prevents dephosphorylation of ArlR as shown by the presence of phosphorylated ArlR up to 60 minutes in the reaction. This is in contrast to ArlS wild type that showed loss of phosphorylated ArlR at the same time point (Fig. 8D, left panel). Both the ArlS H242A and T246A proteins contain the expected secondary structure as assessed by circular dichroism spectra, suggesting that they are properly folded (Fig. S5). Altogether, the ArlS H242 residue is essential for kinase activity, while the T246 residue controls the phosphatase activity of this histidine kinase.

Alternative activation of ArlR

Although the ArlRS TCS was required for expression of *mgrA* P2, we observed that ArlR alone, in the absence of ArlS, was still able to drive expression of MgrA at ~15–20% of wild type levels (Fig. 9C, Fig. 11). Mutating the phosphorylation site in *arlR* to an alanine decreased *mgrA* expression to levels similar to the *arlRS* mutant (Fig. 11). This has been observed previously with other response regulators and suggests non-specific phosphorylation from other histidine kinases or inorganic phosphate sources such as acetyl phosphate (Gao & Stock, 2013, Klein *et al.*, 2007). It has recently been shown that the histidine kinase GraS may be able to substitute for ArlS *in vivo*, although direct phosphorylation of ArlR by GraS was not examined (Villanueva *et al.*, 2018). We investigated if GraS could act as a non-cognate histidine kinase for ArlR using our *mgrA* P2-GFP reporter plasmid. Deletion of *graRS* in the *arlR*⁺ strain did not significantly decrease

expression of *mgrA* (Fig. 11), suggesting that there is not significant cross talk between ArlRS and GraRS.

Discussion

The ArlRS TCS was connected with autolysis and biofilm phenotypes nearly 20 years ago (Fournier & Hooper, 2000, Toledo-Arana *et al.*, 2005), and its importance in virulence has been observed in several animal models of infection (Benton *et al.*, 2004, Walker *et al.*, 2013, Radin *et al.*, 2016, Burgui *et al.*, 2018, Kwiecinski *et al.*, 2019). Yet the molecular details of gene regulation by ArlRS have remained largely unknown. In this study we clarified the ArlRS regulon, defined a likely ArlR binding site, and investigated the kinase and phosphatase kinetics of ArlS. It has been shown previously that ArlRS activates expression of the global regulator MgrA (Luong & Lee, 2006, Crosby *et al.*, 2016), and in this work RNA-sequencing analysis of the ArlRS regulon demonstrated that there is considerable overlap with the MgrA regulon (Crosby *et al.*, 2016). In total, 70% of the genes regulated by ArlRS were also regulated in the same direction by MgrA (Table S2), and 96% of genes with 4-fold change in expression showed overlap with the MgrA regulon (Fig. 1). We demonstrate for the first time that ArlR directly activates *mgrA* expression, and identify a potential ArlR binding site (Fig. 6). The ArlRS TCS was also recently shown to directly regulate *spx* expression (Bai *et al.*, 2019). We show that this regulation occurs at the *spx* P2 promoter, which contains a similar ArlR binding site (Fig. 7).

These results indicate that ArlRS functions at the top of a regulatory cascade (Fig. 12) in which ArlR directly activates expression of the important global transcriptional regulators MgrA and Spx. Both MgrA and Spx are redox sensitive (Hillion & Antelmann, 2015), suggesting that ArlRS may be involved in the oxidative stress response. MgrA is in the MarR/OhrR family and contains a conserved redox-sensing cysteine residue. Oxidation of this cysteine residue by reactive oxygen species results in MgrA dissociation from DNA (Chen *et al.*, 2006). Spx is a thiol stress sensor, and it is primarily regulated at the posttranslational level via degradation by the ClpXP proteasome (Pamp *et al.*, 2006, Donegan *et al.*, 2014). The Spx protein is only stabilized when a CXXC motif near its N-terminus is oxidized, and in this oxidized state it binds to RNA polymerase to affect gene expression (Hillion & Antelmann, 2015). Spx is a key stress-response regulator that controls expression of thioredoxin reductase (*trxB*), the *ica* locus for poly-N-acetylglucosamine synthesis, and TrfA, a Clp proteasome adaptor involved in resistance to cell wall-active antibiotics (Pamp *et al.*, 2006, Jousselin *et al.*, 2013, Bai *et al.*, 2019). Under the conditions of our RNA-seq experiment we did not see regulation of *trxB*, *icaADBC*, or *trfA*. This could be due to the relatively low fold-change in *spx* expression in the *arlRS* mutant, combined with the strong posttranslational regulation of Spx protein levels (Pamp *et al.*, 2006, Donegan *et al.*, 2014). It is possible that the effect of an *arlRS* mutant on Spx function would be more apparent under redox stress conditions.

In contrast to previous studies (Liang *et al.*, 2005, Harper *et al.*, 2018, Ingavale *et al.*, 2005, Yan *et al.*, 2019), we did not see evidence that ArlRS controls expression of the *agr* quorum-sensing system. We speculate that some of the apparent down-regulation of *agr* seen in other studies could result from strain- and media-dependent growth delays in the *arlRS* mutant

(Liang *et al.*, 2005, Radin *et al.*, 2016), since *agr* expression is highly dependent on growth phase. There is partial overlap in the ArlRS and *agr* regulons, such as *lukSF*, *lukAB*, and the urease and capsule operons (Thoendel *et al.*, 2011). However, there are many genes in the *agr* regulon that are not regulated by ArlRS, suggesting that the overlap may be indicative of complex regulation of virulence factors in *S. aureus*, rather than direct regulation of *agrBDCA* expression by ArlRS.

Genes repressed by ArlRS-MgrA

A variety of genes and operons are strongly repressed by the ArlRS-MgrA cascade (Fig. 1 and 12). These include two direct targets of MgrA repression, the transcriptional regulator *sarV* (Manna *et al.*, 2004) and the giant surface protein *ebh* (Crosby *et al.*, 2016). Other cell surface adhesins regulated by this cascade include *sdrD*, *fntB*, and *sasC* (Fig. 1). We have previously shown that regulation of surface proteins by the ArlRS-MgrA cascade has a large effect on multicellular behavior, including clumping, adhesion to host proteins, and biofilm formation (Walker *et al.*, 2013, Crosby *et al.*, 2016, Kwiecinski *et al.*, 2019). ArlRS-MgrA also affects extracellular polysaccharide production. In agreement with previous work (Luong & Lee, 2006), we see activation of several genes in the capsule biosynthesis operon (Fig. 1). ArlRS-MgrA appears to simultaneously repress expression of two uncharacterized putative polysaccharide biosynthesis gene clusters, *capIABC* and SAUSA300_0130–0133. Whether these hypothetical clusters are actually involved in capsule or polysaccharide biosynthesis remains to be determined.

The ArlRS TCS was originally identified as a regulator of autolysis (Fournier & Hooper, 2000), although the exact mechanism remains unknown. Consistent with previous results, we observe ArlRS-MgrA mediated repression of the autolysin *lytN*, but not *lytM* or *atl* (Luong *et al.*, 2006, Memmi *et al.*, 2012). Similarly to Luong *et al.* (Luong *et al.*, 2006), we saw that AtlR is overexpressed in the *arlRS* mutant. AtlR is a repressor of Atl expression (Houston *et al.*, 2011), however this should result in decreased autolytic activity in the *arlRS* mutant. In addition, we see modest regulation of the LysM/CHAP domain containing enzyme SAUSA300_0739 (Osipovitch *et al.*, 2015) by ArlRS but not MgrA.

Our results show that the ArlRS-MgrA cascade represses expression of the urease gene cluster, consistent with previous observations (Liang *et al.*, 2005, Luong *et al.*, 2006). *S. aureus* relies on urease for maintaining pH homeostasis in acidic conditions (Zhou *et al.*, 2019). ArlRS and MgrA also appear to be important in controlling divalent cation acquisition. Genes encoding the ferrous iron transporter FeoA, the catechol siderophore transporter SstABCD (Morrissey *et al.*, 2000, Beasley *et al.*, 2011), and the staphyloferrin B receptor SirA (Grigg *et al.*, 2010) were all repressed by ArlRS. In addition, the manganese transporter MntH (Horsburgh *et al.*, 2002) was up ~8-fold in the *arlRS* mutant. Divalent cation acquisition is critical inside the host, where metals are often limiting (Cassat & Skaar, 2012), but the contribution of ArlRS-dependent regulation of these processes during *S. aureus* disease is not yet known.

Genes activated by ArlRS-MgrA

The genes that are activated by ArlRS are predominantly enzymes involved in anaerobic metabolism or secreted virulence factors. In all cases these genes were also down-regulated in the *mgrA* mutant, suggesting that regulation occurs via MgrA (Fig. 1 and 12). Regulation by both ArlRS and MgrA was confirmed using transcriptional reporters for a subset of these genes: *nuc*, *lukA*, *lukS*, *esxA*, and *gehB* (Fig. 2). Previous work has shown that MgrA can act as a repressor, and the MgrA binding site has been identified (Manna *et al.*, 2004, Crosby *et al.*, 2016). It is not clear if MgrA can also function as an activator, but apparent activation by MgrA could be the result of indirect regulation. MgrA is known to repress expression of the transcriptional regulator SarV (Manna *et al.*, 2004), and our results suggest that the DNA-binding proteins AtlR and SAUSA300_1202 are also repressed by ArlRS-MgrA (Fig 1). It is also possible that stable regulatory RNAs play a role in mediating expression of secreted virulence factors (Morrison *et al.*, 2012, Das *et al.*, 2016).

Not all secreted virulence factors are regulated by ArlRS-MgrA, though. Notably, alpha hemolysin (*hla*) showed no change in gene expression in the *arlRS* or *mgrA* mutants. This is in contrast to reports that MgrA regulates Hla expression (Ingavale *et al.*, 2005, Sun *et al.*, 2011), but consistent with an earlier microarray analysis of the MgrA regulon in strain Newman (Luong *et al.*, 2006). We also observed a 2.6-fold increase in protein A (*spa*) gene expression in the *arlRS* mutant, consistent with the 54-fold increase in *spa* expression in the *mgrA* mutant and previous reports that *spa* is repressed by ArlRS (Merino *et al.*, 2009) or MgrA (Ingavale *et al.*, 2005, Luong *et al.*, 2006). However, this is in contrast to the large decrease in protein A expression recently reported for an *arlRS* mutant grown in chemically defined medium (Villanueva *et al.*, 2018), suggesting that regulation of protein A expression is complex and highly dependent on growth conditions.

Identification of the ArlR binding site

By using a series of site directed mutants in the *mgrA* P2 reporter we have identified what is likely to be the ArlR binding site (Fig. 6). ArlR is a member of the OmpR family of response regulators, which typically bind to direct repeats separated by a 4–5 bp spacer (de Been *et al.*, 2008). This spacing allows the DNA binding domains of each monomer to bind in a head-to-tail orientation along one face of the DNA helix (Blanco *et al.*, 2002). Consistent with this, the potential ArlR binding site appears to be almost a direct repeat separated by a 4 bp gap (TTTTCTCAT-N₄-TTTAAATAA in the *mgrA* P2 promoter). This sequence is nearly identical in the *spx* P2 promoter, and in both promoters, there is an 11 bp gap between the –10 sequence and the potential ArlR binding site (Fig. 7F). This binding site appears to be in place of a –35 sequence, as neither *mgrA* P2 or *spx* P2 has a recognizable –35 sequence. This spacing and localization is similar to that of the PhoB response regulator in *E. coli* (Makino *et al.*, 1993), another member of the OmpR family. PhoB-dependent promoters also lack a consensus –35 sequence and require PhoB for expression (Makino *et al.*, 1988). PhoB appears to interact directly with the housekeeping sigma factor to facilitate gene expression, rather than binding farther upstream in the promoter region and interacting with the alpha subunit of RNA polymerase (Makino *et al.*, 1993).

A search of the *S. aureus* USA300 genome for 90% matches to the consensus binding sequence TTTTCTCAT-N₄-TTTTAATNN resulted in only four hits that occurred in the same orientation within likely promoter regions. Besides *mgrA* and *spx*, the other two hits were upstream of the putative UDP-glucose 4-epimerase SAUSA300_0130. While expression from *mgrA* P2 and *spx* P2 is activated by ArlR binding, it appears that expression of SAUSA300_0130 may be directly repressed by both ArlR and MgrA.

Kinase and phosphatase activity of ArlS

Our *in vivo* and *in vitro* results demonstrate that ArlS has both kinase and phosphatase activity (Fig. 8, 9, and 10), which is typical of HisKA family histidine kinases (Willett & Kirby, 2012). ArlS appears to have slow autophosphorylation kinetics. Interestingly, the HAMP domain inhibited the kinetics of phosphotransfer and phosphatase activity. Although the biochemical and structural bases for these observations are not entirely clear, it may be that ArlS requires a lipid membrane environment for full activity. In addition, the *in vitro* experiments did not include the extracellular Cache domain and the signal(s) needed to activate ArlS. Thus, the presence of the lipid membrane, the extracellular domain and bound ligand likely affect the kinetics of ArlS activity in a physiologically relevant manner.

ArlS may function predominantly as a kinase during normal *in vitro* growth. Elimination of ArlS kinase activity by mutating H242 decreased *mgrA* expression ~5-fold, whereas mutation of T246, which is critical for phosphatase activity, increased *mgrA* expression ~2-fold (Fig. 9C). Consistent with this, Ebh and LukS protein levels in the ArlS T246A phosphatase mutant appear to be similar to wild-type levels (Fig. 9D, 9E), indicating that phosphatase activity is not playing a large role in gene expression under these conditions. For any TCS the phosphatase activity is finely tuned to suppress non-specific phosphorylation by non-cognate histidine kinases and inorganic phosphate donors (Gao & Stock, 2017). Indeed, in the absence of ArlS we do see some non-specific activation of ArlR (Fig. 9C, Fig. 11). A previous report suggested that there may be cross talk between ArlRS and the GraRS TCS (Villanueva *et al.*, 2018), however our *in vivo* reporters did not indicate that GraS is involved in activation of ArlR (Fig. 11). Future work could investigate if this low-level background activation of ArlR is due to chemical phosphorylation of ArlR or cross talk with a different TCS or kinase.

In this work we clarified how ArlRS regulates gene expression and ultimately virulence. ArlRS appears to be at the top of a regulatory cascade, and it affects gene expression through activation of the transcriptional regulators MgrA and Spx. In our RNA-seq experiment MgrA regulated >100 genes (Crosby *et al.*, 2016), although it is not yet clear how many of these genes are directly regulated by MgrA. There are most likely several levels of regulation in this cascade, potentially including both transcriptional regulators like SarV and small RNAs. In contrast, we did not observe changes in expression of genes in the Spx regulon under the growth conditions used here. Examining the effect of ArlRS under redox stress conditions may aid in identifying new roles for this TCS.

Experimental Procedures

Reagents and growth conditions

S. aureus strains and plasmids used in this work are listed in Table 1. *S. aureus* was cultured in tryptic soy broth (TSB) and *E. coli* was cultured in lysogeny broth (LB) at 37 °C with shaking at 200 rpm. Antibiotics were added to the media at the following concentrations: chloramphenicol (Cam), 10 µg/mL; erythromycin (Erm), 5 µg/mL; and tetracycline (Tet), 1 µg/mL. *E. coli* strains with plasmids were maintained on media supplemented with ampicillin at 100 µg/mL; kanamycin, 50 µg/mL; or spectinomycin at 50 µg/mL.

Recombinant DNA and genetic techniques

E. coli DH5α and DC10B were used as a cloning hosts for plasmid constructions. Restriction enzymes, DNA ligase, and Phusion DNA polymerase were purchased from New England Biolabs. The plasmid mini-prep and gel extraction kits were purchased from Invitrogen or Qiagen. Lysostaphin, used for *S. aureus* DNA extractions, was purchased from Sigma. Plasmids were purified from *S. aureus* RN4220 or *E. coli* DC10B and electroporated into *S. aureus* LAC strains as described previously (Monk *et al.*, 2012, Lofblom *et al.*, 2007). Bacteriophage transductions between *S. aureus* strains were performed with phage 11 as described previously (Novick, 1991). All oligonucleotides were ordered from IDT (Coralville, IA) and are listed in Supplementary Table 4. DNA sequencing was performed at the University of Iowa DNA Core Facility or the Molecular Biology Service Center at the University of Colorado Anschutz Medical Campus.

RNA-sequencing

Triplicate cultures of LAC and LAC *arlRS* were grown to an OD600 of 1.5 in TSB, harvested by centrifugation, and washed briefly with RNeasy Protect Bacterial Reagent (Qiagen) to stabilize the RNA. Cell pellets were then resuspended in PBS and lysed with lysostaphin for 1 h at room temperature prior to RNA purification using the Qiagen RNeasy Mini kit. RNA was then treated with Turbo DNA-free (Invitrogen), and assessed for quality using a Bioanalyzer (Agilent). rRNA was depleted using the Ribo-Zero rRNA Removal Kit for Gram-positive bacteria (Illumina). cDNA libraries were generated at the University of Iowa Genomics Division using the TruSeq Stranded mRNA Library Prep Kit (Illumina). Samples were barcoded, pooled, and sequenced in 100×100 paired end reads using a HiSeq 2000 sequencer (Illumina). The resulting sequences were aligned to the USA300_FPR3757 genome sequence and differential expression was quantified using CLC Genomics Workbench ver. 12 (Qiagen). Genes were considered differentially expressed if they showed a 2-fold change and a false discovery rate (FDR)-corrected P value < 0.05. Raw data from an identical comparison of gene expression in LAC *mgrA::tet* and LAC (Crosby *et al.*, 2016) were reanalyzed using CLC Genomics Workbench. Sequence files and analysis have been deposited in the NCBI GEO database under accession numbers GSE130777 (*mgrA* vs LAC) and GSE130887 (*arlRS* vs LAC).

Promoter fusion plasmids

All *mgrA* promoter-sGFP transcriptional reporters were generated in the shuttle vector pCM11 (Lauderdale *et al.*, 2010). Fragments of the *mgrA* promoter were amplified from *S. aureus* strain LAC genomic DNA using the primers listed in Table S4, and digested with HindIII and KpnI before ligating into pCM11. Promoter fusions for all other genes were generated in plasmid pCM29 (Pang *et al.*, 2010). Promoter fragments were amplified using the primers listed in Table S4 and digested with XbaI and KpnI before ligating into pCM29. In all cases promoter fragments were cloned upstream of an optimized ribosome binding site and codon optimized gene for superfolder GFP. Site directed mutagenesis was performed using modified versions of the QuikChange (Stratagene) method (Liu & Naismith, 2008, Zheng *et al.*, 2004) and the primers listed in Table S4.

To assess expression, overnight cultures were diluted 1:100 in TSB containing the appropriate antibiotic in a black 96-well plate, and plates were incubated at 37 °C with shaking in a humidified microtiter plate shaker (Stuart). A Tecan Infinite M200 plate reader was used to periodically measure OD₆₀₀ and fluorescence intensity with excitation at 495 nm and emission at 515 nm. For each experiment values from triplicate wells were averaged after subtracting background fluorescence from strains carrying an empty vector control (pCEMCS for erythromycin resistant reporters and pCM28 for chloramphenicol resistant reporters). For graphs of 24 h time points, averages and standard deviations were calculated from at least three independent experiments. Statistical significance was determined using a one-way ANOVA with a Dunnett test for correction for multiple comparisons (GraphPad Prism).

arlRS chromosomal complementation

The *arlRS* chromosomal complementation strain AH3244 has been described previously (Kwiecinski *et al.*, 2019). Point mutations were introduced into *arlR* or *arlS* using the QuikChange mutagenesis method (Stratagene), using the primers listed in Table S4 and pHC24 (pLL29-*arlRS*) as a template. A construct expressing *arlR* alone was also generated by amplifying *arlR* with primers HC84 and HC708. The product was cloned into pLL29 using BamHI and HindIII. In all cases the plasmids were electroporated into *S. aureus* RN4220 containing pLL2787, resulting in integration of pLL29 into the phage 11 attachment site. Integrated *arlRS* constructs were then transduced into *S. aureus* LAC, and integration was confirmed by PCR as described previously (Luong & Lee, 2007).

Protein Expression and Purification

The DNA fragments of HAMP-DHpCA region (amino acids 187–451) and DHpCA domain (amino acids 231–451) of ArlS from *S. aureus* LAC Strain were amplified by PCR and sub-cloned into a modified pET21a plasmid (Novagen) containing an N-terminal 6× His-tag followed by a recombinant tobacco etch virus (rTEV) protease cleavage site (ENLYFQG). The DNA fragment of full-length ArlR (amino acids 1–219) was amplified by PCR and cloned into a modified pET31b plasmid (Novagen) (pTEV5), which has an N-terminal 6× His-tag followed by a rTEV protease cleavage site. The ArlS HAMP-DHpCA H242A and ArlS HAMP-DHpCA T246A mutants were created by oligonucleotide-directed mutagenesis. The mutants were confirmed by Sanger sequencing (University of Iowa, Iowa

Institute of Human Genetics Genomic Division). *E. coli* strain BL21(DE3) (Novagen) cells containing ArlS or ArlR plasmids were grown in Luria Broth with 100ug/mL ampicillin and the protein expression was induced with 0.5 mM isopropyl-1-thio-D-glactopyranoside at 18 °C overnight. The cells were harvested by centrifugation and re-suspended in buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl and 10 mM imidazole. All proteins were purified by nickel-affinity chromatography (Nickel Sepharose 6 fast flow media, GE Healthcare). The N-terminal 6x His-tag of all proteins was removed by incubation with rTEV protease at 4 °C overnight. Size-exclusion chromatography (Superdex 75, GE Healthcare) was performed as a final purification step and eluted in a buffer containing 25 mM Tris pH 7.5, 150 mM NaCl, 5 % glycerol, 0.5 mM EDTA and 1 mM dithiothreitol (DTT). Proteins were ~95% pure as judged by SDS-PAGE as shown in Fig. 8B.

Kinase and phosphotransfer assays

Enzyme assays were followed by radioactivity (^{32}P) using previously described protocols (Willett & Kirby, 2011). Briefly, auto-phosphorylation reactions used 5 μM ArlS HAMP-DHpCA or ArlS DhpCA incubated with ATP mix [0.25 μM [γ - ^{32}P]-ATP and 250 μM cold ATP (Sigma) in 1X kinase buffer (25 mM Tris pH 7.6, 1 mM MgCl_2 , 1 mM MnCl_2 , 1 mM CaCl_2 , 50 mM KCl, 1 mM DTT)] for 1 hour. The reactions were quenched at desired time points by addition of SDS-PAGE loading buffer. The samples were then resolved by SDS-PAGE and the radioactive bands visualized using a phosphor screen (GE healthcare Typhoon FLA 9500). The bands were quantified using ImageJ software.

Phosphotransfer reactions used 5 μM of auto-phosphorylated ArlS HAMP-DHpCA and ArlS DhpCA prepared as described above. Auto-phosphorylated ArlS HAMP-DHpCA or DhpCA was then incubated with 5 μM full-length ArlR. The phospho-transfer reaction was allowed to proceed for 1 hour and individual samples quenched at desired time points by addition of SDS-PAGE loading buffer. The samples were resolved by SDS-PAGE and the radioactive bands visualized on a phosphor screen (GE healthcare Typhoon FLA 9500). The autophosphorylation and phosphotransfer assays were repeated at least three times and representative images are shown.

Electrophoretic mobility shift assay

Full-length *arlR* was cloned into the overexpression vector pET28a using primers HC41 and HC42 (Table S4) and transformed into the overexpression strain ER2566. This strain was grown in LB supplemented with kanamycin and expression was induced using 0.5 mM IPTG at 25 °C overnight. Cells were harvested and re-suspended in buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 10 mM imidazole, and 5% glycerol. Cells were lysed using BugBuster (Millipore) and sonication, before purification of His₆-ArlR by nickel-affinity chromatography (HIS-Select Nickel Affinity Gel, Sigma). Purified ArlR was concentrated and dialyzed against storage buffer containing 25 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 0.5 mM EDTA.

EMSA experiments were modified from the protocol described by Bai *et al.* (Bai *et al.*, 2019). DNA probes 5' end-labeled with IRDye700 were generated by annealing 50 nt oligos HC656 and HC657 (probe 2), and HC789 and HC790 (probe 3). Prior to DNA binding,

ArlR was phosphorylated for 20 min using 50 mM acetyl phosphate. Increasing concentrations of ArlR (0 to 2 µg) were incubated with 10 nM DNA probe for 20 min at 25 °C in 10 µL reactions containing 20 mM HEPES pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol, 0.2 % Tween 20, and 30 mM KCl. Reactions were then separated on a 0.5X TBE/8% polyacrylamide gel at 4 °C, using 0.75X TBE running buffer, before imaging on a Licor Odyssey CLx Imaging System. EMSA experiments were repeated twice and a representative image is shown.

Western blotting

S. aureus LAC strains lacking protein A (*spa*) were grown overnight in TSB, and then subcultured 1:100 into RPMI medium (Gibco) supplemented with 1% casamino acids. After growing at 37 °C for 8 h with shaking, 1.6 ml of culture were removed, the cells were pelleted, and the supernatant was transferred to a new tube. Secreted proteins were precipitated by adding 178 µL of trichloroacetic acid, vortexing briefly, and leaving at 4 °C overnight. Precipitated proteins were then centrifuged for 10 min at 17,000g, washed twice with 100% ethanol, and allowed to air dry. Pellets were resuspended in SDS-PAGE loading buffer and run on 4–20% gradient SDS-PAGE gels (Bio-Rad). Proteins were then transferred to nitrocellulose membranes using the high molecular weight setting on a Trans-blot Turbo transfer system (Bio-Rad). Membranes were blocked with TBST plus 5% milk, and then probed using a 1:10,000 dilution of either anti-Ebh antibodies (Walker *et al.*, 2013) or anti-LukS antibodies (IBT Bioservices). Blots were then washed and probed with a 1:10,000 dilution of IRDye 680 goat anti-rabbit secondary antibody (LiCor) before imaging on a LiCor Odyssey CLx Imaging System.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. RNA-seq analysis of the ArlRS regulon and comparison to that of MgrA. All genes with a fold change ≥ 4 in the *arlRS* mutant are shown, grouped by predicted function. For comparison, the change in expression of these genes in the *mgrA* mutant grown under the same conditions is also shown (Crosby *et al.*, 2016).

Fig. 2. Verification of selected RNA-seq results using transcriptional reporters.

Plasmids containing the promoter regions fused to GFP were introduced into the wild-type LAC strain (black), *arlRS* mutant (blue), chromosomally complemented *arlRS* mutant (gray), and *mgrA* mutant (red). Gene name or locus tag (strain USA300_FPR3757) is indicated above each graph. Cultures were grown for 24 h and the average fluorescence signal and standard deviation was calculated from at least three independent experiments. Statistical significance relative to LAC was calculated with Prism using a one-way ANOVA with Dunnett's test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Fig. 3. Determining minimum *mgrAP2* promoter length required for activation by ArlRS.

Sequentially smaller fragments of the *mgrA* P2 promoter region were cloned into pCM11 in front of GFP (A), and expression was measured at 24 h in wild-type strain LAC (B).

Numbers correspond to the plasmid numbers in (A). The average fluorescence signal and standard deviation was calculated from at least three independent experiments. Statistical analysis is as described for Fig. 2. (C) Sequences of the four shortest reporters, showing only the region upstream of the transcription start site (TSS). The predicted -10 site is indicated above the sequences.

Fig. 4. Mutating potential ArIR binding site.

6 bp mutations were introduced into pHC137, an *mgrA* P2 reporter with full activity, and expression was tested at 24 h in wild-type strain LAC (A). (B) Sequences of each promoter fragment, showing the 6 bp mutation in red. The inferred ArIR binding site is indicated by the gray box, along with the predicted -10 site and transcription start site. The average fluorescence signal and standard deviation was calculated from at least three independent experiments.

Fig. 5. Refining the ArlR binding site.

2 bp mutations were introduced into the *mgrA* P2 reporter pHC137, based on the results of Fig. 4. Expression was measured at 24 h in strain LAC as before. The refined ArlR binding site and -10 sequence are indicated by the gray boxes. The average fluorescence signal and standard deviation was calculated from at least three independent experiments.

Fig. 6. EMSA showing binding of ArlR to the *mgrA* P2 promoter.

(A) Purified, His-tagged ArlR was incubated with 10 nM IRDye700-labeled probes, and then separated by PAGE. (B) Location of probes 2 and 3 within the *mgrA* P2 promoter, as well as the predicted ArlR binding site. The images are representative of two replicates.

Fig. 7. ArlRS regulate expression of *spx* P2.

(A) Expression of *spx*-GFP reporter pHC169 in the indicated *S. aureus* strains after 24 h of growth. The average fluorescence signal and standard deviation was calculated from three independent experiments. (B) Schematic of the major *spx* promoters determined by Prados *et al.* (Prados *et al.*, 2016), as well as the transcriptional reporters used below. (C-E) Time course of each *spx* reporter in wild-type LAC (black) or the *arlRS* mutant (blue). (F) Alignment of *mgrA* P2 and *spx* P2, showing the potential ArlR binding site. The growth curve is representative of two replicates.

Fig. 8. Characterization and activity of ArlS and ArlR proteins.

(A) Domain architecture of ArlS and ArlR proteins. ArlS is a membrane bound histidine kinase with extracellular sensing domain, transmembrane HAMP domain and cytoplasmic DHpCA domain. ArlR is predicted to be an OmpR-like response regulator with receiver domain and winged helix-turn-helix DNA binding domain. (B) Coomassie staining showing purified ArlS (DHpCA, ~26 kDa; HAMP-DHpCA, ~31 kDa) and ArlR (~26 kDa) proteins. (C) Autophosphorylation of ArlS HAMP-DHpCA (left panel) and DHpCA (right panel). (D) Phosphotransfer of ArlS HAMP-DHpCA (left panel) and DHpCA (right panel) to full-length ArlR. All assays were performed at least three times and the images are representative data.

Fig. 9. Identification of residues important for ArlS kinase and phosphatase function.

(A) Alignment of the Dimerization and Histidine Phosphotransfer (DHp) domains of histidine kinases from various two-component systems. Numbering corresponds to the ArlS sequence. (B) Alignment of a portion of the receiver domains of response regulators centered on the phosphorylated aspartic acid residue, D52 in ArlR. Numbering corresponds to ArlR sequence. (C) *S. aureus* LAC strains with an *arlRS* deletion and chromosomal complementation with various versions of *arlR* and *arlS* as indicated. ArlS H242 lacks kinase activity *in vitro*, and ArlS T246A is deficient in phosphatase activity *in vitro*. Bar graph shows expression of *mgrA* from the *mgrA* P2-GFP promoter fusion pHC68 after 24 h of growth in TSB. Values represent averages and standard deviations of three independent experiments. Lower panels show Western blots of Ebh (D) and LukS (E) from culture supernatants, and are representative of two replicates.

Fig.10. ArlS has both kinase and phosphatase activity.

(A) Auto-phosphorylation of the ArlS HAMP-DhpCA T246A mutant. (B) Phosphotransfer from the ArlS HAMP-DhpCA T246A mutant to full-length ArlR. Arrow indicates the phosphorylated ArlR band at 60 min, demonstrating the loss of ArlS phosphatase activity in the T246A mutant. The assays were performed at least three times and the images are representative data.

Fig. 11. Alternative activation of ArlR.

S. aureus LAC strains with deletions in *arlRS* and *graRS*, and chromosomal complementation with various versions of *arlR* and *arlS* as indicated. Expression of *mgrA* from the *mgrA* P2-GFP promoter fusion pHC68 was measured after 24 h of growth in TSB. Values represent averages and standard deviations of three independent experiments. Significance was determined by ANOVA using Tukey's test for multiple comparisons.

Fig. 12. Scheme for the ArlRS regulon.

ArlR appears to directly activate expression of the transcriptional regulators *mgrA* and *spx*. MgrA then either directly or indirectly regulates over 100 genes, some of which are indicated.

Table 1**Bacterial strains and plasmids**

Strain/plasmid	Genotype/properties	Reference
<i>E. coli</i>		
DH5α	Cloning strain	Protein Express
DC10B	Cloning strain (<i>dcm</i> ⁻)	(Monk <i>et al.</i> , 2012)
ER2566	Protein expression strain	NEB
BL21 (DE3)	Protein expression strain	Novagen
<i>S. aureus</i>		
RN4220	Restriction deficient cloning host	(Nair <i>et al.</i> , 2011)
AH1263	USA300 CA-MRSA Erm ^S (LAC [*])	(Boles <i>et al.</i> , 2010)
AH1975	LAC [*] <i>arlRS</i>	(Walker <i>et al.</i> , 2013)
AH3052	LAC [*] <i>spa</i>	(Ibberson <i>et al.</i> , 2014)
AH3056	LAC [*] <i>arlRS spa</i>	(Crosby <i>et al.</i> , 2016)
AH3244	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlRS</i>	(Kwiecinski <i>et al.</i> , 2019)
AH3275	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i> D52A, <i>arlS</i>	This work
AH3277	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i> D52E, <i>arlS</i>	This work
AH3278	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlRS</i>	This work
AH3280	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlR</i> D52A, <i>arlS</i>	This work
AH3281	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlR</i> D52E, <i>arlS</i>	This work
AH3455	LAC [*] <i>mgrA</i> ::tetM	(Crosby <i>et al.</i> , 2016)
AH5242	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i>	This work
AH5243	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i> , <i>arlS</i> H242A	This work
AH5244	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i> , <i>arlS</i> T246A	This work
AH5271	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i> , <i>arlS</i> H242A T246A	This work
AH5360	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlR</i>	This work
AH5361	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlR</i> , <i>arlS</i> H242A	This work
AH5362	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlR</i> , <i>arlS</i> T246A	This work
AH5363	LAC [*] <i>arlRS spa</i> Φ11::LL29 <i>arlR</i> , <i>arlS</i> H242A T246A	This work
AH5407	LAC [*] <i>arlRS graRS</i> Φ11::LL29 <i>arlR</i>	This work
AH5434	LAC [*] <i>arlRS</i> Φ11::LL29 <i>arlR</i> D52A	This work
Plasmids		
pCEMCS	Empty vector control for pCM11, Erm ^R	(Malone <i>et al.</i> , 2009)
pCM11	sGFP expression vector, Erm ^R	(Lauderdale <i>et al.</i> , 2010)
pCM28	Empty vector control for pCM29, Cam ^R	(Boles <i>et al.</i> , 2010)
pCM29	sGFP expression vector, Cam ^R	(Pang <i>et al.</i> , 2010)
pCM38	P _{nuc} -GFP reporter, Cam ^R	(Yan <i>et al.</i> , 2019)
pLL29	<i>S. aureus</i> integration vector, Tet ^R	(Luong & Lee, 2007)

Strain/plasmid	Genotype/properties	Reference
pET21a	Overexpression vector, Amp ^R	Novagen
pET28a	Overexpression vector, Kan ^R	Novagen
pTEV5	Overexpression vector, N-terminal Tev-cleavable His6 tag, Amp ^R	(Rocco <i>et al.</i> , 2008)
pHC07	pET28a- <i>arlR</i> , Kan ^R	This work
pHC24	pLL29- <i>arlRS</i> , Spec ^R	(Kwiecinski <i>et al.</i> , 2019)
pHC28	pLL29- <i>arlR</i> D52A, <i>arlS</i> , Spec ^R	This work
pHC29	pLL29- <i>arlR</i> D52E, <i>arlS</i> , Spec ^R	This work
pHC35	pTEV5- <i>arlR</i> , Amp ^R	This work
pHC68	<i>mgrA</i> P2-sGFP fusion, 251 bp insert, Erm ^R	(Crosby <i>et al.</i> , 2016)
pHC136	<i>mgrA</i> P2-sGFP fusion, 156 bp insert, Erm ^R	This work
pHC137	<i>mgrA</i> P2-sGFP fusion, 123 bp insert, Erm ^R	This work
pHC138	<i>mgrA</i> P2-sGFP fusion, 97 bp insert, Erm ^R	This work
pHC139	<i>PureA</i> -sGFP fusion, Cam ^R	This work
pHC140	<i>P1566</i> -sGFP fusion, Cam ^R	This work
pHC141	<i>P0130</i> -sGFP fusion, Cam ^R	This work
pHC144	<i>PesxA</i> -sGFP fusion, Cam ^R	This work
pHC145	<i>mgrA</i> P2-sGFP fusion, 110 bp insert, Erm ^R	This work
pHC146	<i>mgrA</i> P2-sGFP fusion, 83 bp insert, Erm ^R	This work
pHC147	<i>PlukA</i> -sGFP fusion, Cam ^R	This work
pHC148	<i>PlukS</i> -sGFP fusion, Cam ^R	This work
pHC149	<i>Pebh</i> -sGFP fusion, Cam ^R	This work
pHC150	<i>PmntH</i> -sGFP fusion, Cam ^R	This work
pHC151	<i>PsdR</i> -sGFP fusion, Cam ^R	This work
pHC152	<i>PlytN</i> -sGFP fusion, Cam ^R	This work
pHC159	pLL29- <i>arlR</i> , Spec ^R	This work
pHC161	pLL29- <i>arlR</i> , <i>arlS</i> H242A, Spec ^R	This work
pHC162	pLL29- <i>arlR</i> , <i>arlS</i> T246A, Spec ^R	This work
pHC164	<i>mgrA</i> P2-sGFP scramble 2, Erm ^R	This work
pHC166	pLL29- <i>arlR</i> , <i>arlS</i> H242A T246A, Spec ^R	This work
pHC167	<i>mgrA</i> P2-sGFP scramble 3, Erm ^R	This work
pHC168	<i>mgrA</i> P2-sGFP scramble 4, Erm ^R	This work
pHC169	<i>PspX</i> -sGFP fusion, Cam ^R	This work
pHC170	<i>mgrA</i> P2-sGFP scramble 5, Erm ^R	This work
pHC171	<i>mgrA</i> P2-sGFP scramble 6, Erm ^R	This work
pHC172	<i>mgrA</i> P2-sGFP scramble 7, Erm ^R	This work
pHC173	<i>mgrA</i> P2-sGFP scramble 8, Erm ^R	This work
pHC174	<i>mgrA</i> P2-sGFP scramble 9, Erm ^R	This work

Strain/plasmid	Genotype/properties	Reference
pHC176	<i>spx</i> P1-sGFP fusion, Cam ^R	This work
pHC177	<i>spx</i> P2-sGFP fusion, Cam ^R	This work
pHC178	<i>PgehB</i> -sGFP fusion, Cam ^R	This work
pHC183	<i>mgrA</i> P2-sGFP scramble 10, Erm ^R	This work
pHC184	<i>mgrA</i> P2-sGFP scramble 11, Erm ^R	This work
pHC185	<i>mgrA</i> P2-sGFP scramble 12, Erm ^R	This work
pHC186	<i>mgrA</i> P2-sGFP scramble 13, Erm ^R	This work
pHC188	<i>mgrA</i> P2-sGFP scramble 14, Erm ^R	This work
pHC189	<i>mgrA</i> P2-sGFP scramble 15, Erm ^R	This work
pHC190	<i>mgrA</i> P2-sGFP scramble 16, Erm ^R	This work
pHC191	<i>mgrA</i> P2-sGFP scramble 17, Erm ^R	This work
pHC192	<i>mgrA</i> P2-sGFP scramble 18, Erm ^R	This work
pHC194	<i>mgrA</i> P2-sGFP scramble 19, Erm ^R	This work
pHC195	<i>mgrA</i> P2-sGFP scramble 20, Erm ^R	This work
pHC196	<i>mgrA</i> P2-sGFP scramble 21, Erm ^R	This work
pHC198	<i>mgrA</i> P2-sGFP scramble 22, Erm ^R	This work
pHC199	<i>mgrA</i> P2-sGFP scramble 23, Erm ^R	This work
pHC200	<i>mgrA</i> P2-sGFP scramble 24, Erm ^R	This work
pHC201	<i>mgrA</i> P2-sGFP scramble 25, Erm ^R	This work
pHC202	<i>mgrA</i> P2-sGFP scramble 26, Erm ^R	This work
pHC203	<i>mgrA</i> P2-sGFP scramble 27, Erm ^R	This work
pHC204	<i>mgrA</i> P2-sGFP scramble 28, Erm ^R	This work
pHC205	<i>mgrA</i> P2-sGFP scramble 29, Erm ^R	This work
pHC206	<i>mgrA</i> P2-sGFP scramble 30, Erm ^R	This work
pET21-ArlS1	ArlS HAMP-DHpCA (amino acids 187–451) in pET21a-His-Tev, Amp ^R	This work
pET21-ArlS2	ArlS DHpCA (amino acids 231–451) in pET21a-His-Tev, Amp ^R	This work
pET21-ArlS3	ArlS HAMP-DHpCA H242A, Amp ^R	This work
pET21-ArlS4	ArlS HAMP-DHpCA T246A, Amp ^R	This work
pET21-ArlS5	ArlS HAMP-DHpCA H242A T246A, Amp ^R	This work