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Characterization of MroQ-Dependent Regulation of Staphylococcus Aureus Quorum Sensing

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CHARACTERIZATION OF MROQ-DEPENDENT REGULATION OF *STAPHYLOCOCCUS AUREUS* QUORUM SENSING

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIP	autoinducing peptide
Agr	accessory gene regulatory
MroQ	membrane regulator of quorum sensing
MLST	multi-locus sequence typing
ST	sequence type
CC	clonal complex
MRSA	methicillin-resistant *S. aureus*
SCC	staphylococcal cassette chromosome
PBP	penicillin-binding protein
VSSA	vancomycin-susceptible *S. aureus*
VISA	vancomycin-intermediate *S. aureus*
VRSA	vancomycin-resistant *S. aureus*
Spa	staphylococcal protein A
Clf	clumping factor
CHIPS	chemotaxis inhibitory protein of *S. aureus*
PSM	phenol soluble modulin
TCS	two component system
Sae	*S. aureus* exoprotein expression
Arl	autolysis-related locus

Srr	staphylococcal respiratory response
Sar	staphylococcal accessory regulator
Hla	alpha-hemolysin
Hlg	gamma-hemolysin
Luk	leucocidin
PVL	Panton-Valentine leucocidin
QS	quorum sensing
AI	autoinducer
AHL	acyl-homoserine lactone
SAM	*S*-adenosyl-methionine
ACP	acyl carrier protein
DPD	4,5-dihydroxy-2,3-penanedionine
CSP	competence stimulating peptide
SHP	short-hydrophobic peptide
GAS	Group A *Streptococcus*
RHK	receptor histidine kinase
HK	histidine kinase
DHp	dimerization and histidine phosphorylation
CA	catalytic and ATP
ATP	adenosine triphosphate
RR	response regulator
DBD	DNA-binding domain
DNA	deoxyribonucleic acid

RNA	ribonucleic acid
RNAp	RNA polymerase
SD	Shine Dalgarno
Rot	repressor of toxins
Aur	aureolysin
SspA	staphylococcal serine protease A
Fsr	*E. faecalis* regulator
GBAP	gelatinase biosynthesis-activating pheromone
AFC	alpha-factor converting enzyme
Abi	abortive infectivity
CPBP	CAAX proteases and bacteriocidin-processing enzymes
Spd	surface protein display
Lyr	lysostaphin resistance
LB	lysogeny broth
TSB	tryptic soy broth
RPMI	Roswell Park Memorial Institute
Cm	chloramphenicol
Amp	ampicillin
Erm	erythromycin
Tet	tetracycline
AnTet	anhydrous tetracycline
BHI	brain heart infusion
OD	optical density

TSM	Tris, sucrose magnesium chloride
IDT	Integrated DNA Technologies
PCR	polymerase chain reaction
SOE	splicing by overlap extension
$CaCl_2$	calcium chloride
$MgSO_4$	magnesium sulfate
TMG	Tris, magnesium chloride, gelatin
CY	casitone yeast
TSA	tryptic soy agar
GFP	green fluorescent protein
rpm	rotations per minute
mL	milliliter
μL	microliter
L	liter
mM	millimolar
M	molar
μM	micromolar
min	minute
hr	hour
˚C	degrees Celcius
sod	superoxide dismutase
Ssp	*Synechocystis* species PCC6803
FWD	forward

REV	reverse
SDS	sodium dodecyl sulfate
PBS	phosphate buffered saline
$NaCl_2$	sodium chloride
DDM	N-Dodecyl-beta-Maltoside
PMSF	phenylmethylsulfonyl fluoride
V	volts
PVDF	polyvinylidene difluoride
TBS	Tris-buffered saline
TBST	TSB-Tween 20
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3idoyl-phosphate
NBT	nitroblue tetrazolium
TCA	trichloroacetic acid
PAGE	polyacrylamide gel electrophoresis
CM	conditioned media
RBC	red blood cell
PCV	packed cell volume
mg	milligram
kg	kilogram
CFU	colony forming units
IACUC	Institutional Animal Care and Use Committee
USDA	United States Department of Agriculture

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
OLAW	Office of Laboratory Animal Welfare
ANOVA	one-way analysis of variance

ABSTRACT

Gram-positive bacteria produce small autoinducing peptides (AIPs), which act to regulate expression of genes that promote adaptive traits including virulence. The Gram-positive pathogen *Staphylococcus aureus* generates a cyclic AIP that controls expression of virulence factors via the accessory gene regulatory (Agr) system. *S. aureus* strains belong to one of four Agr groups (I, II, III, and IV), and each group harbors allelic variants of AgrD, the precursor of AIP. In a prior screen for *S. aureus* virulence factors, the Alonzo lab identified MroQ, a putative peptidase. A Δ*mroQ* mutant closely resembled a Δ*agr* mutant and had significant defects in AIP production in an Agr-I strain. I show that expression of AgrD-I in a Δ*mroQ* mutant leads to accumulation of an AIP processing intermediate at the membrane that coincides with a loss of secreted mature AIP, indicating MroQ promotes maturation of AgrD-I. MroQ is conserved in all Agr sequence variants, suggesting either identical function amongst all Agr types or activity specific to Agr-I strains.

My data indicate that MroQ is required for AIP maturation and activity in Agr-I, -II, and -IV strains irrespective of background. However, MroQ is not required for Agr-III activity despite an identifiable role in peptide maturation. My work suggests that these results may be due to the ability of an AIP-III intermediate to serve as an active AIP. Isogenic Δ*agr* and Δ*agr* Δ*mroQ* strains complemented with Agr-I-IV validated the critical role for MroQ in the generation of active AIP-I, -II, and -IV, but not AIP-III. These

findings were reinforced by skin infection studies in mice. Exploration of other Gram-positive bacteria identified MroQ homologues which may also contribute to respective Agr system function. Together, my data substantiate the prevailing model that MroQ is a mediator of cyclic peptide maturation and highlight a role for Type II CAAX proteases in Gram-positive bacteria.

CHAPTER ONE

LITERATURE REVIEW

Section 1: *Staphylococcus aureus*

Staphylococcus aureus is a major bacterial human pathogen responsible for nearly 1.3 million infections a year in the United States (1). While many of these cases are community acquired and occur in healthy individuals, the rest are categorized as hospital acquired and are an important cause for concern due to the rise of antibiotic resistant strains (2–6). Though *S. aureus* is part of the normal human microbiota, innocuously colonizing the skin and mucous membranes of nearly half of all adults, serious infections may arise if *S. aureus* is introduced to the bloodstream or internal tissues (7). These severe infections cause almost 750,000 deaths worldwide (8). Given its capability for significant morbidity, increasing antibiotic resistance, and recurrent infections, the Centers for Disease Control considers *S. aureus* a serious threat to human health (9).

S. aureus strains are differentiated into clonal groups using a technique called multi-locus sequence typing (MLST), which analyzes the sequence of seven housekeeping genes to stratify isolates based on sequence type (ST) (10). Strains with similar STs are grouped into a clonal complex (CC). Most clinical isolates belong to one of 10 clonal complexes (CC1, CC5, CC8, CC15, CC22, CC30, CC45, CC97, CC93, and CC121). Amongst these CCs, strains belonging to CC8 and CC5 are predominantly

responsible for hospital acquired infections, and many strains in these groups are methicillin-resistant *S. aureus* (MRSA) (11–18). Further, most instances of vancomycin resistance are in strains within CC5 (19).

Antibiotic Resistance in *S. aureus*

S. aureus is infamous for its ability to acquire antibiotic resistance. The first instance of antibiotic resistant *S. aureus* was reported as early as 1942, just a year after the introduction of penicillin, a beta-lactam drug which targets the bacterial cell wall (20). Soon after, reports of *S. aureus* strains resistant to streptomycin, erythromycin, and tetracyclines arose (21–23). Even methicillin, the drug expected to overcome penicillin resistance, was unable to escape the incredible capacity of *S. aureus* to evolve, and cases of MRSA were reported after just two years (24).

Though the mechanism of penicillin resistance was found to be based on an inducible beta-lactamase in the mid-1940s, that for methicillin resistance was not identified until 1981 (25,26). MRSA strains are characterized by the presence of the chromosomal gene *mecA,* which is located in a mobile genetic element called the staphylococcal cassette chromosome (SCC*mec*) (27). Thus far, 11 types of SCC*mec* have been identified, and each confers varied resistance to several antibiotics (28). *mecA* encodes the protein PBP-2a (penicillin-binding protein 2a), which is an essential bacterial cell wall enzyme that catalyzes the production of peptidoglycan, a critical cell wall component (26). Because PBP-2a has a lower affinity for beta-lactams than its other PBP counterparts, it continues to catalyze the synthesis of the cell wall, thus allowing for growth despite the presence of antibiotics (26). As such, strains with this protein tend to be resistant to several antibiotics.

Since the introduction of methicillin and the subsequent development of resistant *S. aureus* strains, several new antibiotics have been generated to try to limit infection. Amongst these are vancomycin and daptomycin, which are considered last resort treatment options for antibiotic resistant strains. Vancomycin interrupts cell wall synthesis by binding the terminal D-alanyl-D-alanine of the precursor lipid II, preventing incorporation into the growing peptidoglycan chain (29–31). Isolates of vancomycin resistant *S. aureus* are divided into groups based on the level of reduced susceptibility: vancomycin-susceptible *S. aureus* (VSSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA) (32). Of these, VRSA presents the largest concern, as vancomycin remains an important last resort antibiotic treatment option. VRSA strains are characterized by the presence of the *vanA* gene cluster, which modifies the D-alanyl-D-alanine of the precursor lipid II to D-alanyl-D-lactone (33,34). This modification substantially reduces the affinity of vancomycin for the precursor lipid II (35). To date, 52 VRSA strains have been reported worldwide (36–52).

VISA strains are associated with long-term infection, hospitalization, and persistent vancomycin use, though the reasons for this intermediate susceptibility are not well understood (53). It is suggested that VISA results from gradual accumulation of mutations within genes that contribute to common VISA phenotypes, such as thicker cell walls, decreased autolytic activity, and reduced virulence (53,54).

Daptomycin affects cell wall homeostasis via a mechanism similar to the cationic antimicrobial peptides through interaction with phospholipids (55,56). *S. aureus* develops resistance to daptomycin through changes in cell surface charge via expression of genes related to evasion of antimicrobial peptides, such as *mprF* and *dlt*

(57–66). Another notable phenotype of daptomycin resistant strains is the increased cell wall thickness seen in VISA (67,68). Several studies have suggested a link between VISA and daptomycin resistant strains (69–78). Given that vancomycin and daptomycin are the final options for antibiotic-based treatment, the ability of *S. aureus* to develop resistance is alarming. As such, those developing therapeutics have begun to consider alternative targets for limiting infection.

S. aureus and the Host

Pathogenic *S. aureus* is the causative agent for myriad human infections including infective endocarditis, osteomyelitis, bacteremia, pulmonary infections, and vascular catheter-related infections (7,9,79). *S. aureus* also infects the skin and soft tissue, resulting in disease manifestations such as impetigo, furuncles, cellulitis, and scalded skin syndrome (7). Origins of infection depend on the type of disease. Most systemic infections occur because of a breach of asymptomatically colonized bacteria through the epithelial protective layer, usually following minor scratches on the skin (80). In hospitals, *S. aureus* can adhere to indwelling medical devices soon after insertion and form a biofilm, which allows for bacterial growth and dissemination into the bloodstream and tissues (81). Often, those susceptible to severe *S. aureus* infection have predisposed conditions or tissues compromised by previous infection (82–84).

Systemic infection usually follows the introduction of *S. aureus* to the bloodstream. Within the bloodstream, there are several agents of cellular and humoral defense. Of these, the phagocytes, such as macrophages and neutrophils, pose a significant threat to *S. aureus* survival, as they are equipped to rapidly recognize and eliminate pathogens (85–87). *S. aureus* shields itself from phagocyte-mediated killing

through the production of several factors that facilitate evasion of the host immune system. *S. aureus* employs a two-sided attack, with some virulence factors interrupting host defense mechanisms and others directly targeting host cells. Protein A (Spa), staphylokinase, clumping factors (ClfA/B), and CHIPS (chemotaxis inhibitory protein of *S. aureus*) prevent opsonization, migration of leukocytes, and oxidative stress, whereas α-toxin, δ- and γ-hemolysin, leukocidins, and phenol soluble modulins (PSMs), target red blood cells (RBCs), leukocytes, monocytes, neutrophils, and macrophages. (Table 1) (88,89).

The expression of several of these virulence factors is regulated by two-component systems (TCSs), which sense environmental signals. TCSs consist of a sensor histidine kinase and a response regulator. Binding of the sensor kinase triggers a phosphorylation cascade that results in a phosphorylated response regulator that acts as a transcription factor to control gene expression. These include the *sae* (*S. aureus* exoprotein expression) locus, *arlSR* (autolysis-related locus) and *srrAB* (staphylococcal respiratory response), which control the expression of virulence factors and promote resistance to oxidative stress, respectively (Table 1 and (90–101)).

Two major regulators, *sarA* (staphylococcal accessory regulator) and the *agr* (accessory gene regulator) locus, are responsible for controlling the expression of most *S. aureus* virulence factors. SarA is a DNA- and RNA-binding protein that promotes the expression of several cell-wall associated proteins, such as Spa, and exoproteins, including α-toxin (Table 1 and (102–104)). In addition, SarA regulates the expression of the *agr* locus and several *agr*-regulated virulence factors (104). The *agr* locus encodes a quorum sensing system that regulates the expression of almost every major virulence

factor, including α-toxin, Protein A, the hemolysins, and leukocidins (Table 1 and (104–110)). Given its widespread effect on virulence factor gene expression, the *agr* locus is very well studied, and has been highlighted as a key target for possible therapeutic strategies that do not rely on antibiotics (111–113).

Table 1. Mediators of Immune Evasion in *S. aureus*

Effector	Contribution to Immune Evasion	Regulated by	Reference
Fur	Promote resistance to oxidative stress	SrrAB	(99,114)
Hla	Pore-forming toxin, targets several eukaryotic cells, including erythrocytes and neutrophils	Agr, SarA, Sae	(91,106,115,116)
HlgAB, HlgCB	Leukocidin and hemolysin, targets erythrocytes	Agr, SarA, Arl	
LukDE	Leukocidin, targets monocytes, neutrophils, and macrophages	Agr, SarA, Arl	(98,106,121)
LukAB (LukGH)	Leukocidin, targets monocytes, neutrophils, and macrophages	Agr	(106,117,119)
PVL (LukSF)	PVL (LukSF)	Agr, SarA	(106,125–127)
PSMa	Cytolytic toxins that target leukocytes	Agr	(106,128,129)
Spa	Binds FC region of IgG to resist phagocytosis	Agr, SarA, Arl	(91,94,95,106,107,130–132)
ClfA/B	Binds fibrinogen to prevent phagocytosis	Arl	(133–136)
Staphylokinase	Activates plasminogen to degrade complement	Agr	(106,137)
CHIPS	Prevents neutrophil migration	SarA, Sae	(138,139)
Dlt, MprF	Partially neutralize cell surface charge to avoid antimicrobial peptides	Agr, Arl	(60,140–143)

Section 2: Quorum Sensing in Bacteria

Quorum sensing (QS) is a means of cell-to-cell communication that allows for bacteria to regulate gene expression in response to cell density. As such, genes controlled by QS are often most beneficial to bacteria carrying out group-specific behaviors. Many processes are controlled by QS, including sporulation, competence, and biofilm formation (144–146). In pathogenic bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Vibrio cholerae*, QS controls the expression of several virulence factors that contribute to host infection (106,108,147–151).

While QS systems have myriad differences in mechanisms of action, the basis for each remains similar. All QS systems rely on the production and response to autoinducers (AIs), extracellular molecules that induce QS activity. AI concentration is a function of cell density and spatial containment. At low cell density, AIs will be diffuse and at concentrations below the threshold required for detection by QS receptors, which exist in the cytoplasm or membrane. As cell density increases, local concentrations of AI increase above this threshold, and receptors can detect and respond to the signal (152). Importantly, AI production is a positive-feedback loop i.e., detection and response to AI results in more AI being made (153,154).

There are two major ways in which AIs act as a signal to QS receptors. In certain bacteria, AIs bind directly to cytoplasmic receptors, which act as transcription factors to modulate expression of the QS regulon. In other cases, AIs serve as a signal for a membrane-bound TCS which controls gene expression from the QS regulon. These differing mechanisms of AI detection exist in both Gram-negative and Gram-positive bacteria.

Quorum Sensing in Gram-negative Bacteria

QS in Gram-negative bacteria depends on small molecules, often acyl-homoserine lactones (AHLs), that act as AIs (155). These AIs can freely diffuse across the inner and outer cell membranes (156,157). In most Gram-negative bacteria, QS is driven by binding of AI to cytoplasmic transcription factors that modulate QS regulon expression, though certain species employ a TCS as means of detection (158).

Most QS systems in Gram negative bacteria are homologous to the LuxI/LuxR system, which was first described in *Vibrio fischeri* (159,160). In this system, LuxI catalyzes a reaction between *S*-adenosyl-methionine (SAM) and an acyl carrier protein (ACP) to give rise to an AHL AI (Figure 1 and (156,157,161)). Once appropriate cell density is reached, AIs bind cognate LuxR receptors, which exist in the cytoplasm (Figure 1 and (156,162,163)). Upon binding, LuxR activates the expression of *luxCDABEG* operon, leading to luminescence, and promotes the transcription of *luxI,* allowing for the production and release of more AI (156,162–164).

LuxI/LuxR homologues exist in numerous Gram-negative bacteria, and the activity of these homologues depends on AHLs that vary by side chain length and decorations (157,165,166). The AHL-LuxR interaction is highly specific, as each LuxR contains a unique binding pocket that only permits interaction with particular AHL ligands (167–171). LuxI-mediated AI synthesis is similarly stratified, with LuxI structure conferring accommodation of specific acyl-ACPs that give rise to a particular AI (172,173). As such, bacterial cell-to-cell communication becomes intraspecies specific.

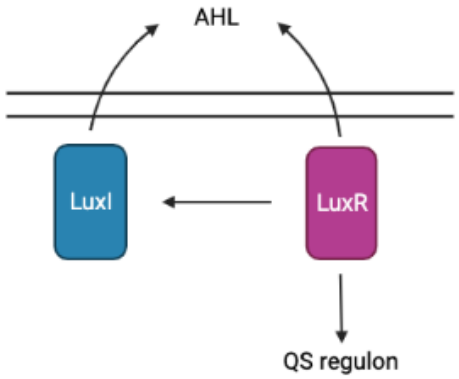

Figure 1. The LuxI/LuxR Quorum Sensing System Mediates Gene Expression in *V. fischeri.* LuxI produces an AHL AI that can bind the LuxR receptor. Following activation, LuxR acts as a transcription factor to promote expression of genes in the QS regulon.

Though many Gram-negative bacterial QS systems are similar to LuxI/LuxR, others are more complex. A well-studied example of this is *Vibrio cholerae,* which utilizes two parallel QS circuits which produce and respond to two different AIs (Figure 2 and (158)). The first AI, (S)-3-hydroxytridecan-4-one (CAI-1), is synthesized by the CqsA enzyme using SAM and decanoyl-coenzyme A as substrates (Figure 2 and (155,174,175)). The other, AI-2, is produced following the conversion of SAM cycle intermediate *S*-riobsylhomocysteine to 4,5-dihydroxy-2,3-penanedionine (DPD) by LuxS (Figure 2 and (158)). AI-2 is produced following a spontaneous conversion of DPD (176–178). These AIs are detected by parallel two-component receptors, CqsS and LuxPQ. Both act upon LuxO via the phosphotransfer protein, LuxU, in response to an AI. CqsS responds to CAI-1, whereas LuxPQ responds to AI-2 (Figure 2 and (175,179–185)). Phosphorylated LuxO promotes the expression of *qrr* genes, which give rise to Qrr sRNAs that target the mRNAs encoding HapR and AphA, the master QS regulators (Figure 2 and (186,187)). In essence, phosphoryolated LuxO gives rise to high levels of *aphA* and low levels of *hapR.* Importantly, AphA activates *tcpPH,* whose protein

activates *toxT* (158,188–190). ToxT then activates the expression of major virulence factors (191,192). In contrast, HapR represses the activity of AphA, shutting down virulence factor production (Figure 2 and (187,193)).

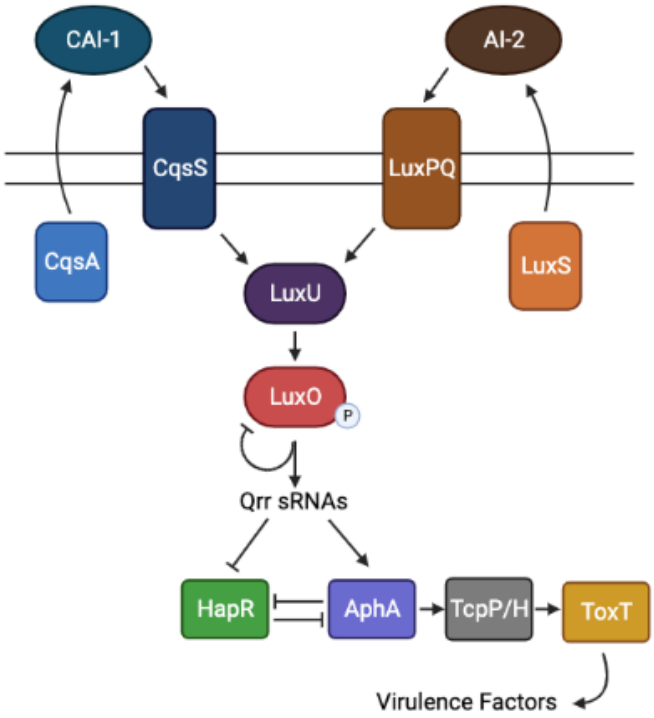

Figure 2. Quorum Sensing Systems in *V. cholerea* Produce and Respond to Two Different Ais. CAI-1 is produced by CqsA, released into the extracellular space, and recognized by CqsS. AI-2 is generated by LuxS, released, and recognized by LuxPQ. Upon activation, both CqsS and LuxPQ act upon LuxO via LuxU. LuxO regulates the expression of Qrr sRNAs which target HapR and AphA, the master regulators of the QS regulon. Active AphA represses HapR and leads to the production of TcpP/H, a protein that activates the expression of *toxT.* ToxT promotes the expression of virulence genes. Active HapR represses AphA, preventing expression of virulence genes.

Interestingly, *luxS* homologues exist in several species of both Gram-negative and Gram-positive bacteria. Each homologue produces a unique AI-2. As such, AI-2 has been associated with interspecies communication: various AI-2 producing bacteria in a particular environment can recognize the presence of their own and other AI-2s.

This enables bacteria to gain knowledge of their microbial community and its overall cell-density (194,195).

Quorum Sensing in Gram-positive Bacteria

For Gram-positive bacteria, QS signaling depends on the production, processing, and secretion of peptides called autoinducing peptides (AIP). AIPs are encoded as precursor peptides (pro-AIPs), which are subsequently processed and secreted. These pro-AIPs are diverse in sequence and structure and require specific proteins for processing (196–202). Because the cell membrane does not confer peptide permeability, AIPs are often secreted via dedicated transporters. Mature AIPs range in size from 5 to 17 amino acids and are linear or cyclic (201–205).

In some cases, once extracellular AIP reaches a threshold concentration, the peptide binds to a cognate TCS comprised of a membrane-bound histidine kinase receptor and a cytoplasmic response regulator (Figure 3A and (206–208)). Binding by AIP catalyzes an ATP-mediated autophosphorylation of the histidine kinase receptor. This phosphate is transferred to a cognate response regulator, thus allowing it to activate the transcription of genes in the QS regulon (Figure 3A). Typically, the pro-AIP, histidine kinase, response regulator, and proteins required for AIP processing are encoded in an operon (209,210). The phosphorylated response regulator activates the expression of this operon, thus giving rise to a positive feedback loop of QS activity (Figure 3A). QS systems that exhibit this mechanism of action to promote virulence factor expression exist in several Gram-positive bacteria, including *S. aureus, Listeria monocytogenes, Enterococcus faecalis,* and *Clostridium perfringens* (200,211–214).

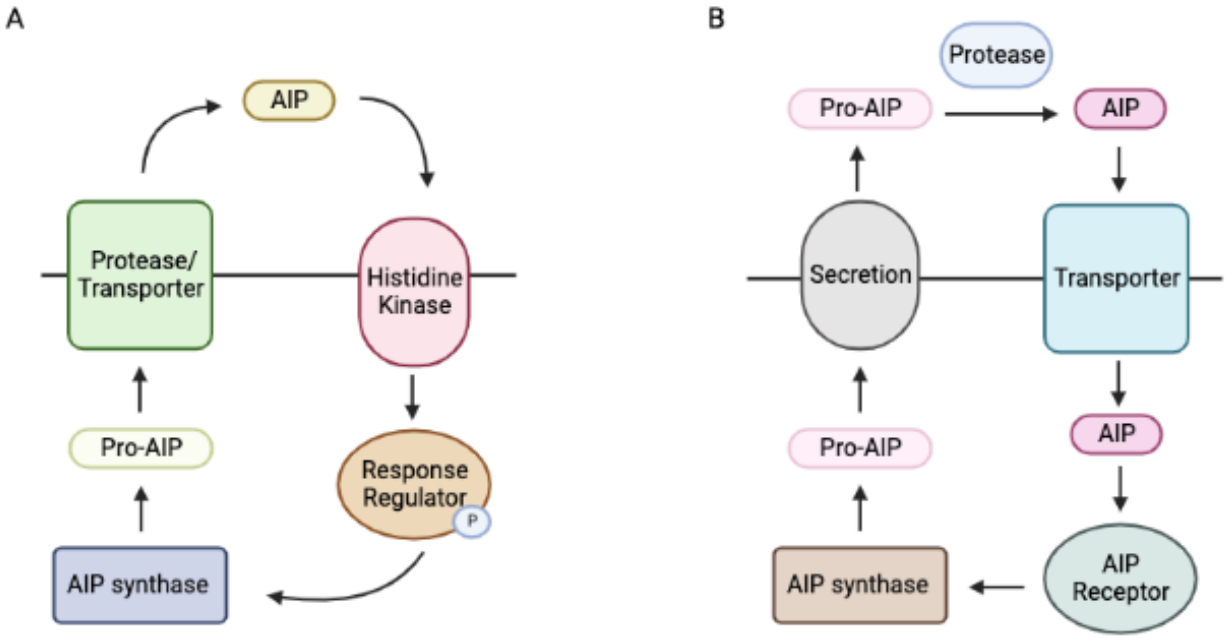

Figure 3. QS Circuits in Gram-positive Bacteria. (A) Here, an AIP synthase produces a pro-AIP peptide that is processed into mature AIP and secreted via dedicated proteins. Once in the extracellular space, mature AIP can act as a signal for a membrane bound TCS consisting of a histidine kinase and a response regulator. Upon activation, the response regulator promotes expression of the AIP synthase. (B) In this system, an AIP synthase produces a pro-AIP peptide that is secreted from the cell before maturation. Once outside the cell, the pro-AIP is processed into mature AIP by a dedicated protease. Mature AIP is then transported back into the cell, where it binds a receptor that acts as a transcription factor to regulate the expression of genes.

Streptococcus pneumoniae utilizes a similar QS system to regulate competence. Here, the QS system generates and responds to the competence stimulating peptide (CSP) and is comprised of genes in two operons, *comAB* and *comDEF,* where ComAB is an ABC transporter/protease that cleaves and exports ComC, the CSP precursor peptide (Figure 4 and (201,215)). Once outside the cell, CSP binds to the histidine kinase, ComD, causing ComD to autophosphorylate (216). This phosphoryl group is then transferred to the response regulator, ComE (216). Phosphorylated ComE binds to specific promoter regions upstream of *comAB, comDEF, comX*, and several other genes (Figure 4 and (217–219)). In *S. pneumoniae,* competence is induced in two

separate phases, called the early and late phases. The early phase is regulated by phosphorylated ComE, whereas ComX regulates the late phase (210,219). Several other genes whose expression is regulated by ComE have also been implicated in the development of the competent state, highlighting the importance for this QS system in competence (219).

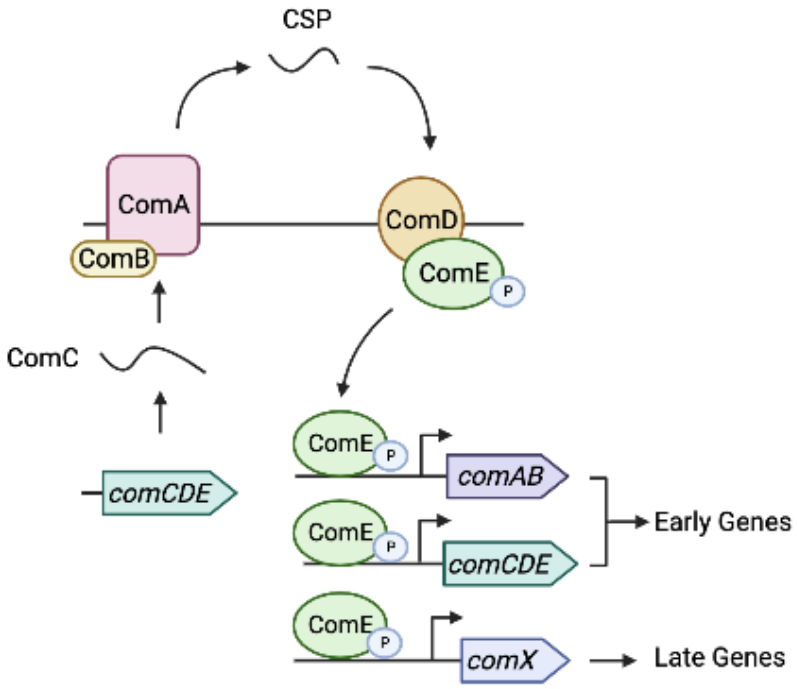

Figure 4. The ComABCDE QS System in *S. pneumoniae.* In *S. pneumoniae,* competence is regulated by a QS system encoded by the *comAB* and *comCDE* loci. Competence stimulating peptide (CSP) is matured from its precursor, ComC, and released from the cell following proteolysis and export by ComAB. CSP is recognized by a TCS consisting of the histidine kinase, ComD, and its response regulator, ComE. Once phosphorylated, ComE acts as a transcription factor to promote the expresson of *comAB, comCDE, comX,* which are important for early and late phase induction of competence, respectively, and several other genes also important for the development of competence (not pictured).

Other Gram-positive bacteria utilize a QS system in which pro-AIP is released from the cell, processed into mature AIP by extracellular proteases, then transported back into the cell, where the AIP can bind to and alter the activity of a transcription

factor (Figure 3B and (220)). A well-studied example of this type of QS system is the Rgg/short-hydrophobic peptide (SHP) system found in *Streptococcus pyrogenes* (Group A *Streptococcus*, GAS) which regulates biofilm formation and lysozyme resistance (Figure 5 and (221)).

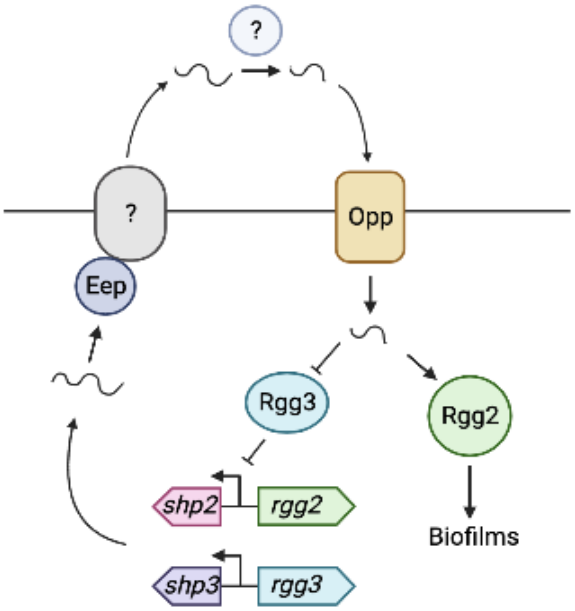

Figure 5. The Rgg/SHP Quorum Sensing System in GAS. Pro-SHP2 and -SHP3 undergo initial cleavage Eep before secretion from the cell by an unknown protein. Once outside the cell, the SHP intermediates are further processed by a dedicated protease. SHP2 and SHP3 are transported back into the cell by Opp where they bind Rgg2 and Rgg3, respectively. Rgg2 positively regulates the transcription of *shp2* and *shp3*, whereas Rgg3 negatively regulates their expression.

In general, this system consists of two different pro-SHPs (SHP2 and SHP3), which undergo cleavage by a trans-membrane peptidase and are subsequently exported from the cell, where a second cleavage event occurs, giving rise to mature SHP (Figure 5 and (221)). An oligopeptide permease then facilitates transport back into the cell, where SHPs act on Rgg2 and Rgg3 to activate transcription of *shp* promoters or inhibit repression of *shp* promoter transcription, respectively (Figure 5 and (221,222)).

An AIP import system is also used to regulate sporulation and competence in *Bacillus subtillis* and virulence factor production in the *B. cereus* group (205,220,223).

Concluding Remarks

In this section, I have provided an overview of the mechanisms of QS in Gram-negative and Gram-positive bacteria. The model QS system for most Gram-negative bacteria is the LuxI/LuxR system, which produces and responds to an AHL AI. In *V. fischeri,* where this system was first identified, response to AI gives rise to bioluminescence, an important factor for the bacteria's symbiotic relationship with the Hawaiian bobtail squid. Other Gram-negative bacteria, such as *V. cholerae*, utilize a form of cell-to-cell communication where two distinct but parallel QS systems produce and respond to CAI-1 and AI-2 Ais, which regulate expression of important virulence factors. Further, diverse AI-2 homologues exist in several bacteria and have been implicated in communication between bacteria in a niche.

Gram-positive bacteria rely on peptide-based QS systems, where a pro-AIP is made, matured and secreted. Whether the maturation occurs before or after release from the cell depends on bacterial species. Mature AIP can either act as a TCS signal or be transported back into the cell, where it binds to a transcription factor. In *S. pneumoniae,* competence is regulated by the ComABCDE QS system. Here, the precursor AIP is matured and released from the cell, where it acts upon a TCS to activate gene expression. In contrast, in the Rgg-SHP QS system in GAS, the pro-AIP is matured extracellularly, then mature AIP is transported back into the cell where it acts as a transcription factor to regulate the production of genes involved in biofilm

formation. In the next section, I will detail a major QS system in *S. aureus,* the Accessory Gene Regulator (Agr) system.

Section 3: The Accessory Gene Regulatory (Agr) System

The most well-studied quorum sensing system in Gram-positive bacteria is the *S. aureus* Agr system, which regulates the expression of several important virulence factors (Table 1). The Agr system is encoded by the *agr* locus, which is comprised of two divergent transcriptional units that are subject to regulation by AgrA: RNAII and RNAIII (Figure 6 and (224,225)).

RNAII: The *agr* Operon

The P2 promoter drives the expression of RNAII, which encodes the components of the Agr system. These include AgrC and AgrA, a histidine kinase-response regulator pair, the protease AgrB, and the peptide AgrD, which is post-translationally processed and exported as AIP. AIP serves as a signal for AgrC, causing a phosphorelay event that results in phosphorylation of AgrA (Figure 6 and (153,224,226)). Once phosphorylated, AgrA binds to the P2 promoter and facilitates the transcription of RNAII, thus causing positive feedback (153). *S. aureus* isolates harbor one of four Agr variants. These variants are stratified based on hypervariable regions within RNAII. Regions of variability include sequences encoding AgrD, parts of AgrB, and the sensor domain of AgrC (200,227–234). As such, each variant is able to produce and respond to its own AIP.

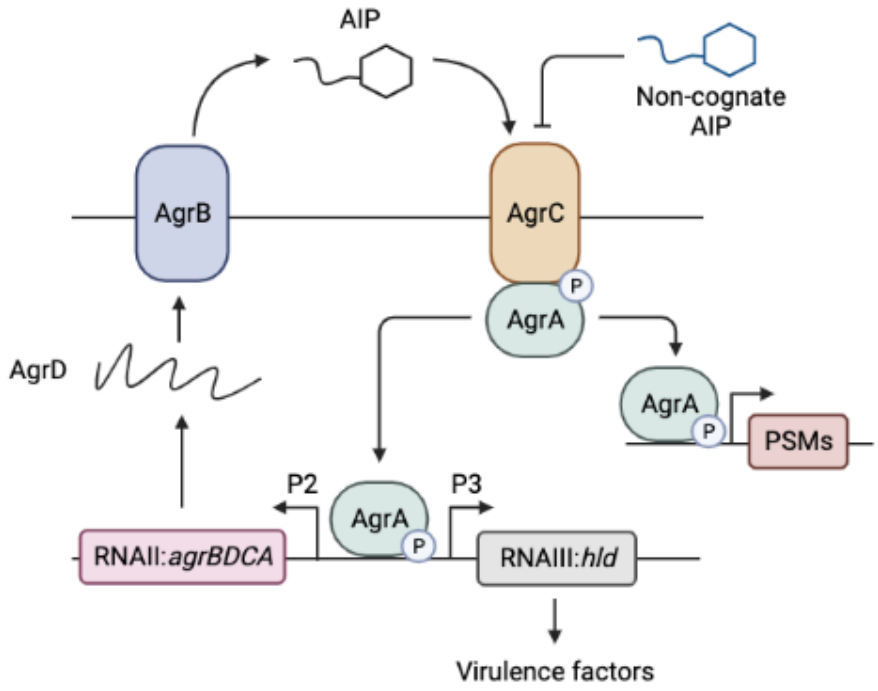

Figure 6. The Agr System in *S. aureus.* In this system, AIP is generated following a series of post-translational modifications to the pro-AIP, AgrD. The first step in this processing is C-terminal cleavage by AgrB. Mature AIP is released from the cell following subsequent modification of AgrD at the N-terminal end. Mature AIP can act as a signal for a TCS comprised of AgrC and AgrA, where AgrC is the histidine kinase and AgrA is the response regulator. Upon activation, AgrA promotes the expression of two divergent loci: RNAII and RNAIII. RNAII encodes all members of the *agr* locus, promoting a mechanism of positive feedback, as the recognition of AIP results in the production of additional AIP. RNAIII regulates virulence factors. AgrA can also directly promote the expression of PSMs. Non-cognate AIPs are able to bind to AgrC and prevent Agr system activation.

RNAII: AgrD and AIP

AgrD is the precursor peptide for AIP. It contains an N-terminal amphipathic α-helical leader, followed by AIP and a charged C-terminal tail (Figure 7). The N- and C-termini are critical for AIP maturation (235,236). The N-terminal α-helix localizes the precursor peptide to the membrane, where subsequent events in peptide maturation occur (235). This maturation begins with proteolytic activity at the C-terminal tail by AgrB (237), and site-directed mutagenesis studies suggest a requirement for two

conserved residues within the C-terminus of AgrD, glutamate (E34) and leucine (L41) in this cleavage event (238).

Mature AIP is made up of a cyclic thioester ring at its C-terminus and an N-terminal tail consisting of two to four exocyclic residues, depending on the *agr* allelic variant (Figure 7). AIP from *S. aureus* is able act as both an activator and an inhibitor, functioning as an agonist for its own AgrC, but as an antagonist for non-cognate AgrCs (204,229,239). Truncation analysis and site-directed mutagenesis studies highlight the requirement for key residues in the exocyclic tail and thiolactone ring for proper orientation of the peptide in the binding pocket of its group-specific AgrC (236,240). In some cases, substitution of as little as one amino acid is sufficient to switch the AIP from agonist to antagonist status (239). This suggests that proper maturation of AIP is essential for activation of the Agr system.

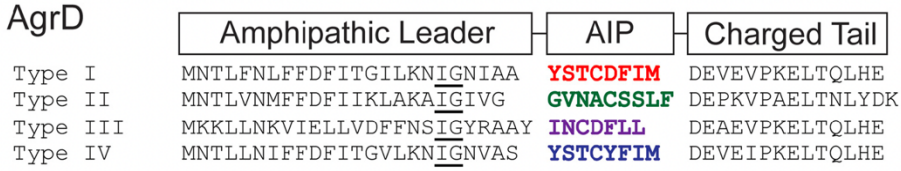

Figure 7. AgrD in *S. aureus* Allelic Variants. Comparison of the amino acid sequences of AgrD in LAC (Type I), SA502 (Type II), MW2 (Type III), and RN4850 (Type IV). Type I and Type IV AIPs differ by one amino acid. Underlined “IG”, conserved “helix breaker” region; colored regions correspond to AIPs I to IV.

RNAII: AgrB

Initial cleavage of AgrD at its C-terminal charged tail is carried out by AgrB (Figure 8 and (227,237,238,241,242)). Proteolytic cleavage occurs over several steps: 1) The sulfhydryl group of cystine residues in AgrB attack the amide carbonyl of the C-terminal amino acid of the AIP sequence, forming an acyl-enzyme thioester intermediate. 2) The sulfhydryl group of the conserved cystine residue in AgrD (C28)

catalyzes thio-ester exchange, resulting in the formation of a lactone ring (227,237,238,241,242). The proteolytic function of AgrB is derived from a catalytic dyad between a conserved histidine (H77) and cysteine (C84); these residues are required for AgrB-mediated cleavage of AgrD. The resulting intermediate, consisting of the leader peptide linked to AIP, then undergoes N-terminal proteolytic processing and export to give rise to mature AIP in the extracellular space (Figure 8 and (237,241,242)).

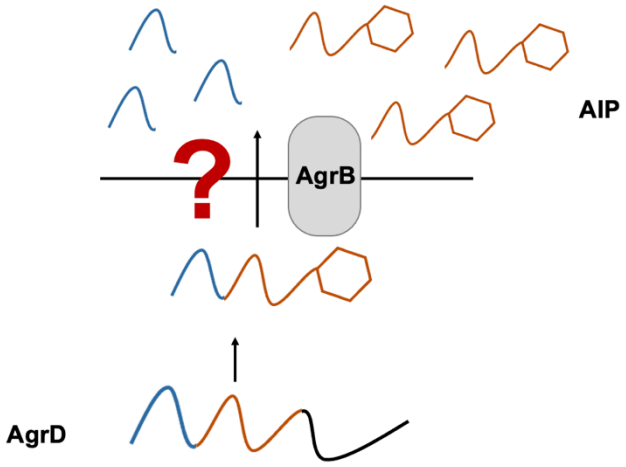

Figure 8. AgrD is Post-Translationally Modified to Become AIP. Modification of AgrD occurs over a series of steps. AgrB cleaves the C-terminus, causing formation of a thiolactone ring. The N-terminus is cleaved, and the mature AIP and N-terminal leader peptide intermediate are released into the supernatant. The mediators of cleavage at the N-terminus are not well understood.

The final steps in AIP maturation are yet to be fully understood. One study proposed a role for the canonical signal peptidase, SpsB, in removal of the N-terminal α-helix (243). This work defined a model of processing that requires translocation of the Leader-AIP intermediate to the outside of the cell before SpsB-mediated cleavage (243). Though cleavage of the AIP leader peptide would be in line with the general proteolytic function of SpsB, conclusions about the role of SpsB were drawn from

enzymatic studies that employed a synthetic, truncated AgrD peptide. Due to this experimental strategy, questions remained about the ability of this protein to cleave full length AgrD. More recent publications have demonstrated that SpsB is likely not the major protease involved in AIP maturation and suggest a role for additional proteases in this process (244–246). Further, whether AIP maturation is completed inside or outside the cell remains unclear.

RNAII: AgrC

Mature AIP binds to and signals through a receptor histidine kinase (RHK), AgrC (204,236,247,248). Like many other RHKs, AgrC contains an N-terminal sensor domain and a C-terminal histidine kinase (HK) domain, with all residues required for enzymatic activity present in the HK domain (249). At least two subdomains exist within the HK domain, with the dimerization and histidine phosphorylation (DHp) and catalytic and ATP binding (CA) domains being of high importance (250). The DHp subdomain includes a region that can fold into an α-helical hairpin, which is responsible for obligate dimerization via helix-bundle formation, and the histidine phosphoacceptor residue (250). The CA subdomain binds ATP and catalyzes autophosphorylation (250).

An elegant model has been described in which AgrC phosphatase activity is mediated by helical movements which position the DHp and CA subdomains in or out of proximity (236). Studies show that binding of cognate AIP causes helical movement that places the DHp and CA subdomains in a position conducive to autophosphorylation, whereas binding by non-cognate AIP shifts these subdomains out of proximity, thus preventing autophosphorylation and subsequent phosphoryl transfer to AgrA (236).

RNAII: AgrA

As the response regulator (RR), AgrA coordinates expression of genes in response to AIP recognition by binding cognate DNA sequences (251). Like many RRs, AgrA contains an N-terminal CheY-like domain, which contains the phosphoacceptor (252). This domain is connected by a linker to the C-terminal DNA binding domain (DBD) belonging to the LytTR domain family (252). This $AgrA_{DBD}$ binds to imperfect direct repeats of consensus DNA sequences located upstream of the -35 promoter region recognized by RNA polymerase (RNAp) to initiate transcription of RNAII and RNAIII (252,253).

Work has demonstrated that phosphorylated AgrA is essential for RNAp localization to P3, but not P2 (254). Further, this AgrA-mediated RNAp localization to P3 depends on dimerization that occurs following phosphorylation (253). The reasons why these promoters exhibit AgrA-dependent and AgrA-independent transcription initiation are not well understood; however, it has been suggested that AgrA-independent initiation of transcription from P2 allows for the production of low levels of AgrA and AgrC that are readily available for response to signals (153).

Additionally, low levels of AgrA may allow for expression of certain virulence factors independent of *agr* activation (129). For example, it has been shown that AgrA directly binds to the promoter regions of *psm*α and *psm*β, regulating their expression in an RNAIII-independent manner (Figure 6 and (129)). As such, it is possible that these virulence factors may be expressed even before quorum is sensed.

RNAIII

The P3 promoter controls the expression of RNAIII, which regulates the switch between virulence factor and surface protein expression (Figure 6 and (255)). RNAIII contains a short open reading frame that encodes δ-hemolysin, a virulence factor that targets and lyses red blood cells (248). The remaining non-coding sequence of RNAIII has been implicated in translational regulation (116,143,256,257). RNAIII is characterized by 14 hairpin structures and three long-distance interactions that bring the 5' and 3' ends close together (258). These hairpin motifs are thought to be a means by which RNAIII exerts regulatory RNA activity, as at least three contain C-rich regions within the apical loop that could facilitate binding to the Shine Dalgarno (SD) sequence of target mRNA (248,258).

Most well studied is the RNAIII-dependent control of *hla, spa* and their respective translational products. Here, RNAIII acts as an antisense RNA, binding mRNA to facilitate or prevent ribosomal binding and subsequent translation (116,259). In the absence of RNAIII, translation of *hla* mRNA is prevented by intramolecular base pairing that occludes the SD sequence. When RNAIII binds *hla* mRNA, a conformational change occurs that makes the SD sequence available for ribosomal binding (116). RNAIII prevents translation of *spa* mRNA by binding the ribosome binding site, causing rapid degradation of *spa* mRNA (259). RNAIII has also been shown to modulate translation of *rot* mRNA (108). Rot (repressor of toxins) has been implicated in the decreased production of hemolysins, proteases, and lipases, and the increased production of surface proteins (108,256). As such, RNAIII-regulated translation of *rot* mRNA has a significant impact on virulence factor production.

Amongst the proteases whose expression is repressed by Rot are aureolysin (*aur*) and staphylococcal serine protease (*sspA*) (248,260). Work has demonstrated that these proteases are required for biofilm detachment (145). Indeed, strains with dysfunctional *agr* typically exhibit robust biofilm formation (145,261,262). Other *agr*-regulated factors such as additional proteases, nucleases, and surfactants, such as δ-hemolysin, may also contribute to biofilm development. However, in-depth studies examining the contributions of these factors to biofilm formation are yet to be done (145,263).

Agr Allelic Variants

Within *S. aureus,* four *agr* specificity groups exist on account of hypervariable regions in *agrB, agrC,* and *agrD,* and each allelic variant exhibits specific AgrC-AIP interactions which result in AgrC phosphorylation (204,229,236,239,264). While AIP acts as an agonist to its cognate receptor, studies demonstrate that non-cognate AIPs antagonize AgrC activity by preventing autophosphorylation and phosphoryl transfer to AgrA, thereby inhibiting Agr system function (236,240). The exception to this phenomenon is Agr-I and Agr-IV, which differ by a single amino acid at position 5 and have been shown to cross-activate AgrC-IV and AgrC-I, respectively (Figure 7 and (229,230,236)).

AIP production and signaling kinetics vary among Agr allelic variants. At least one study has demonstrated differences in the timing and amount of signal generated from P3, suggesting a possible divergence in rates of AIP maturation and accumulation or AgrC signaling between *agr* variants. These differing magnitudes of response resulted in variant-specific expression of *agr*-regulated virulence factors (230). Between

the four *agr* allelic variants, *agr*-I and -IV are induced earliest and strongest in broth culture, followed by *agr*-II then *agr*-III (230,246). While the reasons for this delayed induction in *S. aureus* strains harboring an *agr*-III allelic variant are not well understood, it has been suggested it may be in part due to decreased production of AIP-III (230).

S. aureus **Agr as a Therapeutic Target**

S. aureus strains containing any of the four Agr variants can cause disease and at least one study has suggested associations with disease outcome: Type I variants are enriched in cases of bacteremia, Type II variants are overrepresented in infective endocarditis, and Type III variants are increased in menstrual toxic shock syndrome (228,229,264). Given the propensity of each *agr* variant to cause disease, several efforts have been made to generate therapeutics which target all variant Agr systems (265–271).

In particular, studies have tried to harness the cross-inhibitory features of allelic variant AIPs to generate an AIP derivative capable of broadly inhibiting AgrC activity across strains harboring any variant (265,266,270,271). In these studies, an AIP analogue, typically with a tail truncation or amino acid substitution, is generated for use as a competitive binder of AgrC (265,266,270,271). Binding by this analogue prevents native AIP from binding, thereby inhibiting Agr system activity. While these analogues are brilliant in principle, they have been less successful in practice, partially due to their large size, expense to produce, and relative instability. Recent efforts have tried to develop more stable compounds with similar inhibitory effects, though the use of these analogues has not reached a clinical level (270).

Agr Homologues in Other Gram-positive Bacteria

Agr homologues exist in several other Gram positive bacteria, including *Clostridiodes spp, Listeria monocytogenes, Enterococcus faecalis,* and *Staphylococcus epidermidis* (200,211–214).

Several QS systems in Gram-positive bacteria maintain a core homology to *S. aureus* Agr, but do not mimic all aspects of the staphylococcal system. Most *Clostridiodes spp.* contain two variant *agr* loci that produce AIP: *agr2*, encoding for *agrACDB,* and *agr1,* which encodes a partial *agr*-like locus containing *agrDB* (Figure 9 and (272,273)). Both loci have been shown to be important for pathogenesis and virulence factor production, as they control expression of genes associated with toxin production, sporulation and motility (213,274–277). However, while *agr1* is present in all sequenced *Clostridiodes spp., agr2* is present in only a few clinical isolates. The reasons for this divergence are unclear (272,273).

The *L. monocytogenes* genome contains a complete *agr* operon, where *agrC* and *agrA* encode a histidine kinase and its response regulator, respectively, *agrB* encodes a protease, and *agrD* encodes the propeptide (211,214,278). Here, mature AIP is a cyclic pentapeptide that contains the characteristic central cysteine found in most AIPs (Figure 9 and (278,279)). Of note, there is little structural diversity amongst AIPs from *Listeria* species, unlike the tremendous divergence seen in AIPs from the staphylococci (279). Studies demonstrate the importance of this Agr system in production of virulence factors and biofilm formation (211,214,278).

The QS system in *E. faecalis* is perhaps the most divergent from *S. aureus* Agr. The *fsr* operon encodes members of this system: FsrC and FsrA, a histidine kinase-

response regulator pair; FsrD, the propeptide, and FsrB, the protease involved in peptide maturation (198,280,281). FsrD is matured into an autoinducer called GBAP, which is cyclic in nature, but differs from the AIP of *S. aureus* in that it has a larger size and contains a lactone ring rather than a thiolactone ring (Figure 9 and (280)). While the relevance of a serine lactone ring versus cystine thiolactone ring has not been well defined, it is suggested that peptides with a lactone ring may be better protected from degradation (248). The TCS components of this system, FsrA and FsrC are highly homologous to AgrA and AgrC, and reports have suggested a similar requirement for these proteins in QS-mediated control of virulence factor expression (282–287).

The Agr system in *S. epidermidis* maintains the fundamental Agr system features seen in *S. aureus*: AgrB, AgrD, AgrC, and AgrA (196,288). Furthermore, studies have determined that the mechanisms by which *S. epidermidis* processes and responds to AIP are nearly identical to those reported in *S. aureus* (288–290). In *S. epidermidis,* three Agr allelic variants exist, and cross-inhibitory patterns have been described for at least two of these systems (Figure 9 and (233,291)). Upon activation, *S. epidermidis* Agr systems regulate the expression of several proteases with reported roles in biofilm formation, immune evasion, and polymicrobial interactions within the skin niche.

AIP from *S. epidermidis* has been shown to inhibit three of the four *S. aureus* Agr types (I, II and III), while only one *S. aureus* Agr type (IV) inhibits *S. epidermidis agr* activity (265). *S. epidermidis* is not unique in its ability to inhibit *S. aureus* Agr systems: Studies have demonstrated that AIPs from several staphylococcal species exhibit cross-talk with *S. aureus agr* (265,292,293).

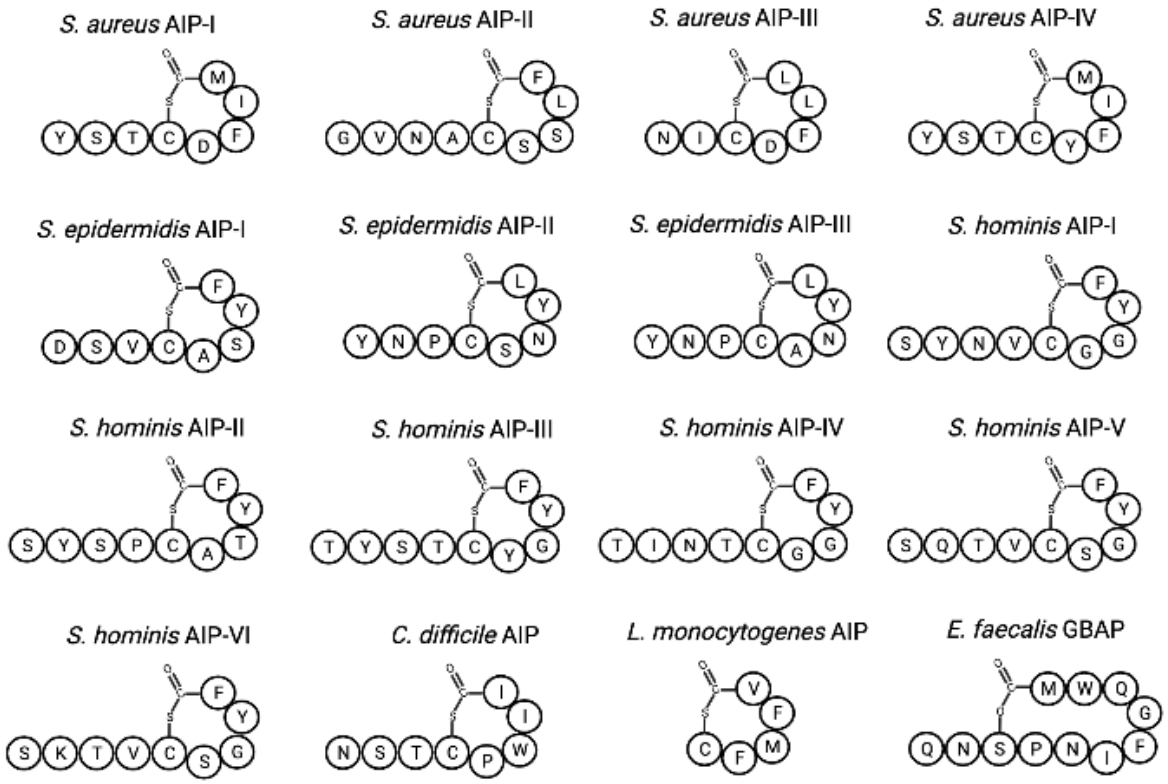

Figure 9. *S. aureus* AIP and Similar Gram-positive QS Peptides. Cyclic AIPs from *S. aureus, S. epidermidis, S. hominis, C. difficile, L. monocytogenes,* and *E. faecalis*. All peptides share a similar heterocyclic ring structure and serve as signal for Agr or Agr-like QS systems.

Recent work has described six *agr* allelic variants present in *S. hominis,* an important skin commensal, that are able to inhibit non-cognate Agr systems, including *S. aureus* Agr (Figure 9 and (293)). These data suggest a role for *S. hominis, S. epidermidis,* and staphylococcal cross-talk in protecting the skin from pathogenic species.

Concluding Remarks

In this section, I have described the Agr QS system in *S. aureus.* This system is a master regulator, promoting the expression of its own locus and the expression of RNAIII, which regulates virulence factor production. Clinical isolates of *S. aureus* harbor

one of four allelic variants of the Agr system, and each variant is associated with specific disease states. Agr-like systems exist in several other Gram-positive bacteria, including *S. epidermidis, S. hominis, C. difficile, L. monocytogenes,* and *E. faecalis.* While it is well understood that Agr QS systems contribute to bacterial pathogenesis, the mechanisms of AIP maturation are less defined. In the next section, I will introduce a class of proteins, called CAAX proteases that have been shown to facilitate peptide processing.

Section 4: CAAX Proteases

CAAX proteases facilitate post-translational modifications of proteins within the membrane of eukaryotic and prokaryotic cells. This family of proteins is stratified into two types based on the presence of a HexxH sequence motif (294–296). The major Type I CAAX protease, which contains the HexxH motif, is a metalloprotease called a-factor converting enzyme (AFC1, or Ste24p in yeast) that facilitates membrane localization of proteins by covalently attaching lipid molecules in a process called prenylation (294). Here, a prenyl group is attached to the C-terminal cysteine of the CAAX motif. The “AAX” tripeptide of the prenylated CAAX is then liberated by endoproteolysis, where A is typically an aliphatic amino acid, and X can be one of several amino acids that defines specificity for the protease (297). Additionally, studies demonstrated that AFC1 modifies C- and N-terminal ends of a-factor, suggesting an additional role for CAAX proteases in maturation of pheromone peptides (295),.

Type II CAAX proteases have been more broadly studied (298,299). In bacteria, roles for Type II CAAX proteases in bacteriocin maturation and peptide hydrolysis have been demonstrated (300–302). These functions depend on glutamate and histidine

residues, which belong in highly conserved motifs characteristic to the Type II CAAX proteases: motif 1, EEXXXR; motif 2, FXXXH; and motif 3, an invariant histidine (Figure 9 and (303)). Proteins that contain the highly conserved Type II CAAX motifs are also called Abi (abortive infectivity) or CPBP (CAAX proteases and bacteriocin-processing enzymes) family proteins.

```
SpdA      FTVTIIPAIVEEIVFRG-----FASLHESDTW
SpdB      LLIVIVGPIVEEIVFRH-----FAFIHVTDAK
SpdC      LIGHILMAFVVEFGFRS-----FLFSEEIGDL
MroQ      FFVSIIGPLLEEYVFRK-----FALAHNDFKF
```

Figure 10. Known Type II CAAX Proteases in *S. aureus.* Amino acid sequence alignments of the EEXXXH and FXXXH Type II CAAX protease motifs in SpdA, SpdB, SpdC, and MroQ.

S. aureus Type II CAAX Proteases: SpdA, SpdB, and SpdC

In *S. aureus,* at least four Type II CAAX proteases have been identified (Figure 10 and (244,245,304)). Of those, SpdA, SpdB, and SpdC (surface protein display) have been shown to be important for production of secreted proteins and cell wall homeostasis (304). Specifically, work demonstrates a role for SpdA, SpdB, and SpdC in the trafficking of surface proteins with YSIRK/G-S signal peptides, such as protein A; however, how exactly these proteins contribute to surface display remains unknown (304). Amongst the Spd proteins, SpdC has been extensively studied (305–307).

S. aureus strains lacking *spdC* have increased resistance to lysostaphin (306). As such, several reports have annotated this gene and its protein *lyrA* and LyrA (lysostaphin resistance A), respectively. This increased resistance is suggested to be due to a role for SpdC in the peptidoglycan synthesis process (307). In general, peptidoglycan consists of repeating units of disaccharide, pentapeptide stem, and a

bridge structure (308). These units are polymerized into glycan chains that are incorporated into existing peptidoglycan (309,310). In *S. aureus,* glycan chain length is regulated by the glucosaminidase, SagB (311,312). SpdC has been shown to be required for optimal SagB activity, possibly by presenting nascent glycan chains to SagB for cleavage (307). Of note, this role for SpdC does not seem to be due to its CAAX protease annotation, as none of the residues within the CAAX motif are required for SpdC to promote SagB activity (307).

Other studies into SpdC revealed a role in activation of the WalKR TCS, where SpdC negatively controls the expression of WalKR-regulated genes via an interaction with the histidine kinase, WalK (305). In addition to its ability to regulate WalKR function, SpdC was also shown to interact with 10 of the 16 known *S. aureus* histidine kinases, including SaeS, SrrB, and PhoR (305). A similar Type II CAAX protease-mediated regulation of bacterial TCS signaling has been demonstrated with the Group B *Streptococcus* protease, Abx1 (313). Here, Abx1 acts as a positive regulator of the CovSR TCS through an interaction with the histidine kinase CovS (305). Unlike its Spd counterparts, the fourth Type II CAAX protease encoded by *S. aureus,* MroQ, does not appear to be important for cell wall homeostasis (244,245,304).

S. aureus **Type II CAAX Proteases: MroQ**

Recent studies have identified MroQ (membrane regulator of quorum sensing) as an important mediator of *S. aureus* pathogenesis in strains harboring a Type I Agr variant (244–246). A Δ*mroQ* mutant phenocopies a Δ*agr* mutant for reduced levels of secreted proteins, decreased toxin production, and attenuated skin and soft tissue infection (244,245). These shared phenotypes suggest a link between MroQ function

and Agr system activation (244,245). This idea is further supported by global transcriptome profiling, which revealed that expression of all Agr system genes, including those for RNAII and RNAIII, were downregulated in a Δ*mroQ* mutant (245). The substantial contribution of MroQ to the transcription of Agr system genes suggests a role in controlling Agr system function, perhaps through direct interaction with Agr proteins.

Loss of MroQ does not impact AgrC-AgrA signaling capacity, as Δ*mroQ* mutants still exhibit gene expression from the P3 promoter upon activation of AgrC with native AIP (244). In contrast, separation of *agrBD* from its regulatory circuit revealed defective AIP production in the absence of MroQ. Further, accumulation of full-length C-terminal 6x-His-tagged AgrD was observed in the cell lysate of a Δ*mroQ* mutant, suggesting compromised AIP maturation in the absence of MroQ (244). Indeed, recent work from Zhao et. al. found that AgrD maturation required only AgrB, MroQ, and AgrD, supporting the model that MroQ functions as a protease that cleaves AgrD (246). In accordance with its annotation as a Type II CAAX protease, loss of Agr system function upon mutation of predicted active site residues E141, E142, and H180 to alanine was observed, suggesting a requirement for catalytic activity in MroQ-mediated AIP maturation (244). Despite these observations which suggest a role for MroQ in AIP maturation, the exact ways in which MroQ interfaces with the Agr system are less well defined. Further, the role for MroQ in *S. aureus* strains harboring a Type II, III or IV *agr* allelic variant has not been fully explored.

Summary

S. aureus is an important human pathogen that can cause severe infection on account of several virulence factors. The expression of many of these important virulence factors is regulated by peptide-based QS, which allows for the coordination of pathogenic response with high cell density. Of these QS systems in *S. aureus,* the Agr system is widely recognized for its role in regulating the expression of myriad important immune evasion factors that facilitate disease, such as Spa, Hla, and the Luk proteins. Though there is an established connection between Agr peptide signaling and *S. aureus* pathogenesis, gaps remain in our understanding of the exact mechanisms behind AIP synthesis. Notably, AgrD processing among all Agr allelic variants has not been investigated, the proteins required for the final maturation steps of AgrD are not known, and the sequence determinants of N-terminal AgrD processing remain unclear.

Thus, this thesis first sought to investigate the role of MroQ in the maturation and export of AIP. Through immunoblot analysis, I determined that MroQ promotes the processing and release of AIP-I. This contribution to AIP-I maturation is dependent on conserved residues within the CAAX motif. Second, given the low sequence similarity among allelic variants of *S. aureus* AgrD, I examined the relevance of MroQ in AIP maturation, virulence factor production, and pathogenesis across all Agr types. I found that MroQ facilitates at least one step of AgrD maturation in all variants. MroQ-dependent AgrD maturation was important for the generation of active AIP in Agr-I, -II and -IV strains, however it was not required for activation of the Agr-III system, suggesting that MroQ dependent cleavage of AgrD-III is not required for activation of the Agr system. Similarly, AIP-mediated intraspecies inhibition was MroQ-dependent in

strains harboring Agr-I, -II, and -IV, but not -III. In a skin and soft tissue infection model, MroQ was found to be important for the pathogenesis of Agr-I and Agr-IV, but not Agr-II or Agr-III strains, suggesting complex regulation of Agr-mediated virulence in vivo. Finally, I initiated experiments exploring the function of MroQ homologues in other bacterial species with Agr systems, *L. monocytogenes* and *S. epidermidis.*

In summary, this thesis reinforces the prevailing model that MroQ mediates AIP processing and release in *S. aureus.* Further, this work demonstrates a conservation of function on a range of peptide precursors, a remarkable phenomenon considering the divergent peptide sequence, and suggests a possible role for MroQ-like proteins in other bacterial Agr systems.

CHAPTER TWO

MATERIALS AND EXPERIMENTAL METHODS

Bacterial Strains and Culture Conditions

All bacterial strains used in this work are described in Table 2. *S. aureus* LAC (AH-1263), SA502A, MW2, and RN3984 were used as WT strains for experiments in this study. LAC (AH-1263) is an *S. aureus* USA300 clinical isolate that harbors a Type I Agr system and is cured of its resistance plasmid (314). SA502A is a clinical isolate that harbors a Type II Agr system (315). MW2 is a clinical community-acquired methicillin resistant isolate (316). RN3984 is a natural occurring toxic shock syndrome strain (317). RN4850 is a Type IV Agr strain from an individual with scalded skin syndrome (318). Most recombinant plasmids were maintained in *Escherichia coli* DH5α or BH10C before transformation into *S. aureus* strains RN4220 and RN9011 (RN4220/pRN7023) and subsequent electroporation or transduction into AH-1263, SA502A, MW2, RN3984 or their respective isogenic mutant derivatives. All *E. coli* strains were grown in lysogeny broth (LB; Amresco). *S. aureus* and *S. epidermidis* strains were grown in either tryptic soy broth (TSB; Amresco) or Roswell Park Memorial Institute medium (RPMI; Corning) supplemented with 1% casamino acids (Amresco) and 2.4 mM sodium bicarbonate (Amresco). When required, media were supplemented with the following antibiotics: chloramphenicol (Cm) (Amresco), 10 μg/mL; ampicillin (Amp) (GoldBio) 100 μg/mL; erythromycin (Erm) Amresco) 5 μg/mL; tetracycline (Tet) (Amresco) 2 μg/mL; and anhydrous tetracycline

(AnTet) (Acros Organics) 1 μg/ml. All *L. monocytogenes* strains were grown in brain heart infusion medium (BHI, Amresco) and supplemented with CM (5 μg/mL) when required. To select for pJC1111 transductants, cadmium chloride (Alfa Aesar) was used at 0.1-0.3 mM. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) using a Genesys 10S UV-visible spectrophotometer.

Genetic Techniques

For isolation of genomic DNA from *S. aureus*, bacterial strains were grown overnight in 5 mL TSB at 37˚C, 220 rpm. The next day, 1.5 mL of culture was centrifuged at 8,000 rpm for 10 min and the resulting pellet was resuspended in TSM buffer (50 mM Tris pH 7.5, 0.5 M sucrose, 10 mM $MgCl_2$) followed by incubation with lysostaphin (2 mg/mL in 0.5 M Tris pH 8.0) for 15 min at 37˚C to allow for digestion of the cell wall. Cellular digests were centrifuged at 14,000 rpm for 2 min and supernatants were discarded. Genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega) or Dneasy blood and tissue kit (Qiagen). PCR was performed using Q5 DNA polymerase, GoTaq DNA polymerase, or DreamTaq DNA polymerase (Thermo) and deoxynucleoside triphosphates (Quanta BioSciences). All PCRs were performed in a FlexID Mastercycler (Eppendorf) according to the manufacturer's suggested protocols. Oligonucleotides were purchased from Eurofins or Integrated DNA Technologies (IDT) and are listed in Table 3. Electrophoresis of DNA samples was carried out in 0.8% or 2% agarose (Amresco) gels.

Table 2. List of Strains Used in this Study.

Strain	Description	Designation	Source or reference
AH-1263	*S. aureus* USA300 CA-MRSA strain LAC, Type I Agr	LAC	(314)
SA502A	*S. aureus* clinical isolate SA502A, Type II Agr	SA502A	(315)
MW2	*S. aureus* CA-MRSA strain, Type III Agr	MW2	(316)
RN3984	*S. aureus* clinical isolate, Type III Agr	RN3984	(317)
RN4850	*S. aureus* clinical isolate, Type IV Agr	RN4850	(318)
BH10C	*E. coli* strain that restricts plasmid copy number for cloning of *mroQ*	BH10C	(319)
DH5α	*E. coli* strain used for cloning	DH5α	
RN4220	Restriction negative *S. aureus*	RN4220	(320)
RN9011	RN4220 with pRN7023 expressing the SaPI-I integrase	RN9011	(321)
FA-S922	LAC with in-frame deletion of *mroQ*	Δ*mroQ*	(244)
FA-S982	FA-S922 with integrated pJC1112-*mroQ* for complementation	Δ*mroQ* + *mroQ*	(244)
FA-S1008	LAC with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet*	(244)
FA-S995	FA-S922 with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet* Δ*mroQ*	(244)
FA-S2733	SA502A with in-frame deletion of *mroQ*	Δ*mroQ*	This work
FA-S2764	FA-S2733 with pOS1-FLAG-GG-*mroQ* for complementation	Δ*mroQ* + *mroQ*	This work
FA-S2766	SA502A with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet*	This work
FA-S2741	FA-S2733 with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet* Δ*mroQ*	This work
FA-S2441	MW2 with in-frame deletion of *mroQ*	Δ*mroQ*	This work
FA-S2454	FA-S2441 with integrated pJC1112-*mroQ* for complementation	Δ*mroQ* + *mroQ*	This work

FA-S2735	MW2 with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet*	This work
FA-S3121	MW2 with in-frame deletion of *agrD*	Δ*agrD*	This work
FA-S2738	FA-S2441 with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet* Δ*mroQ*	This work
FA-S2730	RN3984 with in-frame deletion of *mroQ*	Δ*mroQ*	This work
FA-S2734	RN3984 with gene replacement of *agrBDCA* with tetracycline resistance cassette	Δ*agr*::*tet*	This work
FA-S949	LAC with *P3*-GFP reporter plasmid pDB59	LAC + pDB59	(322)
FA-S1972	AH-1263 with an in-frame deletion of *agrB* and *P3*-GFP reporter plasmid pDB59	Δ*agrB* + pDB59	(244)
FA-S1881	SA502A with *P3*GFP reporter plasmid pDB59	SA502A + pDB59	(322)
FA-S2347	MW2 with *P3*GFP reporter plasmid pDB59	MW2 + pDB59	(322)
FA-S3123	FA-S3121 with *P3*GFP reporter plasmid pDB59	Δ*agrD* + pDB59	This work
FA-S2767	RN4850 with *P3*-GFP reporter plasmid pDB59	RN4850 + pDB59	(322)
FA-S2005	AH-1263 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-I*	LAC pOS1-P_{sarA}-sod_{RBS}- *6xHis-GG-agrD-I*	This work
FA-S2010	FA-S922 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-I*	Δ*mroQ* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-I*	This work
FA-S2008	FA-S982 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-I*	Δ*mroQ* + *mroQ* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-I*	This work
FA-S2422	SA502A containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-II*	SA502A pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-II*	This work
FA-S2746	FA-S2733 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-II*	Δ*mroQ* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-II*	This work
FA-S2383	MW2 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG- agrD-III*	MW2 pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-III*	This work
FA-S2452	FA-S2441 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-III*	Δ*mroQ* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-III*	This work
FA-S2762	FA-S2454 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-III*	Δ*mroQ* + *mroQ* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-III*	This work

FA-S2028	FA-S1008 with integrated pJC1111-*agr-I*	Δ*agr*::*tet* + *agr-I*	This work
FA-S2030	FA-S995 with integrated pJC1111-*agr-I*	Δ*agr*::*tet* Δ*mroQ* + *agr-I*	This work
FA-S2032	FA-S1008 with integrated pJC1111-*agr-II*	Δ*agr*::*tet* + *agr-II*	This work
FA-S2034	FA-S995 with integrated pJC1111-*agr-II*	Δ*agr*::*tet* Δ*mroQ* + *agr-II*	This work
FA-S2036	FA-S1008 with integrated pJC1111-*agr-III*	Δ*agr*::*tet* + *agr-III*	This work
FA-S2038	FA-S995 with integrated pJC1111-*agr-III*	Δ*agr*::*tet* Δ*mroQ* + *agr-III*	This work
FA-S2040	FA-S1008 with integrated pJC1111-*agr-IV*	Δ*agr*::*tet* + *ag-rIV*	This work
FA-S2042	FA-S995 with integrated pJC1111-*agr-IV*	Δ*agr*::*tet* Δ*mroQ* + *agr-IV*	This work
FA-S2728	FA-S2735 with integrated pJC1111-*agr-I*	MW2 Δ*agr*::*tet* + *agr-I*	This work
FA-S2750	FA-S2738 with integrated pJC1111-*agr-I*	MW2 Δ*agr*::*tet* Δ*mroQ* + *agr-I*	This work
FA-S2530	LAC with an in-frame deletion of *agrD*	Δ*agrD*	This work
FA-S2564	FA-S922 with an in-frame deletion of *agrD*	Δ*mroQ* Δ*agrD*	This work
FA-S2718	FA-S982 with an in-frame deletion of *agrD*	Δ*mroQ* Δ*agrD* + *mroQ*	This work
FA-S2526	FA-S2530 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-I*	Δ*agrD* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-I*	This work
FA-S2577	FA-S2564 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-I*	Δ*mroQ* Δ*agrD* pOSI-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-I*	This work
FA-S2772	FA-S2718 containing pOSI-P_{sarA}-sod_{RBS}-*6xHis- GG-agrD-I*	Δ*mroQ* Δ*agrD* + *mroQ* pOSI-P_{sarA}-sod_{RBS}-*6xHis-GG-agrD-I*	This work
FA-S2525	FA-S2530 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-Leader-I*	Δ*agrD* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-Leader-I*	This work
FA-S2583	FA-S2564 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis- GG-Leader-AIP-I*	Δ*mroQ* Δ*agrD* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-Leader-AIP-I*	This work
FA-S3125	FA-S2735 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG- Leader-AIP-III-SSP*	Δ*agr* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-Leader-AIP-III-SSP*	This work
FA-S3127	FA-S2738 containing pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG- Leader-AIP-III-SSP*	Δ*mroQ* Δ*agr* pOS1-P_{sarA}-sod_{RBS}-*6xHis-GG-Leader-AIP-III-SSP*	This work

The restriction endonucleases KpnI, SacI, PstI, or EcoRI (New England BioLabs) were used to perform DNA digestions. All reactions were performed according to the manufacturer's suggested protocol and all digested plasmids were further treated with shrimp alkaline phosphatase (Amresco). Ligations were performed with T4 DNA Ligase (New England Biolabs) and were incubated overnight at 16˚C in a ThermoMixer (Eppendorf). DNA gel extraction and PCR purification were performed using Qiagen QIAquick kits. Plasmids were isolated from *E. coli* using a Qiagen miniprep kit. For plasmid isolation from *S. aureus*, strains were grown overnight in 5 ml cultures at 37˚C with shaking at 220 rpm. Bacterial cells were centrifuged at 3,900 rpm for 5 min and the resulting pellet was resuspended in TSM + lysostaphin (2 mg/mL) and incubated for at least 10 min at 37˚C. Following treatment, bacterial cells were centrifuged at 13,000 rpm for 2 min and the remaining miniprep was carried out using a Qiagen miniprep kit, with a five-minute incubation following addition of P1 and P2. Plasmid concentrations were measured using a NanoDrop (Thermo).

Generation of In-frame Deletion Mutants

The temperature-sensitive plasmid pIMAY (323) was used to generate Δ*mroQ,* Δ*agrD* and Δ*SERP_RSO7500* in-frame deletion mutants. To amplify two fragments corresponding to ~500 bp sequence homology immediately upstream or downstream of *mroQ,* oligonucleotides MroQ-1, MroQ-2, MroQ-3, and MroQ-4 were used (Table 3). MroQ-1 and MroQ-2 were designed to amplify the region upstream of *mroQ*, while MroQ-3 and MroQ-4 were designed to amplify the region immediately downstream. To join the fragments, splicing by overlap extension (SOEing) PCR was performed

using the amplicons from the above-mentioned PCR reactions as template along with primers MroQ-1 and MroQ-4.

Table 3. List of Oligonucleotides Used in this Study.

Name	Sequence
MroQ-1	CCC-GGTACC(KpnI)-CCATAAATGATAAACCTCCAT
MroQ-2	GTGTGATTCGTTTTTTTTATTA-GGCGCC(KasI)-CATAATTTTCCTCCAAATATT
MroQ-3	AATATTTGGAGGAAAATTATG-GGCGCC(KasI)-TAATAAAAAAAACGAATCACAC
MroQ-4	CCC-GAGCTC(SacI)-ATTTTTAGCCTTGGCAAATG
MroQFwd	ATGACAAGATTATGGGCATCAT
MroQRev	TTATGGAATAAAAATGTGATAT
pOS1UniSOE1	CCC-CTGCAG(PstI)-CTGATATTTTTGACTAAACCAA
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrD*- SOE2	ACCACCGTGATGGTGATGGTGATGGCTGCTGCCCAT-AAATAATCATCCTCCTAAGGT
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDI*- SOE3	ATGCATCACCATCACCATC-CCTTAGGAGGATGATTATTT
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDI*- SOE4	ATAT-GAATTC(EcoRI)-TTATTCGTGTAATTGTGTTAAT
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDII*- SOE3	ATGGGCAGCAGCCATCACCATCACCATCACGGTGGT-AATACACTTGTTAATATGTTTTTT
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDII*- SOE4	CCC-GAATTC(EcoRI)-CTATTTGTCGTATAAATTCGTT
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDIII*- SOE3	CATCACCATCACCATCACGGTGGT-AAAAAATTACTCAACAAAG
P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDIII*- SO4	CCC-CCATGG(NcoI)-TTATTCGTGTAATTGAGTTAATT
AgrD-1	AAA-GGTACC(KpnI)-TCCATTTTACTAAGTCACCG
AgrD-2	CTCTCTATTTAAATTATTCGTGATTCATTTTAAGTCCTCCTTA
AgrD-3	TAAGGAGGACTTAAAATGAATCACGAATAATTTAAATAGAGAG
AgrD-4	AAA-GAGCTC(SacI)-TCGGGTATTTCGATACTAAT
AgrDMW2 UP F	AAA-GGTACC(KpnI)-AATTTGTTCACTGTGTCGAT
AgrDMW2 UP R	TAGTTATTCGTGTAATTGAGTCATAATTTAGTCCTCCTTTGA
AgrDMW2 DW F	TCAAAGGAGGACTAAATTATGACTCAATTACACGAATAACTA
AgrDMW2 DW R	AAA-GAGCTC(PstI)-GACCTTTAATCTCACGTACT
T1LeaderREV	AAA-GAATTC(EcoRI)-TTAAGCTGCGATGTTACCAATGT
T1Leader-AIPREV	AAA-GAATTC(EcoRI)-TTACATTATGAAGTCACAAGT
T3Leader-AIPREV	TACAAGCTAGCTTGG-CTGCAG(PstI)-CAATAAAAAATCACAATTTATATA

T3Leader-AIPSSP	TGTACCAAATGATAAACCA-CAATAAAAAATCACAATTTATATA
SSPFWDT3Leader-AIP	TGTGATTTTTTATTG-TGTTTATCATTTGGTACA
SSP3	AAAGAATTCTTAATTAAATGCTGCTGC
FLAG-GG-MroQSOE1	CCC-CTGCAG(PstI)-CTGATATTTTTGACTAAACCAA
FLAG-GG-MroQSOE2	ACCACCCTTGTCGTCATCGTCTTTGTAGTCGCTGCTGCCCAT-AAATAATCATCCTCCTAAGGT
FLAG-GG-MroQSOE3	ACAAGATTATGGGCATCATT-ATGGGCAGCAGCGACTACAAAGACGATGACGACAAGGGTGGT
FLAG-GG-MroQSOE4	CCC-GAATTC(EcoRI)-TTATGGAATAAAAATGTGATATA
SepiMroQSOE1	CCC-GGTACC(KpnI)-CTTATTAAATCATTTAATTAATATTT
SepiMroQSOE2	TACATGATGAGATGCTTTTATT-AATTTCCCTCCATCTATATAT
SepiMroQSOE3	ATATATAGATGGAGGGAAATTAATAAAAGCATCTCATCATGTA
SepiMroQSOE4	CCC-GAGCTC(SacI)-ATAGAATGAATAGTAGCGATAA
LmonoMroQSOE1	CCC-GGTACC(KpnI)-ATTTTATATTTCCCTCCGATTA
LmonoMroQSOE2	TGCTTCATTAATGAATTTTTCAT-AAAATACGCATCCTTTCTATAA
LmonoMroQSOE3	TTATAGAAAGGATGCGTATTTTATGAAAAATTCATTAATGAAGCA
LmonoMroQSOE4	CCC-CTGCAG(PstI)-AATAATAATATCAAAATCGTTGCT
LmoMroQSTOPSOE1	CCC-GGTACC(KpnI)-TTACAACACGATCTCCTAAT
LmoMroQSTOPSOE2	TATTTTCTTTCGGTTTTCTTTA-AAGCATTGGAATAATAATCG
LmoMroQSTOPSOE3	CGATTATTATTCCAATGCTT-TAAAGAAAACCGAAAGAAAATA
LmoMroQSTOPSOE4	CCC-CTGCAG(PstI)-TTATCCCCCAATAATTCCTA

The amplicon was subcloned into the multicloning site of pIMAY after digestion with Kpn1 and Sac1 restriction endonucleases. Since *mroQ* and the regions immediately upstream and downstream of the gene share sequence homology amongst Agr Types I-III, the same plasmid was used to generate in-frame deletions in wild type strains harboring these allelic variant Agr systems. To generate Δ*agrD* mutants, primers AgrD-1, AgrD-2, AgrD-3, and AgrD-4 (for Δ*agrD* from LAC) or AgrDMW2 UP F, AgrDMW2 UP R, AgrDMW2 DW F and AgrDMW2 DW R (for Δ*agrD* from MW2) were used to amplify two fragments corresponding to ~500 bp sequence homology immediately upstream or downstream of *agrD.* The resulting amplicons were used

as template for SOEing PCR, which was performed using the primers AgrD-1 and AgrD-4 or AgrDMW UP F and AgrDMW DW R.The resulting amplicon was subcloned into pIMAY as described above. To generate Δ*SERP_RSO7500,* two fragments corresponding to ~500bp homology immediately upstream or downstream of *SERP_RSO7500* were amplified using primers SepiMroQSOE1, SepiMroQSOE2, SepiMroQSOE3, and SepiMroQSOE4. These amplicons were used as templates in a SOEing PCR using primers SepiMroQSOE1 and SepiMroQSOE4. The amplicon was subcloned into pIMAY as described. In each case, mutagenesis was performed according to previously published protocols and mutations were confirmed by PCR and Sanger sequencing (244,324). The temperature-sensitive plasmid pKSV7 was used to generate a Δ*lmo2070* in-frame deletion mutant or *lmo2070*^{STOP} mutant (325). For the Δ*lmo2070* in-frame deletion, two fragments corresponding to ~500 bp sequence homology immediately upstream or downstream of *lmo2070* were amplified using oligonucleotides LmonoMroQSOE1, LmonoMroQSOE2, LmonoMroQSOE3, and LmonoMroQSOE4 were used. LmonoMroQSOE1 and LmonoMroQSOE2 were designed to amplify the region upstream of *lmo2070*, while LmonoMroQSOE3 and LmonoMroQSOE4 were designed to amplify the region immediately downstream. To join the fragments, splicing by overlap extension (SOEing) PCR was performed using the amplicons from the above-mentioned PCR reactions as template along with primers LmonoMroQSOE1 and LmonoMroQSOE4. The amplicon was subcloned into the multicloning site of pKSV7 after digestion with KpnI and PstI restriction endonucleases. To generate a *lmo2070*^{STOP} mutant, a stop codon was inserted 195 nucleotides into the gene using primers LmoMroQSTOPSOE1, LmoMroQSTOPSOE2,

LmoMroQSTOPSOE3, and LmoMroQSTOPSOE4. Resulting amplicons were joined by SOEing PCR using LmoMroQSTOPSOE1 and LmoMroQSTOPSOE4. Subcloning into pKSV7 was performed as described above.

Bacteriophage-mediated Generalized Transduction

Transduction was used to transfer stably integrated complementation plasmids between strains and to mobilize marked mutations within the *S. aureus* chromosome. In this study, *S. aureus* specific bacteriophages ϕ11, 80α, and ϕ85 were used. Donor strains were grown overnight in TSB:LB (1:1) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$, diluted 1:100 in TSB:LB, and grown for 2.5-3 hours at 37˚C, 220 rpm until the OD_{600} reached approximately 0.3 to 0.9. To package donor DNA, 100 μL of serially diluted bacteriophage stock in TMG buffer (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.1% [vol/vol] gelatin) was incubated with 500 μL bacterial culture in microcentrifuge tubes for 30 minutes at room temperature. Melted and cooled CY Top Agar (3 g/L casamino acids, 3 g/L yeast extract, 6 g/L NaCl, 7.5 g/L agar) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$ was added to the bacteria-phage mixture and immediately poured onto prewarmed tryptic soy agar (TSA) plates. Plates were incubated at 30˚C overnight. The following day, phages were harvested from two to four plates with confluent plaques. Phage stocks were stored at 4˚C. To transduce plasmids and marked mutations, recipient strains grown overnight in 20 mL TSB:LB (1:1) supplemented with 5 mM $CaCl_2$ were centrifuged at 3,900 rpm for 15 min and the resulting pellet was resuspended in 3 mL TSB:LB + 5 mM $CaCl_2$. Recipient bacteria were diluted 1:1, 1:10, and 1:100 in fresh TSB:LB + 5 mM $CaCl_2$ in a final volume of 500 μl. 100 μl phage stock was added to each bacterial dilution and

incubated at room temperature for 30 min, inverting the tubes to mix every 10 min. After 30 min, each mixture was supplemented with sodium citrate to a final volume of 40 mM and incubated at room temperature for another 30 min, inverting every 10 min. Bacteria-phage mixtures were centrifuged at 14,000 rpm for 5 min and the resulting pellet was washed two times in TSB:LB + 40 mM sodium citrate, then resuspended in 100 μl TSB:LB + 40 mM sodium citrate and plated on TSA plates with 10 mM sodium citrate and any antibiotics necessary for selection. Plates were incubated at 37˚C overnight and any potential transductants were screened for antibiotic resistance and acquired mutations using PCR and DNA sequencing

Construction of Δ*agr*::*tet* Mutants

To generate Δ*agr*::*tet* mutants, a marked deletion mutant of *agrBDCA* was transduced into AH-1263, SA502A, MW2, and RN3984 by bacteriophage-mediated transduction as described above.

Generation of Complementation Strains

To generate single-copy chromosomal complementation strains expressing *mroQ* under its native promoter, the integrative plasmid pJC1112 was used to generate pJC1112-*mroQ* as previously described (244,324). Complementation of the Δ*mroQ* mutation in SA502A with pJC1112-*mroQ* under the control of its native promoter was unsuccessful, presumably because of altered expression patterns in this strain. To overcome this limitation, I expressed FLAG-GG-*mroQ* under the control of the constitutive P_{sarA} promoter in plasmid pOS1 (326). To generate this plasmid, the P_{sarA} promoter linked to the *S. aureus* superoxide dismutase (*sod*) ribosomal binding site was fused to the coding sequence for *mroQ* with an N-terminal FLAG tag and diglycine

linker. To amplify P_{sarA}-sod_{RBS}, primers FLAG-GG-MroQSOE1 and FLAG-GG-MroQSOE2 were used. FLAG-GG-*mroQ* was generated using FLAG-GG-MroQSOE3 and FLAG-GG-MroQOE4. Amplicons were spliced together using SOEing PCR with primers FLAG-GG-MroQSOE1 and FLAG-GG-MroQSOE4 and cloned into pOS1 using PstI and EcoRI restriction endonucleases. The resulting plasmid was transformed into *E. coli* BH10C and electroporated into *S. aureus* RN4220. The complementation vector was then transduced into an SA502A Δ*mroQ* mutant as described above. To generate complementation strains harboring Agr loci under the control of their native promoters, the integrative plasmid pJC1111 was used. Integrated complementation vectors were transduced into Δ*mroQ* (pJC1112-*mroQ*), Δ*agr* (pJC1111-*agrI-IV*), or Δ*agr*Δ*mroQ* (pJC1111-*agrI-IV*) as defined above. Complement strains were verified using PCR and DNA sequencing. Primers are listed in Table 2.

Construction of *P3-gfp* Reporter Strains

Plasmid pDB59 harboring the Agr-regulated *P3* promoter driving the expression of *gfp* was isolated from *E. coli*, passaged through RN4220, and electroporated into all indicated strains (244,322,327,328).

Construction of pOS1-*PsarA-6x-His-GG-agrD* Expression Plasmid

A 6x-His-GG-*agrD* expression plasmid for each Agr type was generated by fusing the P_{sarA} promoter linked to the *S. aureus* superoxide dismutase (*sod*) ribosomal binding site with the coding sequence for AgrD containing an N-terminal 6x-His tag and diglycine linker. To amplify P_{sarA}-sod_{RBS}, primers pOS1uniSOE1 and P_{sarA}-sod_{RBS}-6x*His-GG-agrD*-SOE2 were used. 6xHis-GG-AgrD-I was amplified using

P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDI*-SOE3 and P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDI*-SOE4. 6xHis-GG-AgrD-II was amplified using P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDII*-SOE3 and P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDII*-SOE4. 6xHis-GG-Agr-III was amplified using P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDIII*-SOE3 and P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDIII*-SOE4. SOEing PCR was used to splice each AgrDI-III to *P*$_{sarA}$-*sod*$_{RBS}$ using the resulting amplicons from the above PCRs as template with primers pOS1uniSOE1 and P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDI*-SOE4, P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDII*-SOE4, or P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDIII*-SOE4. The resulting fusion products were cloned into pOS1 using PstI and EcoRI restriction endonucleases. These products were transformed into DH5α, passaged through RN4220, and electroporated into wildtype, Δ*mroQ*, and Δ*mroQ*+*mroQ* isogenic strains for all Agr Types. To construct pOS1- P_{sarA}-*sod*$_{RBS}$-6x-His-GG-*Leader-AIP-III-SSP* expression plasmid, P_{sarA}-*sod*$_{RBS}$-6x-His-GG-*Leader-AIP-III* was amplified from pOS1-P_{sarA}-*sod*$_{RBS}$-6x-His-GG-*agrDIII* using pOS1uniSOE1 and T3Leader-AIPREV. The fused split-intein from *Synechocystis* species PCC6803 (Ssp) was amplified using SSPFWD T3Leader AIP and SSP3. The resulting amplicons were spliced using SOEing PCR with pOS1uniSOE1 and SSP3. Products were transformed into DH5α, passaged through RN4220, and electroporated into Δ*agr*::*tet* and Δ*agr:tet* Δ*mroQ* strains from MW2. To generate Leader and Leader-AIP peptide controls for Agr Type I, the primers pOS1uniSOE1 and P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrD*-SOE2 were used to amplify *P*$_{sarA}$-*sod*$_{RBS}$. P_{sarA}-*sod*$_{RBS}$-6x*His-GG-agrDI*-SOE3 and T1LeaderREV or T1Leader-AIPREV were used to amplify Leader or Leader-AIP, respectively. SOEing PCR was used to splice the Leader or Leader-AIP to *P*$_{sarA}$-*sod*$_{RBS}$ using the above amplicons as

template with primers pOS1uniSOE1 and T1LeaderREV or T1Leader-AIPREV. All primers are listed in Table 2.

Analysis of 6x-His-AgrD Maturation by Immunoblot

5 mL overnight cultures of *6xHis-GG-agrD* or *6xHis-GG-LeaderAIP-III-SSP* expressing strains were subcultured 1:100 in 50 ml – 800 ml RPMI or TSB at 37˚C, 220 rpm for eight hours or overnight. Bacterial cells were centrifuged at 3,900 rpm for 20 min and supernatants were removed and filter sterilized using a 0.22 μm filter before addition of imidazole (10 mM) and PMSF (1.2 mM), followed by overnight incubation with pre-equilibrated Ni-NTA. Bound 6x-His-AgrD was washed two times with 1 mL 6 M urea buffer followed by addition of 100 μl 4x-SDS Sample Buffer and boiling for 10 min. For isolation of membrane fractions, bacterial pellets were resuspended in 10 ml PBS and treated with lysostaphin for 30 minutes at 37˚C followed by sonication using a Sonifier sonicator (Branson) at 30% power, 20 seconds on, 20 seconds off for a total of 2 min. Cellular debris was removed by centrifugation at 12,000 rpm for 15 min at 4˚C. Lysates were ultracentrifuged at 20,000 rpm for 70 minutes at 4˚C and the resulting pellet was solubilized in PBS + 1 M $NaCl_2$ + 6 M urea + 1% DDM overnight. Equilibrated Ni-NTA was added to the DDM solubilized samples, incubated for 3 hours to allow for binding. Bound 6x-His-AgrD was washed two times with 1 mL 6 M urea buffer and boiled in 4x-SDS Sample Buffer as described above. Samples were resolved on 16% Tris-Tricine gels containing 6 M urea for two hours at 30V to allow for migration into the gel, then overnight at 90V. Proteins were transferred to 0.2-μm-pore-size polyvinylidene difluoride membranes (PVDF; Immobilon, Roche) in 20% methanol transfer buffer at 100V for 30 min. Membranes

were blocked for one hour in Tris-buffered saline (TBS)-Tween 20 (TBST, 0.1% Tween 20 [Amresco] in TBS [Corning]) containing 5% bovine serum albumin (BSA) (GoldBio), incubated with anti-His6 monoclonal mouse antibody (1:5,000 dilution) (Abcam, ab18184) overnight and washed three times in 10-20 mL TBST for 5 min each. Blots were then incubated with goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase (Thermo) for one hour, followed by three washes in 10-20 mL TBST for 5 min each and development using 5-bromo-4-chloro-3-indoyl-phosphate-nitroblue tetrazolium (BCIP/NBT) substrate (GoldBio/VWR).

Exoprotein Preparations

Bacterial strains were subcultured 1:100 in 5 mL TSB or RPMI for 8 hours at 37˚C, 220 rpm. The OD_{600} was measured, cell suspensions were centrifuged at 3,900 rpm for 15 min, and supernatants were removed and filter-sterilized through a 0.22 μm filter. 1.3 mL of supernatant was collected in a 1.5-mL microcentrifuge tube and 150 μl of 100% trichloroacetic acid (TCA) was added. Mixtures were incubated overnight at 4˚C, centrifuged at 12,000 rpm for 15 min to pellet precipitated proteins, incubated with 1 mL 100% ethanol or acetone for at least 30 min at 4˚C, and centrifuged again at 12,000 rpm for 15 min. The resulting pellets were allowed to air dry at room temperature followed by addition of TCA-SDS sample buffer (4% SDS + 0.5M Tris-HCl mixed 1:1 with 2x-SDS loading buffer) and boiling for 10 min. Samples were stored at -20˚C. OD_{600} normalized exoprotein preparations were resolved by SDS-PAGE using 12% acrylamide gels at 120V in a Quadra Mini-Vertical PAGE/blotting system (CBS Scientific). Gels were fixed in a solution containing 50:10:4/Methanol:acetic acid:H_20 for 25 min before washing with 10 mL water three

times for 5 min each. Following fixation, gels were stained in Gel-Code Blue stain reagent (Pierce) for 1 hour at room temperature and subsequently washed in 10-20 mL H_2O for 2-3 hours to allow for destaining.

Immunoblot Analysis for Secreted Virulence Factors

SDS-PAGE separated proteins were transferred to a 0.2-μm-pore-size PVDF membrane as described above. Membranes were blocked with TBST + 5% BSA for one hour before addition of human IgG (Sigma) (1:2,000) in TBST for one hour to block Protein A followed by washing three times with 10 mL TBST for 15 min each before addition of Rb-anti-HlgC (1:5,000) or Ms-anti-Hla (1:5,000) antibodies overnight. The following day, membranes were washed three times with 10 mL TBST and incubated with alkaline phosphatase conjugated goat anti-mouse IgG (H+L) or goat anti-rabbit IgG (H+L) (Invitrogen) for one hour. Membranes were washed three times with 10 mL TBST then developed using BCIP/NBT reagent as described above.

AIP Inhibition Assays

Bacterial strains were subcultured 1:100 in TSB or RPMI for 5 hours at 37˚C, 220 rpm. Cell suspensions were centrifuged at 3,900 rpm for 5 min, and supernatants, designated “conditioned media (CM)”, were filter sterilized through a 0.22 μm filter. Overnight cultures of *P3-GFP* reporter strains were either subcultured 1:100 in 50% CM or 2:1 CM:TSB and allowed to grow for 5 hours at 37˚C, 220 rpm. For assays which required dilution, CM was serially diluted into 4.5 mL fresh TSB and each reporter strains were subcultured into each dilution. Following incubation, 100 μl aliquots were pelleted at 3,900 rpm for 5 min and washed three times with PBS and resuspended in a final volume of 100 μl PBS. GFP fluorescence and OD_{600} were

measured using a SpectraMax ID3 (Molecular Devices) or Synergy 2 (BioTek) plate reader. Relative fluorescence was calculated by normalizing fluorescence units to OD_{600}.

AIP Activation Assay

Bacterial strains were subcultured 1:100 in 5 mL TSB for 5-16 hours at 37˚C, 220 rpm. Cell suspensions were centrifuged at 3,900 rpm for 5 min and supernatant from test strains were collected and filter sterilized through a 0.22 μm filter, whereas bacterial pellets from reporter strains were retained for later application of conditioned medium. The reporter strain pellets were resuspended in 5 ml fresh TSB and 250 μl of this resuspension was mixed with 500 μl of test strain supernatant. 200 μl of the suspension was added in triplicate to a v-bottom plate and incubated for 3-12 hours at 37˚C, 220 rpm. Cell suspensions were centrifuged at 3,900 rpm for 5 minutes, followed by 2 washes and resuspension in 200 μl PBS. GFP fluorescence and OD_{600} was measured as described above.

Rabbit RBC Lysis Assay

Bacterial strains were subcultured 1:50 in 150 μl TSB or RPMI in a 96-well plate for 6 hours for strains LAC, SA502A, and MW2 Δ*agr*::*tet* + pJC1111-Agr-I and MW2 Δ*agr*::*tet* Δ*mroQ* + pJC1111-Agr-I and 16 hours for strains from MW2 and RN3984 at 37˚C, 220 rpm. Cell suspensions were centrifuged at 3,900 rpm for 5 min and OD_{600}-normalized cell-free supernatants were serially diluted 1:1 in PBS in a round bottom 96-well plate followed by 1:1 addition of defibrinated rabbit red blood cells (RBCs, Colorado Serum Company) diluted to 2% packed cell volume (PCV). Following incubation for one hour at 37˚C, samples were centrifuged at 1,500 rpm for 5 min and

supernatants were transferred to flat bottom 96-well plates followed by measuring RBC lysis at OD_{450}.

Murine Skin and Soft Tissue Infection

Bacterial strains were grown overnight in TSB (Criterion) at 37˚C, 220 rpm and subcultured 1:100 in 15 mL TSB for three hours. Strains were washed three times with 5 mL PBS then normalized to an OD_{600} of 0.32 to 0.33 (1×10^8 CFU) followed by mixing 1:1 with sterile Cytodex microcarrier beads (Sigma Aldrich). Mice were anesthetized with 2,2,2-tribromoethanol (Avertin, Sigma) via intraperitoneal injection (250 mg/kg) and 200 μL of the bacteria-Cytodex bead mixture (1×10^7 CFU) was injected intradermally into each side of the shaved flank region of anesthetized mice. After 4 days, mice were euthanized and abscesses were harvested, homogenized, and plated on TSA for enumeration of colony forming units (CFU). Images of representative abscesses were captured and displayed in Figures 17 and 18.

Ethics Statement

All animal experiments followed the ethical standards outlined by the Institutional Animal Care and Use Committee (IACUC) and institutional biosafety committee at Loyola University Chicago, Health Sciences Division. Loyola University Chicago is registered by the USDA (33-R-0024 through 24 August 2023), approved by the Public Health Service (PHS; A3117-01 through 28 February 2026) and is fully accredited by AAALAC International (000180 through November 2022). All animal experiments were performed following USDA and PHS policy guidelines on the humane care and use of animals and were carried out in biosafety level 2 facilities with IACUC-approved

protocols under the guidance of the Office of Laboratory Animal Welfare (OLAW).

Statistical Analysis

All experiments were repeated at least 3 independent times. For AIP reporter assays, statistical significance was analyzed using GraphPad Prism (Version 9.0) with representative data from experiments conducted in triplicate at least three independent times. Statistical tests are specified in the figure legends. For animal studies, statistical analysis was determined by Kruskal-Wallis test with Dunn's *post hoc* test for multiple comparisons.

CHAPTER THREE

EXPERIMENTAL RESULTS

Portions of this Chapter are Reprinted with Modifications from Stock et al. 2022 (329)

Introduction

In Gram-positive pathogens, quorum sensing occurs in response to small signaling peptides called pheromones. These pheromones must be processed and released by the bacterium for signaling to occur (200,330). Following transport outside of the bacterial cell, the peptide activates or inhibits gene expression, either upon import back into the bacterial cell followed by direct interaction with a transcription factor, or via binding of membrane-embedded sensor kinases at the plasma membrane (331). Quorum sensing is an important means of regulating the expression of a wide array of genes including those related to virulence, biofilm formation, and motility (200,255,330,332). As such, understanding how these peptides are processed, transported, and signal is imperative to better combating pathogenic traits of Gram-positive bacteria.

One class of pheromone, the cyclic autoinducing peptide (AIP), is central to the virulence of several Gram-positive pathogens, including *S. aureus* (107,200,333,334). AIP is produced by the accessory gene regulatory (Agr) system to control the expression of virulence genes, including leukotoxins, hemolysins, and tissue-degrading enzymes (107,110,200,255,333,334). The *agrBDCA* operon encodes the components of the Agr system, which include AgrC and AgrA, a histidine kinase-

response regulator pair, the protease AgrB, and the peptide AgrD, which is post-translationally processed and exported as AIP to activate AgrC-AgrA (153,224,226). AgrD contains an N-terminal amphipathic α-helical leader, followed by AIP and a charged C-terminal tail (Figure 11B). The N-terminal α-helix localizes the precursor peptide to the membrane, where subsequent events in peptide maturation occur (235). The first step in AgrD processing is cleavage of the C-terminal charged tail by AgrB (227,237,238,241,242). This proteolytic event triggers thiolactone ring formation between the C-terminal carbonyl and the sulfur atom of a conserved cysteine side chain (237,238,242). Thiolactone ring formation is required to activate AgrC (238,242). The resulting intermediate, consisting of the leader peptide linked to AIP, then undergoes N-terminal proteolytic processing and export to give rise to mature AIP in the extracellular space (237,241,242). These final steps in AIP maturation are yet to be fully understood. One study proposed a role for the canonical signal peptidase, SpsB, in removal of the N-terminal α-helix (243); however, recent publications support a role for additional proteases in this process (244–246). The α-helical leader peptide is separated from the central AIP by a conserved isoleucine/glycine (IG) helix breaker followed by a 3-5 amino acid linker region (Figure 11), which may facilitate presentation of the peptide cleavage site to the active site of a protease (243).

S. aureus isolates harbor one of four Agr variants on account of hypervariable regions within the coding sequences of *agrB, agrC,* and *agrD* (200,227–234). Each Agr variant produces a unique AIP that binds to and signals through its cognate histidine kinase (204,236,247,248). Furthermore, each AIP inhibits the activity of non-cognate

Agr systems, via competitive binding to peptide recognition sites on AgrC (204,226,229,230,236,239,240). The exception to this phenomenon is Agr-I and Agr-IV, which differ by a single amino acid at position 5 and have been shown to cross-activate AgrC-IV and AgrC-I, respectively (229,230,236). AIP production and signaling kinetics vary among variants, with Agr-I, -II, and -IV undergoing activation much sooner than Agr-III in broth culture (230,246). *S. aureus* strains containing any of the four Agr variants can cause disease and at least one study has suggested associations with disease outcome: Type I variants are enriched in cases of bacteremia, Type II variants are overrepresented in infective endocarditis, and Type III variants are increased in menstrual toxic shock syndrome (228,229,264). Strains with defective Agr systems are attenuated for pathogenicity, highlighting the importance of the system to infectious disease (110,111). Though there is an established link between AIP signaling and *S. aureus* pathogenesis, gaps remain in our understanding of the precise mechanisms behind AgrD processing among all allelic variants.

Our previously published work and that of others identified MroQ as an important mediator of *S. aureus* pathogenesis in strains harboring a Type I Agr variant (244,245,324). MroQ is annotated as a putative Type II CAAX protease, a family of multi-pass transmembrane proteins (244,245,324). A Δ*mroQ* mutant phenocopies a Δ*agr* mutant for reduced levels of secreted proteins, decreased toxin production, and attenuated skin and soft tissue infection (244,245). These shared phenotypes suggest a link between MroQ function and Agr system activation. Furthermore, global transcriptome profiling revealed that all Agr system genes were downregulated in a Δ*mroQ* mutant (245). I found that loss of MroQ did not impact AgrC-AgrA signaling

capacity, but rather led to accumulation of full-length C-terminal 6x-His tagged AgrD in the bacterial cell, highlighting a possible link to AIP maturation (244). In accordance with its annotation as a Type II CAAX protease, I observed loss of Agr system function upon mutation of predicted active site residues E141, E142, and H180 to Alanine, suggesting a requirement for catalytic activity in MroQ-mediated AIP maturation (244). Indeed, recent work from Zhao et. al. found that AgrD maturation required only AgrB, MroQ, and AgrD, supporting the model that MroQ functions as a protease that cleaves AgrD (246).

In this work, I investigated the role of MroQ in the maturation and export of each AgrD allelic variant. Furthermore, I examined the relevance of strain background on MroQ activity and its relationship to virulence. I found that MroQ is not only required for AgrD processing, but also export or release from the plasma membrane in an Agr-I sequence variant. Furthermore, examination of the impact of an *mroQ* mutation on Agr system activation in Agr-II and Agr-III variants showed reduced activity in Agr-II, but not Agr-III strains. In contrast, visualization of processing of 6x-Histidine tagged AgrD-II and AgrD-III showed an accumulation of intermediates that closely resembled AgrD-I intermediates and suggest a conserved role for MroQ in at least one step of AIP maturation. However, the generation of isogenic strains harboring Agr-I, -II, -III, or -IV demonstrated that while MroQ mediated AIP maturation is required for the generation of active AIP from Agr-I, -II, and -IV strains, it does not seem to be required for Agr-III system activation. These findings are largely recapitulated in skin and soft tissue infection models. Taken together, these data suggest a requirement for MroQ in at least one step of AgrD maturation that is uniform across all sequence

variants and is indispensable for the generation of active AIP in three of four variants in vitro. This represents a remarkable conservation of function despite significant diversity among the four Agr systems of *S. aureus* and may imply similar conservation exists in other bacteria with Agr peptide signaling systems.

MroQ is Conserved Among *S. aureus* Strains Harboring Agr Variants.

We and others previously tested the hypothesis that MroQ is involved in promoting Agr system activation and found that deletion of *mroQ* disrupted Agr activity, via a presumed defect in peptide maturation that centered on AgrB and AgrD (244,245). These studies were conducted using *S. aureus* strain LAC, which contains an Agr-I allele. The amino acid sequence of AgrB has 50% sequence identity among Agr Types I-IV (Figure 11A). The amino acid sequence of AgrD is similarly hypervariable, with 26% conservation amongst Agr Types I-IV (Figure 11B). In contrast, the amino acid sequence of MroQ is well-conserved among representative strains harboring each Agr allele (LAC, SA502A, MW2, and RN4850) (Figure 11C). Strains LAC, SA502A, and MW2 had 100% identity, while strain RN4850 had 95% identity. My analysis of all sequenced *S. aureus* strains containing Agr-IV in NCBI further indicated MroQ is identical within these strains; however, all had 95% identity relative to LAC, SA502A, and MW2. Thus, despite significant divergence among AgrB and AgrD alleles, MroQ does not share a similar hypervariable sequence.

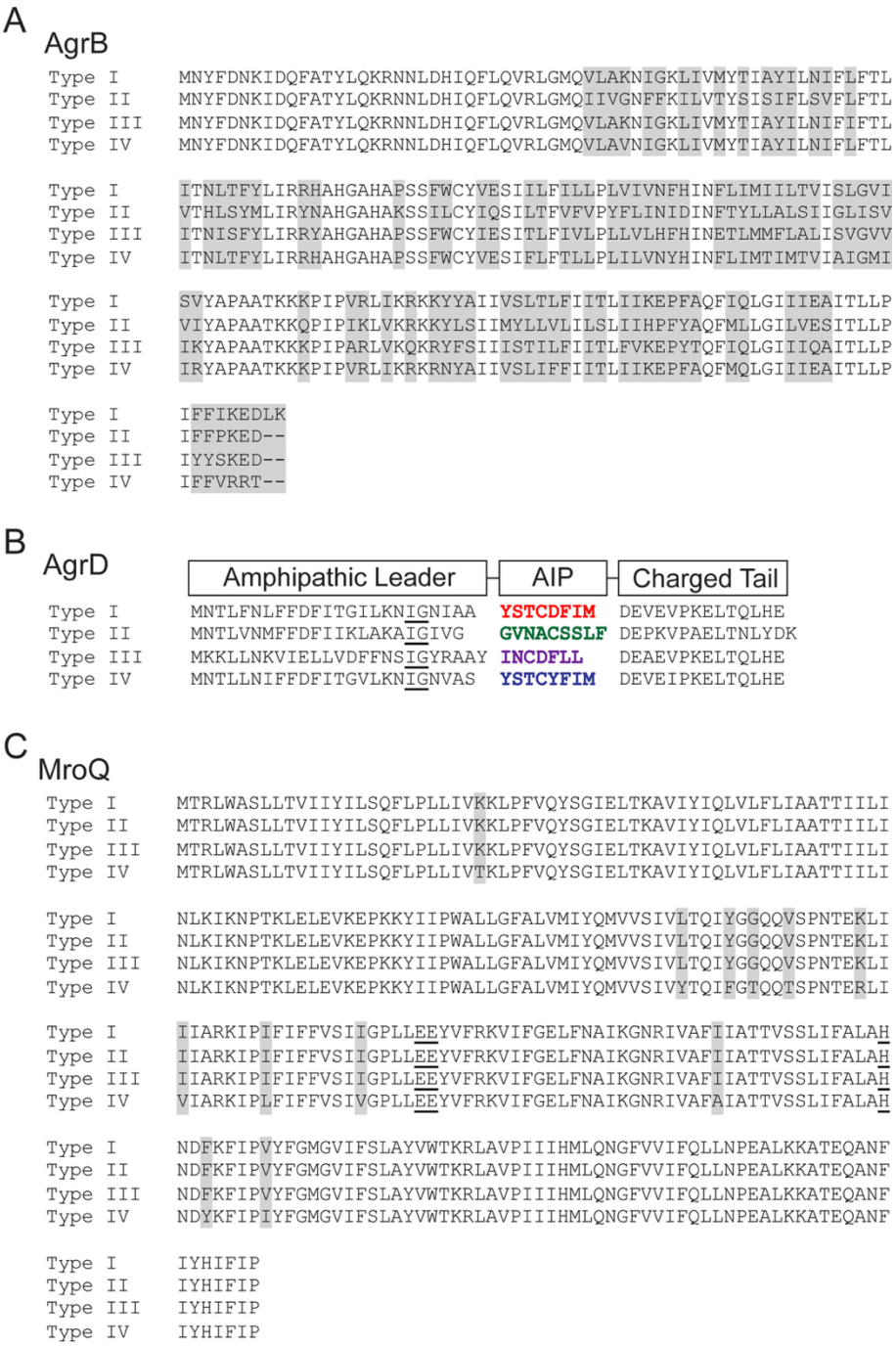

Figure 11. MroQ is Strongly Conserved Among *S. aureus* Strains Harboring Agr Allelic Variants. Comparison of the amino acid sequences of AgrB (A) and AgrD (B) in LAC (type I), SA502A (type II), MW2 (type III), and RN4850 (type IV). An underlined "IG" shows the conserved "helix breaker"; colored regions correspond to AIPs I to IV. Type I and IV AIPs peptides differ by one amino acid. (C) Amino acid sequence alignments of MroQ from LAC (type I), SA502A (type II), MW2 (type III), and RN4850 (type IV). Grayshading shows regions of dissimilarity. Underlined amino acids correspond to conserved active-site residues in MroQ.

A Δ*mroQ* Mutant is Defective for Activation of Agr Type I and Agr Type IV Systems.

Given the conservation of MroQ among sequenced *S. aureus* strains but significant variability among proteins in the Agr locus, I reasoned the role of MroQ in promoting Agr system activation might be restricted to Agr Type I and Agr Type IV, which share the greatest sequence identity (Figure 11A-B and (229,230,236)). To test this hypothesis, I first verified MroQ-dependent defects in Agr system activation in Agr Type I strain, LAC. LAC (WT), Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* strains were evaluated for total exoprotein secretion, leukotoxin production, and hemolytic activity on rabbit red blood cells (RBCs). As expected, I observed reduced exoprotein abundance in a Δ*mroQ* mutant compared to the WT or the Δ*mroQ* + *mroQ* complement strain (Figure 12A, (244)). The decreased exoprotein production phenocopied Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* mutants, validating a role for MroQ in Agr system function (Figure 12A, (244)). Immunoblot analysis of TCA-precipitated supernatant from a Δ*mroQ* mutant showed reduced levels of α-hemolysin (Hla) and γ-hemolysin (HlgC) in accordance with established Agr-regulation patterns (Figure 12B and (224,335)). In agreement with decreased hemolysin levels, a Δ*mroQ* mutant had reduced hemolytic activity against rabbit RBCs (Figure 12C). To further demonstrate perturbation of Agr system function in a Δ*mroQ* mutant, I assessed *agr P3* promoter activity using the fluorescent transcriptional reporter plasmid pDB59-*P3-GFP* (327). Mature AIP-I inhibits the activation of Agr-II and Agr-III, but activates Agr-IV (229,230,236).

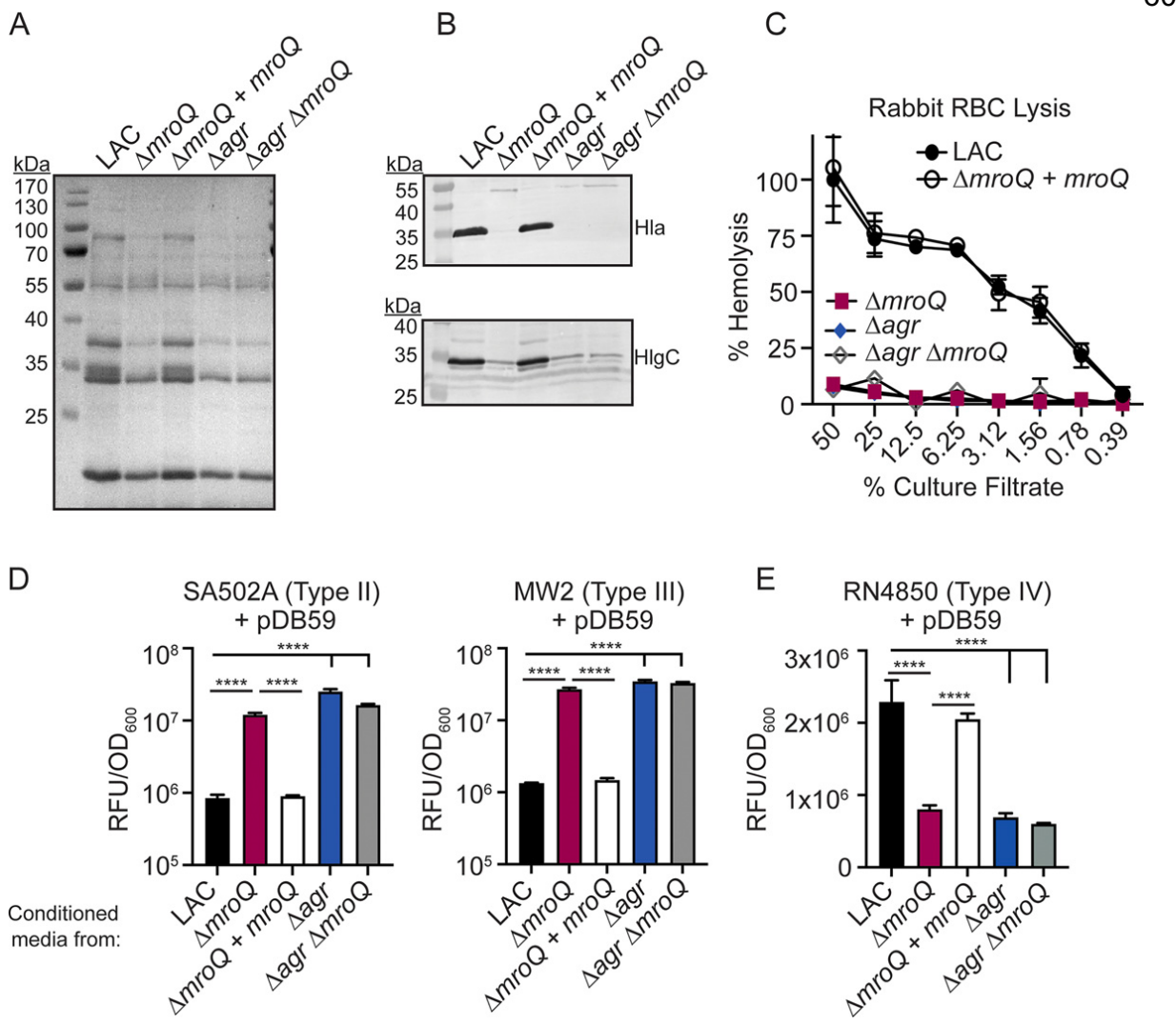

Figure 12. MroQ Contributes to Agr Type I and IV Activation. (A and B) TCA-precipitated exoproteins (A) and Hla and HlgC immunoblots (B) from LAC, Δ*mroQ*, Δ*mroQ* + *mroQ,* Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* strains. (C) Rabbit red blood cell lysis of cell-free culture filtrates derived from LAC, Δ*mroQ*, Δ*mroQ* + *mroQ,* Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (D) pDB59 reporter activity (relative fluorescence units ([RFU]/OD_{600}) in SA502A (left) and MW2 (right) upon addition of conditioned medium from LAC, Δ*mroQ*, Δ*mroQ* + *mroQ,* Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* strains. (E) pDB59 reporter activity (RFU/OD_{600}) in RN4850 (type IV) upon addition of conditioned medium from LAC, Δ*mroQ*, Δ*mroQ* + *mroQ,* Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. Hemolysis and GFP reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots and GelCode blue-stained gels are a representative of at least four replicates. Means ± SD are shown (n = 3). ****, $P < 0.0001$ by one-way analysis of variance (ANOVA) with Tukey's posttest.

I found that addition of cell-free supernatant from WT LAC (Agr-I) inhibited the activation of pDB59-*P3-GFP* in an Agr-II strain (SA502A) and Agr-III strain (MW2), whereas supernatant from a Δ*mroQ* mutant led to robust activation, as determined by GFP fluorescence (Figure 12D). Additionally, supernatant from WT LAC activated an Agr-IV strain (RN4850) containing pDB59-*P3-GFP*, whereas supernatant from a Δ*mroQ* mutant did not, suggesting Agr-I and Agr-IV are likely to be impacted by MroQ in similar ways (Figure 12E and (229,230,236)). Altogether, these data validate prior work indicating Agr-I system activation is defective in the absence of MroQ and established that the highly similar Agr-IV system is activated by AIP-I in a MroQ-dependent manner.

A Δ*mroQ* Mutant is Defective for AIP-I Export and Processing.

Previous work suggested a role for MroQ in the processing and/or export of AgrD to give rise to AIP (244). To determine if loss of MroQ affects AgrD-I maturation or export I expressed 6x-His-AgrD-I in the WT (LAC), Δ*mroQ*, and Δ*mroQ* + *mroQ* Agr Type I strains and monitored the location and generation of processing intermediates via immunoblot. Immunoblot analysis was carried out with assistance from Dr. Liwei Fang, a postdoctoral fellow in the Alonzo laboratory. I observed species which corresponded to AgrB-processed AgrD-I (Leader-AIP) and N-terminally processed AgrD-I (Leader peptide alone) in the membrane fraction of WT and the Δ*mroQ* + *mroQ* complement strains (Figure 13A). In addition, the cell-free supernatant contained significant quantities of Leader peptide (Figure 13A).

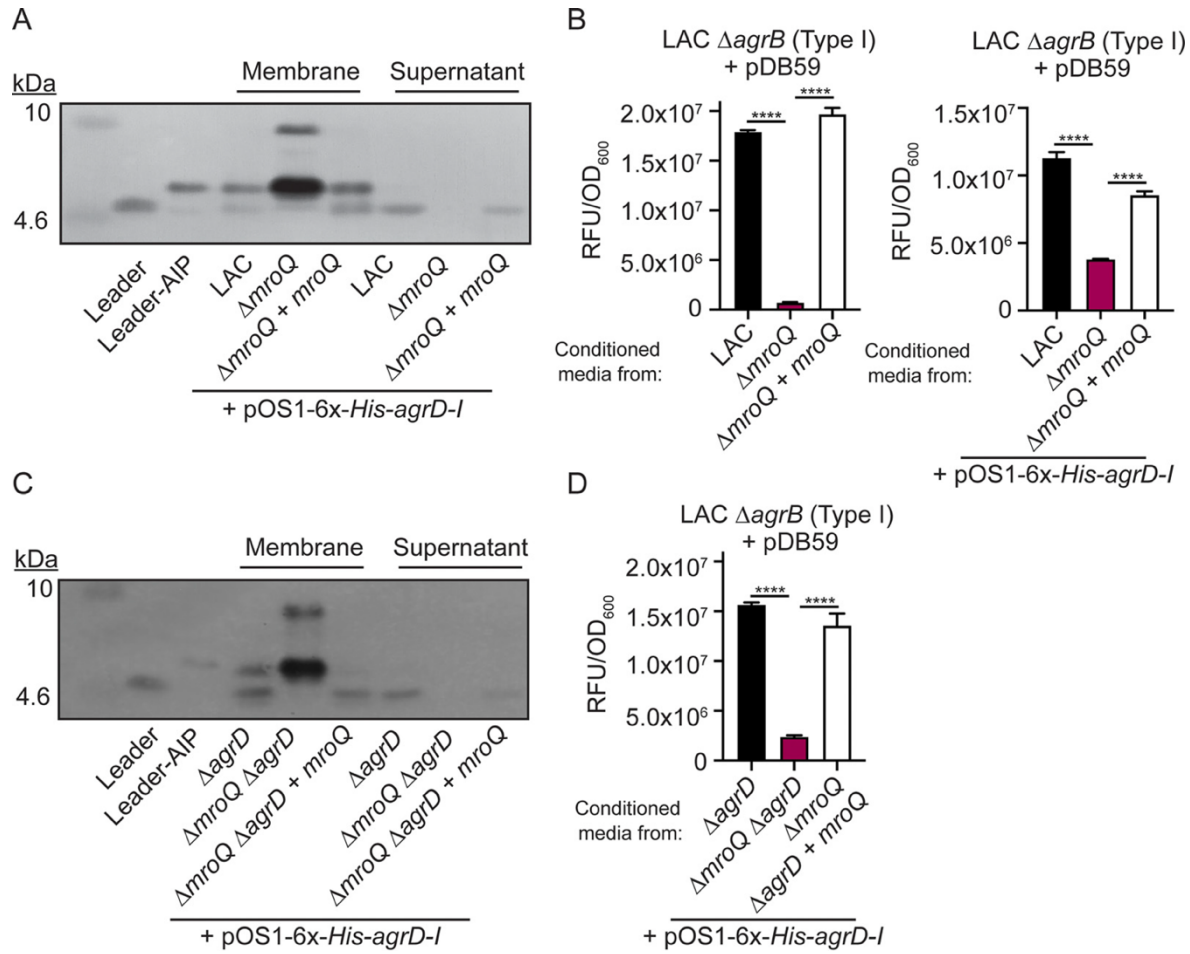

Figure 13. A Δ*mroQ* Mutant is Compromised for AIP-I Export and Processing. (A) Immunoblots of supernatant and membrane fractions of LAC, Δ*mroQ*, and Δ*mroQ* + *mroQ* strains constitutively expressing 6×-His-AgrD-I (pOS1-P_{sarA}-6×-*His-agrD-I*) using anti-His monoclonal antibody. 6×-His-leader-AIP-I (AgrB processing intermediate) and 6×-His-leader-I (AgrD-I leader peptide) were isolated from constitutively expressing *S. aureus and* are included as controls. (B) pDB59 reporter activity (RFU/OD_{600}) in LAC Δ*agrB* upon addition of conditioned medium from LAC, Δ*mroQ*, and Δ*mroQ* + *mroQ* strains or LAC, Δ*mroQ*, and Δ*mroQ* + *mroQ* strains constitutively expressing 6×-His-AgrD-I. (C) Immunoblots of supernatant and membrane fractions of Δ*agrD*, Δ*mroQ* Δ*agrD*, and Δ*mroQ* Δ*agrD* + *mroQ* strains constitutively expressing 6×-His-AgrD-I (pOS1-P_{sarA}-6×-*His-agrD-I*) using anti-His monoclonal antibody. (D) pDB59 reporter activity (RFU/OD_{600}) in LAC Δ*agrB* upon addition of conditioned medium from Δ*agrD*, Δ*mroQ* Δ*agrD*, and Δ*mroQ* Δ*agrD* + *mroQ* strains constitutively expressing 6×-His-AgrD-I. Reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots are representative of at least three replicates. Means ± SD are shown (n = 3). ****, $P < 0.0001$ by one-way ANOVA with Tukey's posttest.

In contrast, a Δ*mroQ* mutant showed accumulation of full length AgrD and Leader-AIP in the membrane fraction and a complete loss of Leader peptide in both the membrane and supernatant fractions (Figure 13A). Complementation of the Δ*mroQ* mutant fully restored AIP maturation and release of the leader peptide, which correlated with activation of an Agr Type-I reporter (Δ*agrB* + pDB59-*P3-GFP*) and was independent of the pOS1-*6x-His-agrD-I* plasmid (Figure 13B). The WT, Δ*mroQ*, and Δ*mroQ* + *mroQ* Agr Type I strains containing the 6x-His-AgrD-I expression plasmid also produce AgrD from the native Agr operon. To rule out the possibility that native AgrD might impact the analysis of 6x-His-AgrD-I processing, Dr. Liwei Fang generated an in-frame deletion of *agrD* in the WT, Δ*mroQ*, and Δ*mroQ* + *mroQ* strain backgrounds. 6x-His-AgrD-I processing and activation of the Δ*agrB* + pDB59-*P3-GFP* reporter was identical to the parental strains (Figure 13C and 13D). These observations support a role for MroQ in the processing and export of AgrD-I and are in keeping with recent biochemical evidence for MroQ processing of AgrD (246).

MroQ Function is Conserved in an Agr-II Allelic Variant.

To test the role of MroQ in Agr-II peptide processing and export, I generated Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* mutants in the Agr Type II strain, SA502A, and assessed total exoprotein secretion, production of leukotoxins, and hemolytic activity on rabbit RBCs. I observed decreased exoprotein abundance in a Δ*mroQ* mutant, similar to Δ*agr* and Δ*agr* Δ*mroQ* mutants (Figure 14A). Further, I saw defective production of Hla and HlgC in Δ*mroQ*, Δ*agr*, and Δ*agr* Δ*mroQ* mutants (Figure 14B).

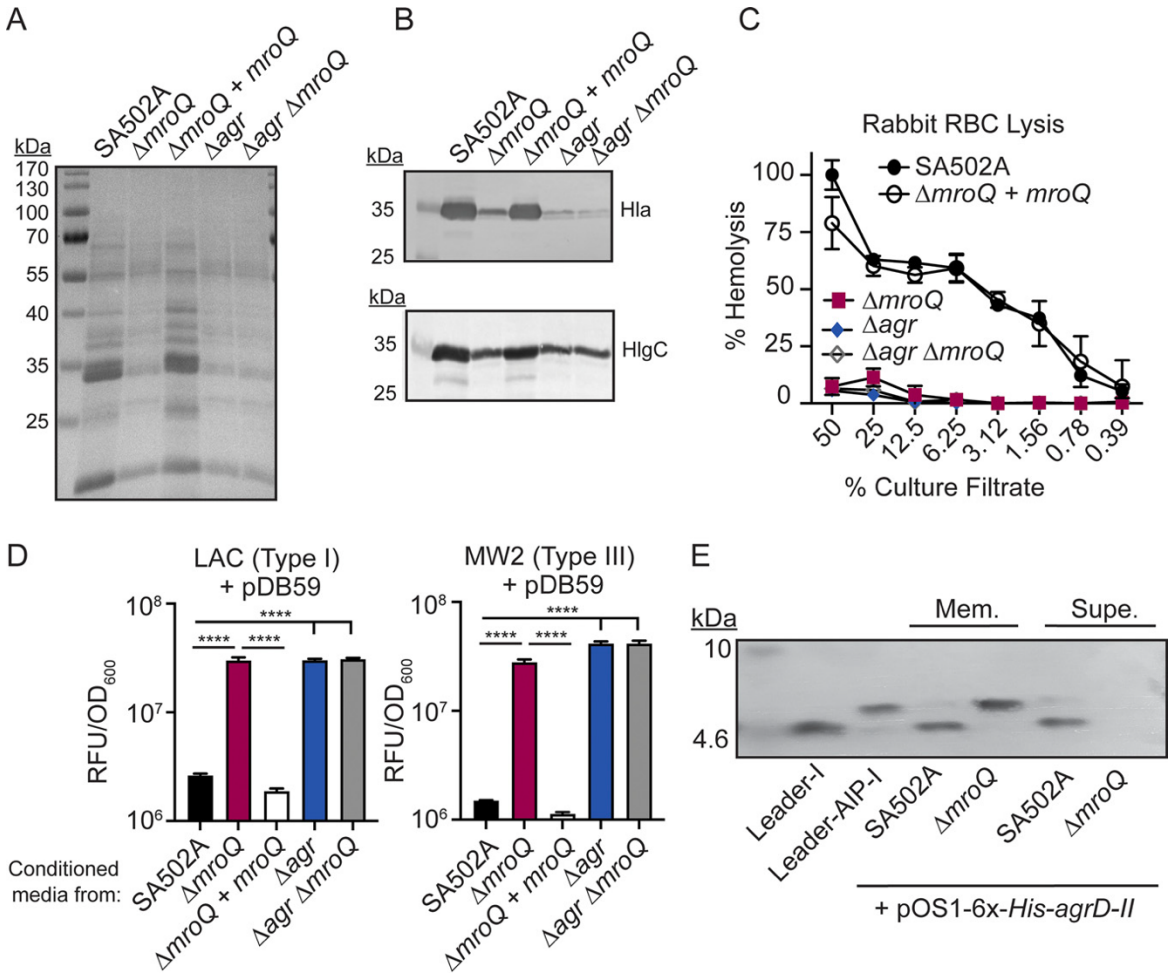

Figure 14. A Δ*mroQ* Mutant is Defective for Agr Type II Activation and AIP-II Maturation and Export. (A) TCA precipitated exoproteins from SA502A, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (B) Hla and HlgC immunoblots from SA502A, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (C) Rabbit red blood cell lysis of cell-free culture filtrates derived from SA502A, Δ*mroQ*, Δ*mroQ* + *mroQ,* Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (D) pDB59 reporter activity (RFU/OD_{600}) of LAC (left) and MW2 (right) upon addition of conditioned medium from SA502A, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (E) Immunoblots of supernatant and membrane fractions from SA502A and Δ*mroQ* strains constitutively expressing 6×-His-AgrD-II (pOS1-P_{sarA}-6×-*His-agrD-II*) using anti-His monoclonal antibody. 6×-His-leader-AIP-I (AgrB processing intermediate) and 6×-His-leader-I (AgrD-I leader peptide) were isolated from constitutively expressing *S. aureus* and were included as controls. Hemolysis and reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots and GelCode blue-stained gels are representative of at least four replicates. Means ± SD are shown (n = 3). ****, $P < 0.0001$ by one-way ANOVA with Tukey's posttest.

Consistent with these observations, I observed decreased hemolytic activity against rabbit RBCs for supernatant from a Δ*mroQ* mutant compared to culture supernatant from WT cells (Figure 14C). The Δ*mroQ* + *mroQ* complementation strain exhibited fully restored mutant phenotypes (Figure 14A-C). Agr-*P3* Reporter activity assays demonstrated inhibition of reporter activation by WT and the Δ*mroQ* + *mroQ* complement strains, whereas cell-free supernatant from a Δ*mroQ* mutant led to activation of the Agr system in both Agr-I and Agr-III reporter strains (Figure 14D). Furthermore, the expression of 6x-His-AgrD-II in SA502A showed Leader peptide alone in both the membrane fraction and the secreted fraction (Figure 14E). In contrast, expression of 6x-His-AgrD-II in a Δ*mroQ* mutant led to accumulation of Leader-AIP in the membrane fraction with no Leader peptide in the membrane or supernatant (Figure 14E). I was unable to monitor 6x-His-AgrD-II maturation in a Δ*mroQ* + *mroQ* strain due to the plasmid required for complementation in this strain background (see Materials and Methods). Taken together, these data indicate MroQ is required for the maturation and export of active AIP-II.

MroQ Contributes to Agr-III Peptide Processing but is Not Required for Agr System Activation.

Given the conservation of MroQ function in Agr-I, -II, and presumably -IV strains, I hypothesized that MroQ would also be required for Agr system activity in an Agr-III strain. I tested the role of MroQ in Agr system activity in two Agr Type III strains, MW2 and RN3984. Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* strains were generated in MW2, whereas Δ*mroQ* and Δ*agr*::*tet* strains were generated in RN3984.

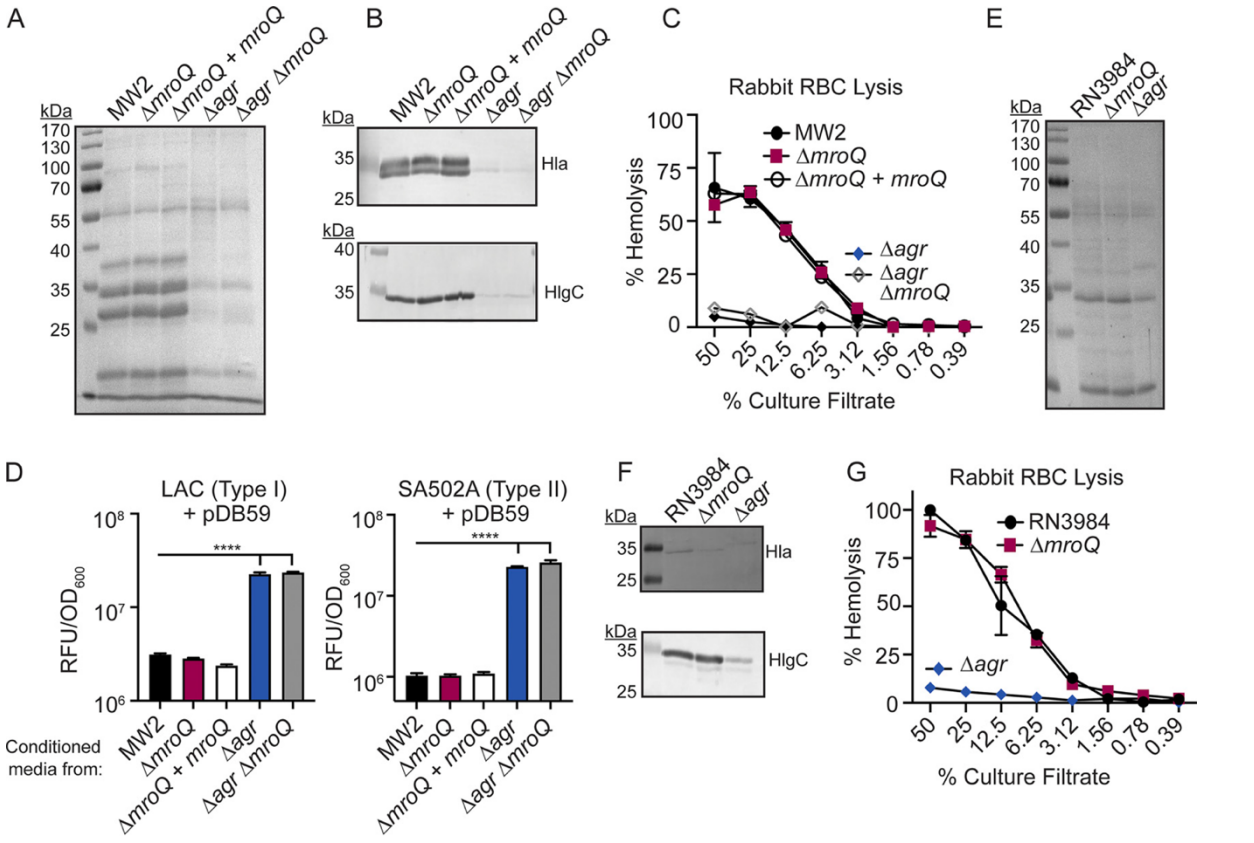

Figure 15. MroQ is Not Required for Agr Type III Activation. (A) TCA-precipitated exoproteins and (B) Hla and HlgC immunoblots from MW2, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (C) Rabbit red blood cell lysis of cell-free culture filtrates derived from MW2, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (D) pDB59 reporter activity (RFU/OD_{600}) of LAC (left) and SA502A (right) upon addition of conditioned medium from MW2, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* ΔmroQ. (E) TCA-precipitated exoproteins from RN3984, Δ*mroQ*, and Δ*agr*::*tet* strains. (F) Hla and HlgC immunoblots of from RN3984, Δ*mroQ*, and Δ*agr*::*tet* strains. (G) Rabbit red blood cell lysis of cell-free culture filtrates derived from RN3984, Δ*mroQ*, and Δ*agr*::*tet* strains. Hemolysis and reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots and GelCode blue-stained gels are representative of at least four replicates. Means ± SD are shown (n = 3). ****, $P <$ 0.0001 by one-way ANOVA with Tukey's posttest.

These strains were grown in broth and monitored for exoprotein production, leukotoxin production, and rabbit RBC hemolysis. For strain MW2, I observed similar levels of exoproteins in WT, Δ*mroQ*, and Δ*mroQ* + *mroQ* strains, whereas exoprotein levels were decreased in Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* strains (Figure 15A). Consistent with the similar exoprotein production, I saw equivalent levels of Hla and HlgC secreted by WT, Δ*mroQ*, and Δ*mroQ* + *mroQ* strains (Figure 15B). Furthermore, I noted identical hemolytic activity against rabbit RBCs in the same strains (Figure 15C). Conditioned medium from a Δ*mroQ* mutant fully inhibited pDB59-*P3-GFP* promoter activation in Agr-I and Agr-II reporter strains, whereas addition of conditioned medium from a Δ*agr*::*tet* or Δ*agr*::*tet* Δ*mroQ* mutant to Agr-I or II reporter strains did not inhibit promoter activation (Figure 15D). Similar results were obtained with strain RN3984 (Figure 15E-G). Together, these data indicate that MroQ is not required for Agr-III activation.

Given the observation that MroQ was not required for Agr-III system activation in MW2 and RN3984, I surmised that MroQ may be dispensable for the generation of AIPs in an Agr Type III strain background. To test this hypothesis, I generated MW2 Δ*agr*::*tet* + pJC1111-Agr-I and MW2 Δ*mroQ* Δ*agr*::*tet* + pJC1111-Agr-I strains and monitored Agr system function via exoprotein production, leukotoxin levels, and rabbit RBC hemolysis. I observed decreased exoprotein production from the MW2 Δ*mroQ* Δ*agr*::*tet* + pJC1111-Agr-I strain compared to the MW2 Δ*agr*::*tet* + pJC1111-Agr-I strain (Figure 16A). The decreased exoprotein production in the MW2 Δ*mroQ* Δ*agr*::*tet* + pJC1111-Agr-I strain corresponded with lower levels of toxins and loss of hemolytic activity against rabbit RBCs (Figure 16B-C), further suggesting that loss of

MroQ in this background caused defective Agr-I system activity. These data were corroborated using Agr reporter assays which showed that conditioned medium from Δ*agr*::*tet* + pJC1111-Agr-I inhibited pDB59-*P3-GFP* expression in Agr-II and Agr-III strains whereas conditioned medium from Δ*mroQ* Δ*agr*::*tet* + pJC1111-Agr-I had robust GFP production (Figure 16D). Thus, MroQ is still required for Agr-I activity when expressed in strain MW2 and strain background presumably does not dictate the requirement for MroQ in AgrD processing. Given this observation, I sought to further explore if MroQ is required for any aspect of AIP-III maturation. To this end, I monitored the expression of 6x-His-AgrD-III in WT, Δ*mroQ*, and Δ*mroQ* + *mroQ* MW2 strains by immunoblot and noted accumulation of a band that resembled Leader-AIP in the membrane fraction of the Δ*mroQ* strain, whereas WT and Δ*mroQ* + *mroQ* strains produced a species that resembled the Leader peptide in both the membrane and supernatant (Figure 16E). Altogether, these data suggest that MroQ processes AIP in a manner that is independent of strain background or Agr Type, yet an active signaling peptide can be generated in the absence of MroQ in Agr-III strains.

MroQ is Required for Virulence in Agr-I Strain LAC.

Animals infected intradermally with a Δ*mroQ* mutant of an Agr-I strain (LAC) have dramatic reductions in abscess pathology and exhibit modest reductions in CFU (~5-fold) (244). To determine if MroQ is required for skin and soft tissue infection of strains with Agr-II and Agr-III alleles, I intradermally infected mice with WT, Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* mutants from LAC (Agr-I), SA502A (Agr-II), and MW2 (Agr-III) and colony forming units (CFU) and gross pathology were assessed 96 hours post-infection.

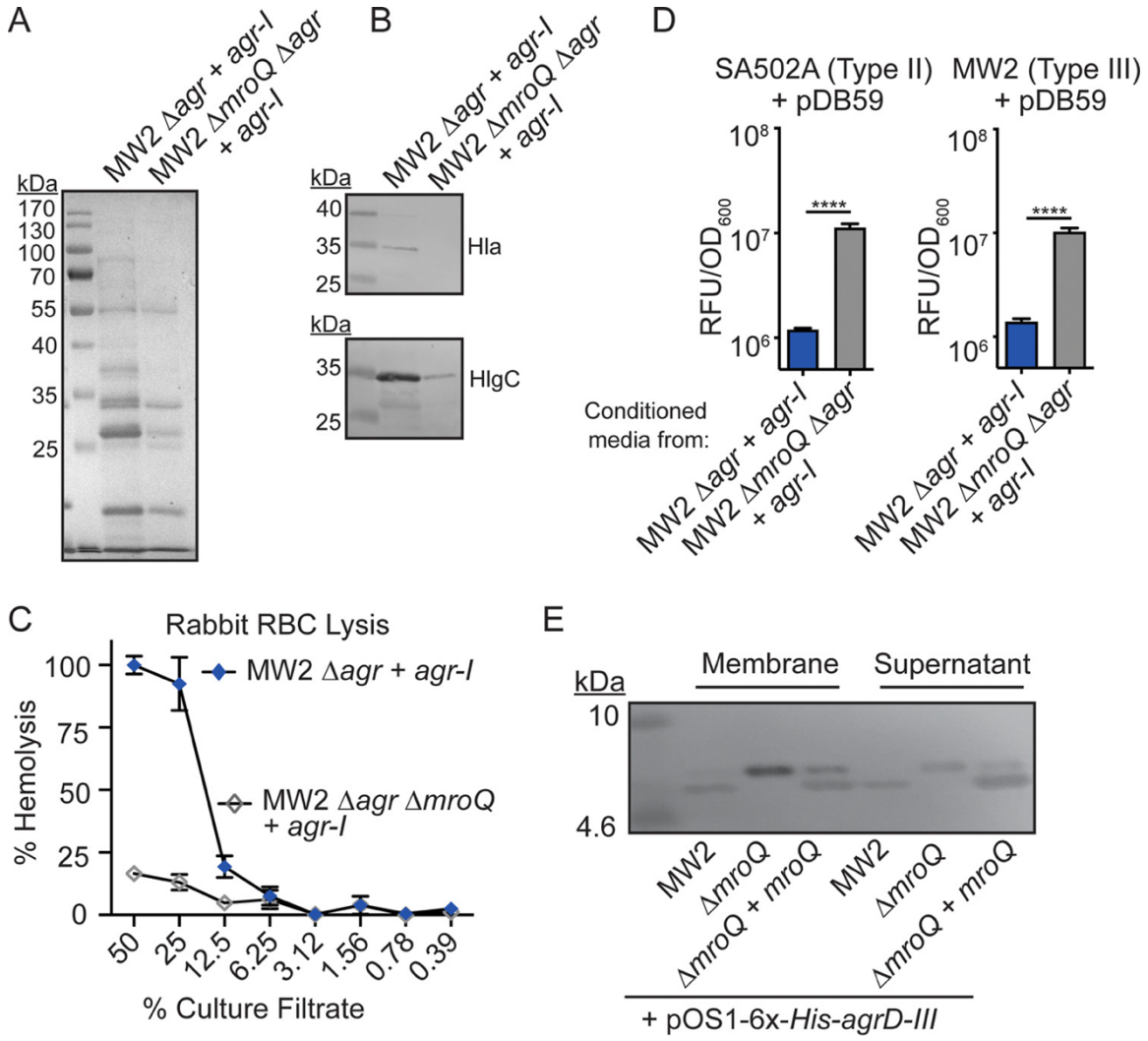

Figure 16. MroQ Contributes to AIP-I Processing in an Agr Type III Strain. (A) TCA-precipitated exoproteins from MW2 Δ*agr*::*tet* + *agr-I* and MW2 Δ*agr*::*tet* Δ*mroQ* + *agr-I* strains. (B) Hla and HlgC immunoblots from MW2 Δ*agr*::*tet* + *agr-I* and MW2 Δ*agr*::*tet* Δ*mroQ* + *agr-I* strains. (C) Rabbit red blood cell lysis of cell-free culture filtrates derived from MW2 Δ*agr*::*tet* + *agr-I* and MW2 Δ*agr*::*tet* Δ*mroQ* + *agr-I* strains. (D) pDB59 reporter activity (RFU/OD_{600}) of SA502A (left) and MW2 (right) upon addition of conditioned medium from MW2 Δ*agr*::*tet* + *agr-I* and MW2 Δ*agr*::*tet* Δ*mroQ* + *agr-I* strains. (E) Immunoblots of supernatant and membrane fractions from MW2, Δ*mroQ,* and Δ*mroQ* + *mroQ* strains constitutively expressing 6×-His-AgrD-III (pOS1-P_{sarA}-6×-*His-agrD-III*) using anti-His monoclonal antibody. Hemolysis and reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots and GelCode blue-stained gels are representative of at least four replicates. Means ± SD are shown (n = 3). ****, $P < 0.0001$ by a two-tailed *t* test.

For strain LAC, I observed significant reductions in pathology after infection with Δ*mroQ,* Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* mutants, although CFU remained high for most infected animals (Figure 17A-B). Though I observed dramatic reductions in pathology for animals infected with Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* mutants from strain SA502A, there was little difference in CFU and pathology in animals infected with the SA502A Δ*mroQ* strain compared to WT SA502A (Figure 17C-D). In agreement with in vitro assays, when animals were infected with the MW2 Δ*mroQ* strain I observed identical CFU and pathology compared to animals infected with WT MW2 (Figure 17E-F). This contrasted with Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ* mutants which had little observable pathology and modest reductions in CFU (Figure 17E-F). These data indicate that MroQ-mediated maturation of AIP is required for virulence in an Agr-I strain but is largely dispensable in an Agr-II or Agr-III strain.

MroQ-mediated AIP Maturation is Required for Agr-I, -II and -IV Activation, but not Agr-III.

As a complementary approach and to further rule out the possibility that strain background drives dependence on MroQ for AIP maturation, I reconstituted the entire Agr locus from each of the four Agr types into isogenic Δ*agr* and Δ*agr* Δ*mroQ* mutants in strain LAC using the site-specific integrational plasmids pJC1111-Agr-I, pJC1111-Agr-II, pJC1111-Agr-III, and pJC1111-Agr-IV (230). To explore the consequences of the Δ*mroQ* mutation in these strains, I assessed exoprotein levels, leukotoxin production, and hemolytic activity on rabbit RBCs.

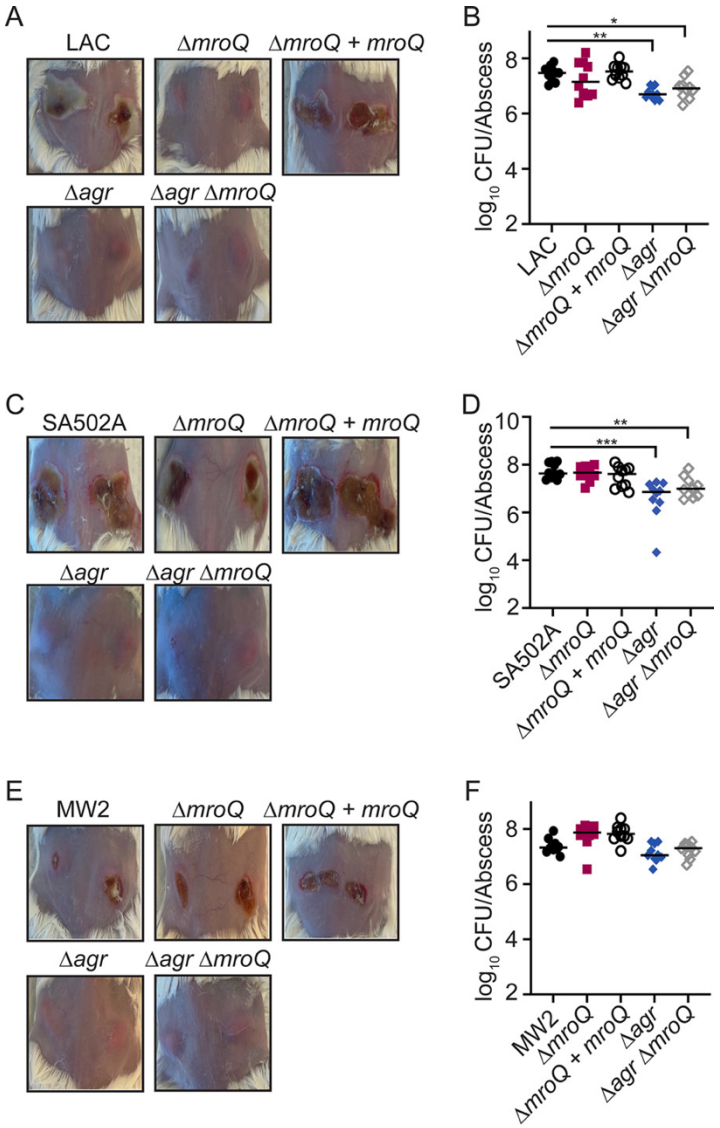

Figure 17. MroQ is Important for *S. aureus* Skin and Soft Tissue Infection in Agr Type I Strains. (A) Representative images of skin abscesses at 96 h after infection with LAC (WT), Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet*, and Δ*agr*::*tet* Δm*roQ* strains. (B) Bacterial burden in skin abscesses of mice at 96 h after infection with LAC (WT) (n = 10), Δ*mroQ* (n = 10), Δ*mroQ* + *mroQ* (n = 10), Δ*agr*::*tet* (n = 10), and Δ*agr*::*tet* Δ*mroQ* (n = 10) strains. (C) Representative images of skin abscesses at 96 h after infection with SA502A (WT), Δ*mroQ*, Δ*mroQ* + *mroQ,* Δ*agr*::*tet*, and Δ*agr*::*tet* Δ*mroQ* strains. (D) Bacterial burden in skin abscesses of mice at 96 h after infection with SA502A (WT) (n = 10), Δ*mroQ* (n = 10), Δ*mroQ* + *mroQ* (n = 10), Δ*agr*::*tet* (n = 10), and Δ*agr*::*tet* Δ*mroQ* (n = 10) strains. (E) Representative images of skin abscesses at 96 h after infection with MW2 (WT), Δ*mroQ*, Δ*mroQ* + *mroQ*, Δ*agr*::*tet,* and Δ*agr*::*tet* Δ*mroQ*. (F) Bacterial burden in skin abscesses of mice at 96 h after infection with MW2 (WT) (n = 10), Δ*mroQ* (n = 10), Δ*mroQ* + *mroQ* (n = 10), Δ*agr*::*tet* (n = 10), and Δ*agr*::*tet* Δ*mroQ* (n = 10) strains. *P* values were determined by a nonparametric one-way ANOVA (Kruskal-Wallis test) with Dunn's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

I observed decreased exoprotein levels and corresponding reductions in Hla and HlgC abundance in Δ*agr* Δ*mroQ* + pJC1111-Agr-I, Δ*agr* Δ*mroQ* + pJC1111-Agr-II, and Δ*agr* Δ*mroQ* + pJC1111-Agr-IV, but not Δ*agr* Δ*mroQ* + pJC1111-Agr-III (Figure 18A). In agreement with decreased hemolysin production, I also observed a reduction in hemolytic activity on rabbit RBCs upon addition of supernatant from Δ*agr* Δ*mroQ* + pJC1111-AgrI, Δ*agr* Δ*mroQ* + pJC1111-Agr-II, and Δ*agr* Δ*mroQ* + pJC1111-Agr-IV, but not Δ*agr* Δ*mroQ* + pJC1111-Agr-III (Figure 18B). These data suggest that MroQ is required for Agr activity of Agr-I, -II and -IV variants, but not Agr-III variants, regardless of strain background. This conclusion was further supported by data from Agr reporter inhibition assays which showed no reporter inhibition when conditioned media from Δ*agr* Δ*mroQ* + pJC1111-Agr-I, Δ*agr* Δ*mroQ* + pJC1111-Agr-II, and Δ*agr* Δ*mroQ* + pJC1111-Agr- IV was added to non-cognate reporter strains. In contrast, conditioned medium from the Δ*agr* Δ*mroQ* + pJC1111-Agr-III fully inhibited Agr reporter activity (Figure 18C).

Mice were infected intradermally with the same strains described above and I observed modest decreases in gross pathology for abscesses of mice infected with Δ*agr* Δ*mroQ* + pJC1111-Agr-I, Δ*agr* Δ*mroQ* + pJC1111-Agr-II, and Δ*agr* Δ*mroQ* + pJC1111-Agr-IV compared to Δ*agr* + pJC1111-Agr-I, Δ*agr* + pJC1111-Agr-II, and Δ*agr* + pJC1111-Agr-IV controls (Figure 19). In contrast, I saw similar ruptured, dermonecrotic abscess formation in animals infected with Δ*agr* + pJC1111-Agr-III and Δ*agr*Δ*mroQ* + pJC1111-Agr-III (Figure 19). These data indicate a potential requirement for MroQ in *S. aureus* virulence in Agr-I, -II and -IV isogenic strains, but not Agr-III.

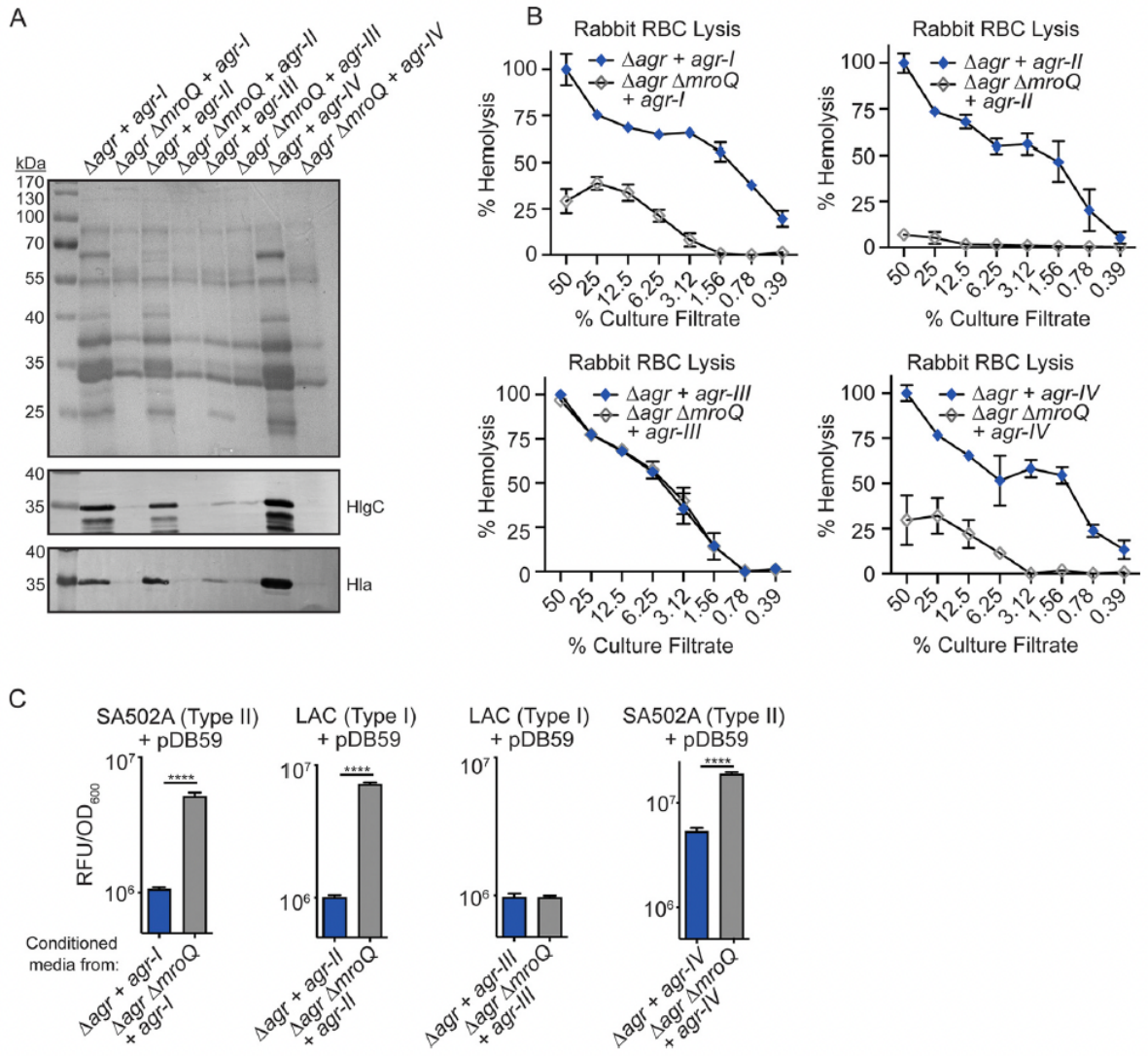

Figure 18. MroQ is Required for Agr Activity of Isogenic Strains Containing Agr-I, -II, and -IV but not Agr-III. (A) TCA-precipitated exoproteins and Hla and HlgC immunoblots from Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* strains in LAC (type I) complemented with the entire Agr locus from each Agr variant (+ *agr-I*, *agr-II*, *agr-III*, or *agr-IV*). (B) Rabbit red blood cell lysis of cell-free culture filtrates derived from Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* strains from LAC (type I) reconstituted with the entire Agr locus from each Agr variant (+ *agr-I*, *agr-II*, *agr-III*, or *agr-IV*). (C) pDB59 reporter activity (RFU/OD_{600}) of SA502A (type II) or LAC (type I) upon addition of conditioned medium from Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* strains in LAC (type I) reconstituted with the entire Agr locus from each Agr variant (+ *agr-I*, *agr-II*, *agr-III*, or *agr-IV*). Hemolysis and reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots and GelCode blue-stained gels are representative of at least four replicates. Means ± SD are shown (n = 3). ****, $P < 0.0001$ by a two-tailed *t* test.

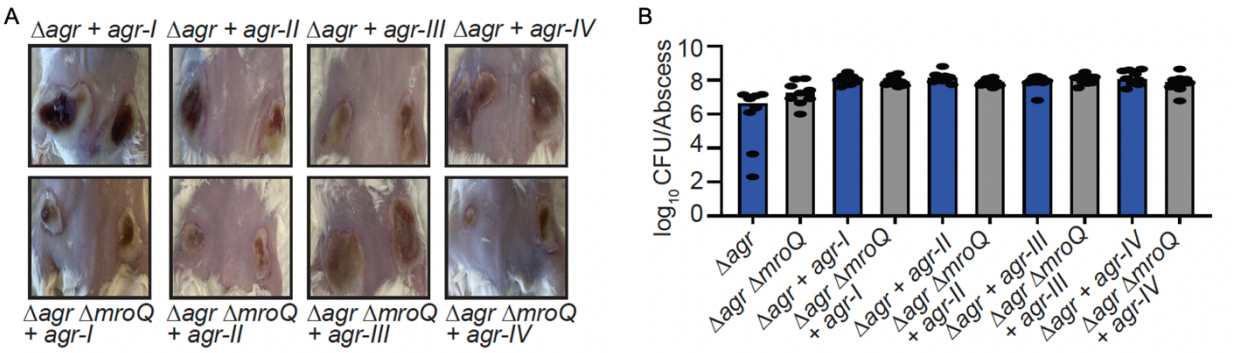

Figure 19. MroQ is Important for *S. aureus* Skin and Soft Tissue Infection in Isogenic Strains Containing Agr-I, -II, and -IV. (A) Representative images of skin abscesses at 96 h after infection with Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* strains in LAC (type I) complemented with the entire Agr locus from each Agr variant (+ *agr-I*, *agr-II*, *agr-III*, or *agr-IV*).. (B) Bacterial burden in skin abscesses of mice at 96 h after infection with Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* strains in LAC (type I) complemented with the entire Agr locus from each Agr variant (+ *agr-I*, *agr-II*, *agr-III*, or *agr-IV*). (n = 10).

A Leader-AIP-III Intermediate May Inhibit Non-Cognate Agr Systems.

A key remaining question is why Δ*mroQ* from MW2 has a functional Agr system despite an observed defect in peptide maturation. One possibility is that mature AIP is produced in Δ*mroQ* mutant at a low amount that would be enough to serve as a signal but not sufficient for visualization in my immunoblot analysis. This point is supported by work from Zhao et al, which identifies mature AIP-III in the supernatant of a Δ*mroQ* mutant from MW2. To ask whether small amounts of AIP-III are present in the supernatant of Δ*mroQ* mutant from MW2, conditioned medium from WT MW2 or a Δ*mroQ* mutant was serially diluted into fresh TSB to a final dilution ratio of $1:10^6$ TSB:conditioned medium. Each 10-fold dilution was then added to a non-cognate reporter strain and GFP activity was measured. Both WT MW2 and Δ*mroQ* strains displayed a similar loss of inhibition as CM concentration decreased, suggesting that both strains contain comparable amounts of inhibitory peptide (Figure 20A).

Another possibility is that an AIP processing intermediate could act as a signal to activate or inhibit Agr system activity. To see if this was the case, I expressed 6x-His-Leader-AIP-III-SSP in Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* mutants from MW2. This construct uses a fused split-intein from *Synechocystis* species PCC6803 (Ssp), which will spontaneously form a thioester ring. Placing SSP at the C-terminus of my construct allows for thiolactone ring formation in the absence of AgrB, which typically carries out cleavage events that result in this phenomenon. To ask whether Leader-AIP-III is sufficient for Agr system activation, I measured the activity of an Agr-III Reporter strain (MW2 Δ*agrD* + pDB59) upon addition of conditioned medium from WT MW2, Δ*mroQ*, Δ*agr*::*tet*, Δ*agr*::*tet* + pOS1-*6x-His-Leader-AIP-III-SSP*, or Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP*. While I observed reporter activation in the presence of conditioned medium from WT and Δ*mroQ*, conditioned medium from Δ*agr*::*tet* + pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP* strains did not cause reporter activation (Figure 20B). However, conditioned medium from Δ*agr*::*tet* + pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP* inhibited the reporter activity of non-cognate strains to similar levels observed with WT (Figure 20C). The reasons for the ability of these strains to inhibit a non-cognate reporter but not activate AgrC-III are currently under investigation. To verify that Leader-AIP-III is the primary species of AIP intermediate responsible for the phenotypes seen in Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP*, I visualized AgrD processing in Δ*agr*::*tet* + pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP* via immunoblot analysis.

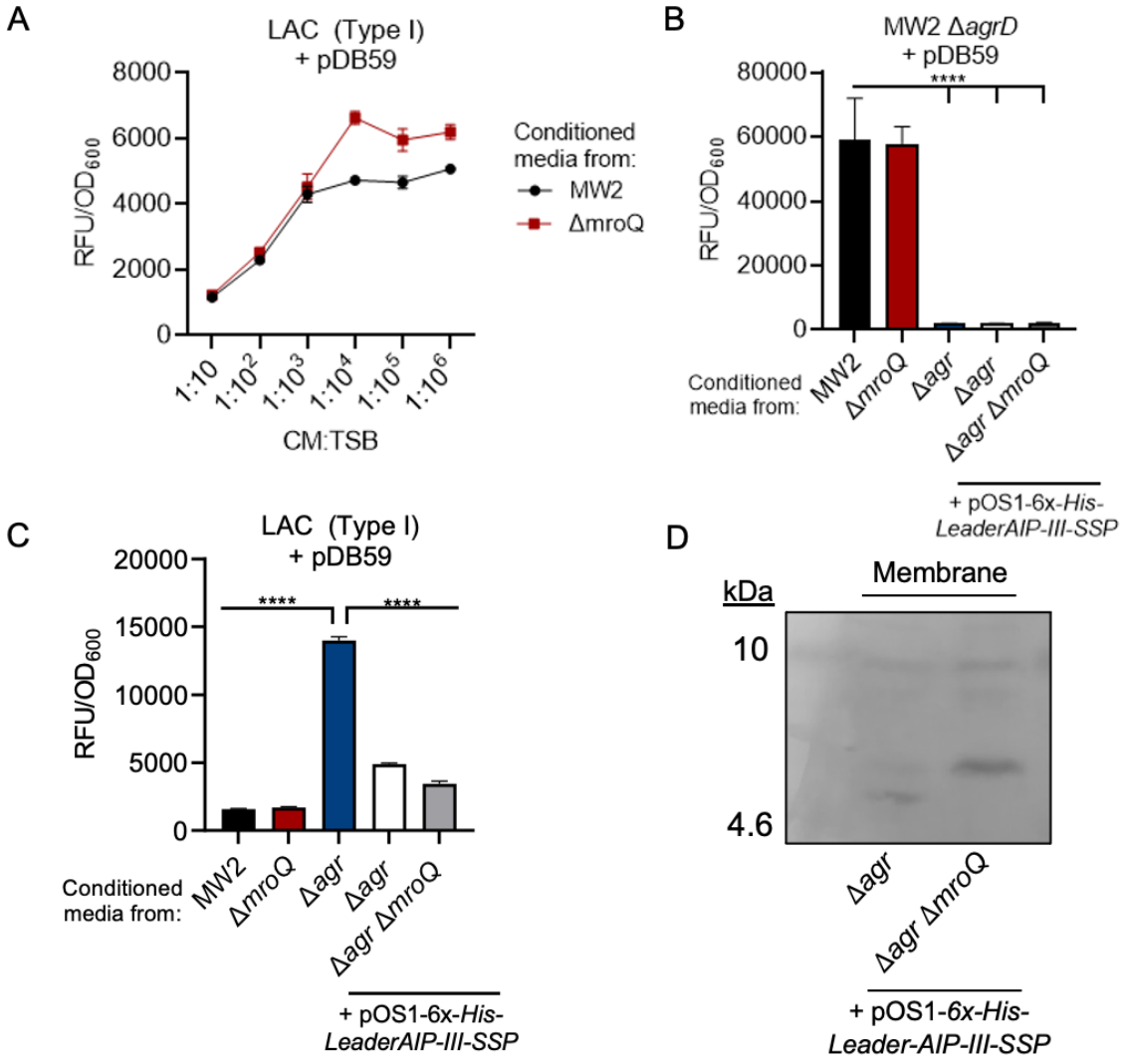

Figure 20. Agr III System Function in Δ*mroQ* May be Due the Presence of an Active Peptide Intermediate. (A) pDB59 reporter activity (RFU/OD_{600}) of LAC (type I) upon addition of conditioned medium from MW2 or Δ*mroQ* (B) pDB59 reporter activity (RFU/OD_{600}) of MW2 Δ*agrD* (type III) upon addition of conditioned medium from MW2, Δ*mroQ*, Δ*agr*::*tet*, Δ*agr*::*tet* + pOS1-*6x-His-Leader-AIP-SSP* and Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-SSP* strains in MW2 (type III). (C) pDB59 reporter activity (RFU/OD_{600}) of LAC (type I) upon addition of conditioned medium from Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* strains in MW2 (type III) expressing pOS1-*6x-His-Leader-AIP-SSP.* (D) Immunoblots of supernatant and membrane fractions from Δ*agr* and Δ*agr* Δ*mroQ* strains constitutively expressing 6×-His-Leader-AIP-III-SSP (pOS1-P_{sarA}-6×-*His-Leader-AIP-III-SSP*) using anti-His monoclonal antibody. Reporter assay data are from one of at least three experiments conducted in triplicate. Immunoblots are representative of at least four replicates. Means ± SD are shown (n = 3). ****, $P < 0.0001$ by one-way ANOVA with Tukey's posttest.

Immunoblot analysis of WT, Δ*mroQ,* and Δ*mroQ* + *mroQ* strains from MW2 revealed accumulation of a band that resembled Leader-AIP in the membrane fraction of the Δ*mroQ* strain, a species that resembled the Leader peptide in both the membrane and supernatant of WT and Δ*mroQ* + *mroQ* strains (Figure 16E). In keeping with these data, I observed the presence of only Leader-AIP-III peptide in the membrane fraction of Δ*agr*::*tet* Δ*mroQ* expressing 6x-His-Leader-AIP-SSP (Figure 20D). Further, Δ*agr*::*tet* expressing 6x-His-Leader-AIP-III-SSP carries out maturation of the peptide as normal, as there are Leader-AIP-III and Leader peptides in the membrane fraction (Figure 20D). Together, these data imply that AIP-III may not require full maturation to function as an antagonist for non-cognate AgrCs, which may partially explain the Agr^+ phenotypes observed in a Δ*mroQ* mutant from MW2. However, Zhao et al show the presence of mature AIP-III in the supernatant of Δ*mroQ* from MW2. Further experiments are required to better understand whether the inhibition observed is due to Leader-AIP-III or if mature AIP-III is still made in these strains.

MroQ Homologues From *S. epidermidis* and *L. monocytogenes* Share Similar Genomic Arrangement.

MroQ has similar proteolytic activity against the widely divergent *S. aureus agr* allelic variants. Given this conservation of function, I asked whether Agr systems in other Gram-positive bacteria rely on a similar protease. To this end, I used NCBI BLAST to determine if the amino acid sequence of MroQ from *S. aureus* shared sequence homology with proteins from other Gram-positive bacteria and identified candidates from *S. epidermidis, L. monocytogenes,* and *E. faecalis.* I chose to focus on possible MroQ homologues in *S. epidermidis* (*SERP_RSO7500*) and *L. monocytogenes*

(*lmo2070*), two Gram-positive bacteria which also rely on a homologous Agr system for production of QS autoinducers. These homologues share the EEXXXH motif present in members of the Type II CAAX protease family (Figure 21A).

In *S. aureus*, *mroQ* is found four genes upstream of the *agr* locus, just downstream of *groES*, which encodes for a protein folding chaperone (Figure 21B). Because its position four genes upstream of agr was an initial indicator to our lab that MroQ may be involved in Agr function, I examined the location of *SERP_RSO7500* and *lmo2070* in their respective genomes. Like *mroQ* in *S. aureus, SERP_RSO7500* is positioned four genes upstream of the Agr system and just downstream of *groES* (Figure 21B). To generate a Δ*SERP_RSOO7500* mutant in *S. epidermidis,* I used a plasmid-based mutagenesis approach with piMAY, a plasmid commonly used for allelic exchange in the Staphylococci. I constructed piMAY-Δ*SERP_RSO7500,* which should generate an in-frame deletion of *SERP_RSO7500* and attempted to transform it into *S. epidermidis.* However, initial efforts resulted in no mutant candidates (Figure 22A). Further, I determined that piMAY-Δ*SERP_RSO7500* did not efficiently integrate into the *S. epidermidis* chromosome (Figure 22B). To date, a Δ*SERP_RSO7500* strain from *S. epidermidis* has not been generated.

The organization of the *L. monocytogenes* genome with respect to *lmo2070* and the Agr system differs from its *Staphylococcus* counterparts. Here, *lmo2070* is not located near the Agr system; however, it maintains proximity to *groES* and is just upstream of a small, unannotated peptide (*lmo2071*) (Figure 21B).

A

```
L. monocytogenes  MAKHYITIVLVYLLLLFSASVGIPIVQHILLSTTSLSAESAASYGMVIWSIFSNLLSLAI
S. epidermidis    MKRLWVSLLTVLFYGLAQILPGLVLGSHLLGDMSKMQTAQTMII--MQVSIFI--IVALL
S. aureus         MTRLWASLLTVIIYILSQFLPLLIVKKLPFVQYSGIELTKAVIY--IQLVLFL--IAATT

L. monocytogenes  IIPMLYRKPKENKIEIGEKTKPLLSIVWIVGGVIGLYVAQIICSVIITMISGGLSNSANT
S. epidermidis    IIFMQATIKNPTQLEQGHKEPKRYIIAWVLLGFCIVMIYQFIISIILFAINGSPQRSPNT
S. aureus         IILINLKIKNPTKLELEVKEPKKYIIPWALLGFALVMIYQMVVSIVLTQIYGGQQVSPNT

L. monocytogenes  ELLVDLTKAAPIFLIFISILGPILEELVFRKVIFGGLSNVT------NIHVAAVISSLFF
S. epidermidis    ERLMDIAKQMPIFIVLISIVGPILEEYVFRKVIFGELYNFIKGSRVVSFIIASVVSSLIF
S. aureus         EKLIIIARKIPIFIFFVSIIGPLLEEYVFRKVIFGELFNAIKGNRIVAFIIATTVSSLIF

L. monocytogenes  GLLHGDISFLLTYFVIGLILCFLYTKTKRIAVSMGAHILMNTIVLLLSLGIIGG------
S. epidermidis    ALAHNDFKFIPVYFGMGVIFSLAYVYTKRIAVPIGIHMLMNGSVVLTQV--IGGDSIKKL
S. aureus         ALAHNDFKFIPVYFGMGVIFSLAYVWTKRLAVPIIIHMLQNGFVVIFQL--LNPEALKKA

L. monocytogenes  -------------
S. epidermidis    QEQATFIFHFIF-
S. aureus         TEQANFIYHIFIP
```

B

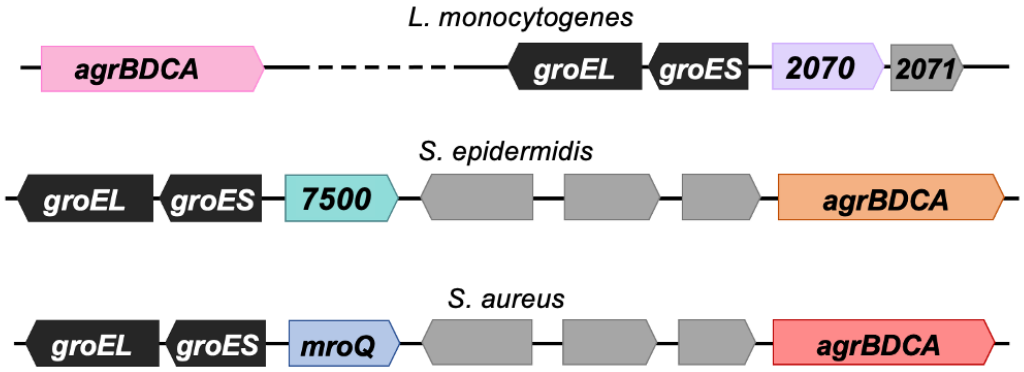

Figure 21. MroQ Homologues in *L. monocytogenes* and *S. epidermidis.* (A) Amino acid sequence alignments of MroQ and homologues from *L. monocytogenes, S. epidermidis,* and *S. aureus.* Underlined amino acids correspond to conserved active-site residues in MroQ. (B) Arrangement of MroQ and homologues in the genome of *L. monocytogenes, S. epidermidis,* or *S. aureus. 2070,* the MroQ homologue in *L. monocytogenes* (*lmo2070*)*; 2071,* small, unannotated peptide in *L. monocytogenes* (*lmo2071*); *7500,* the MroQ homologue in *S. epidermidis* (*SERP_RSO7500*).

Typically, the integration of this plasmid occurs following several passages in liquid growth media; however, *L. monocytogenes* containing pKSV7-Δ*lmo2070* would not grow at the non-permissive temperature at 40˚C in BHI supplemented with chloramphenicol, therefore preventing integration of the plasmid into the chromosome. To address a possible requirement for *lmo2070,* I generated pKSV7-*lmo2070*^{STOP},

which would introduce a stop codon into the gene. Like the in-frame deletion mutant, *L. monocytogenes* containing pKSV7-*lmo2070*^{STOP} would not grow at the non-permissive temperature at 40˚C in BHI supplemented with chloramphenicol. However, I was able to passage *L. monocytogenes* containing pKSV7-*lmo2070*^{STOP} on a BHI agar plate supplemented with CM, and the rest of the mutagenesis protocol was completed (Figure 22C). Following passages which promote excision of the plasmid (and presumably mutation of *lmo2070* at a rate of 50%), I was left with several candidates (Figure 22D). However, none of these candidates contained *lmo2070*^{STOP}*.* I have not yet been able to generate a Δ*lmo2070* or *lmo2070*^{STOP} strain in *L. monocytogenes.*

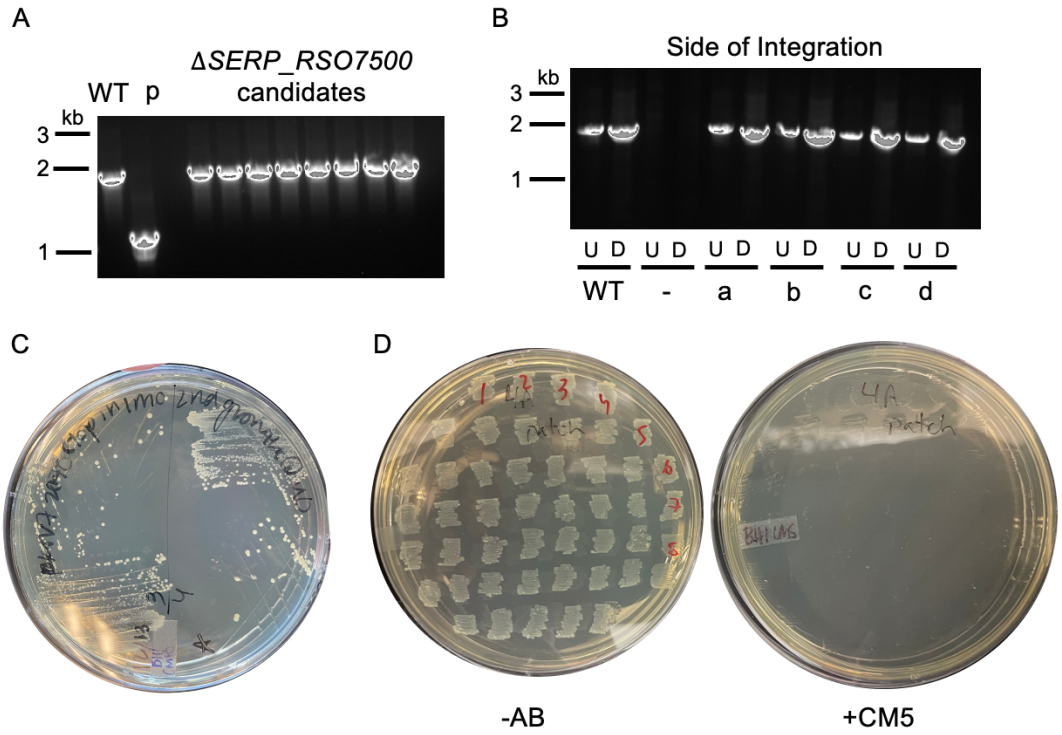

Figure 22. Attempts to Generate Δ*SERP_RSO7500* From *S. epidermidis* or *lmo2070*^{STOP} from *L. monocytogenes* Did Not Yield Mutant Strains. (A) PCR analysis of Δ*SERP_RSO7500* candidates following mutagenesis efforts. All candidates contained a wild-type allele. WT, *S. epidermidis* gDNA; p, pIMAY-Δ*SERP_RSO7500*. (B) Integration of pIMAY Δ*SERP_RSO7500* upstream (U) or downstream (D) of region of homology was checked using PCR. WT, *S. epidermidis* gDNA; a-d, Δ*SERP_RSO7500* candidates. (C) Growth of *L. monocytogenes* following two rounds of integration of pKSV7-*lmo2070*^{STOP}. (D) Growth of *lmo2070*^{STOP} from *L. monocytogenes* candidates on BHI (Left) or BHI + CM5 (Right).

CHAPTER FOUR

DISCUSSION

In this thesis, I explored how the membrane peptidase MroQ promotes virulence traits and maturation/export of AIP in strains of *S. aureus* that harbor four allelic variants of AgrD. My data suggest a requirement for MroQ in at least one step of AgrD processing among all four Agr variants. However, while MroQ-dependent AgrD maturation was important for the generation of active AIP in Agr-I, -II and -IV strains, it was not required for activation of the Agr-III system. These observations were recapitulated in vivo, where virulence was independent of MroQ in an Agr-III strain. Studies suggest that an AIP-III processing intermediate may be able to act as an active AIP, which could explain why a Δ*mroQ* mutant from MW2 maintains an active Agr III system despite defective AIP-III production. Overall, my data argue that MroQ is a mediator of AIP processing and export that promotes quorum sensing and virulence factor gene expression in *S. aureus* with implications for MroQ-like proteins in the function of Agr systems in other bacteria.

MroQ Facilitates AIP Maturation

The final steps of AgrD processing and export have remained elusive. Here, through use of 6x-His-AgrD expression plasmids, Dr. Liwei Fang and I showed an accumulation of a Leader-AIP peptide intermediate in the membrane fraction and a corresponding loss of Leader peptide (reflecting reduced generation of mature AIP) in the supernatant fraction of Δ*mroQ* strains of Agr-I, -II, and -III variants suggesting MroQ

promotes these final steps of peptide maturation and export (Figures 13A and C, 14E, 16E).

While this work was being conducted, Zhao et al provided evidence for a direct role of MroQ in leader peptide cleavage to generate mature AIP-I and AIP-II, but not AIP-III (246). Though these biochemical assays do not demonstrate a role for MroQ in the generation of mature AIP-III, they suggest that MroQ does cleave AgrD-III at the N-terminus; however, the resulting AIP contains an additional amino acid. This work successfully reconstituted the Agr quorum sensing circuit with purified components and proposed a model for how MroQ generates mature AIP-I and AIP-II via amino acid differences in the linker region between the α-helical leader peptide and AIP. Here, I corroborate these findings by demonstrating that MroQ-mediated processing of AIP-I, -II, and -IV is required for Agr system activation, whereas MroQ-mediated AIP-III processing is dispensable for Agr system activation in Agr-III-containing strains (Figures 12-16). In addition, I expand upon these studies by demonstrating MroQ-dependent impacts on peptide processing and export within live bacteria (Figures 12-16, 18).

MroQ-Mediated AIP Maturation is Not Required for Agr III Function

Despite an apparent defect in peptide maturation by immunoblot, Δ*mroQ* mutant strains from MW2 (Agr-III) and RN3984 (Agr-III) maintained the ability to activate AgrC (Figure 15A-G). This unusual observation suggests either an AgrD-III processing intermediate is sufficient to activate the Agr system or the activity of MroQ can be bypassed by an alternative protease to generate sufficient AIP-III for activity. In consideration of the first possibility, Zhao et al recently showed that recombinant AgrB,

MroQ, and AgrD were sufficient for the generation of mature AIP-I and AIP-II, but not AIP-III which contained an intermediate with an additional N-terminal tyrosine (246). In contrast to these biochemical assays, MS-based peptide analysis of supernatant of a Δ*mroQ* mutant from Agr-III strain MW2 identified significant amounts of mature AIP-III (246). Thus, in keeping with my data, the work of Zhao et al suggests MroQ can cleave AgrD-III, yet it is not necessary for the generation of active AIP-III.

Leader-AIP-III May Act as an Inhibitory Peptide

Attempts to ask about the ability of Leader-AIP-III to activate AgrC-III using Δ*agr*::*tet* +pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP* from MW2 were unsuccessful. In particular, conditioned medium from the Δ*agr*::*tet* +pOS1-*6x-His-Leader-AIP-III*-SSP strain should have caused reporter activation, as AIP-III maturation seems to have proceeded as normal based on immunoblot analysis. However, it is possible that while AIP-III was matured, it was not released, as data from Dr. Liwei Fang supports a role for AgrB-I in release of mature AIP-I. To address this possibility, I am currently generating Δ*agrD* +pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agrD* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III*-SSP strains. These strains will not produce their own AIP but will maintain AgrB.

Despite issues with the ability of Δ*agr*::*tet* +pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agr*::*tet* Δ*mroQ* + pOS1-*6x-His-Leader-AIP-III-SSP* from MW2 to activate AgrC-III, I observe that conditioned medium from these strains inhibits AgrC-I. Assuming that Leader-AIP-III is the primary species of inhibitory peptide, these data contrast the idea that an AIP-III processing intermediate is inactive. However, it is possible that mature AIP-III is present. Further, given the issues related to activation of AgrC-III, it is also

possible that no AIP-III species is present at all in the supernatant and that the inhibition observed is due to another inhibitory peptide entirely. Optimization of reporter assays with Δ*agrD* +pOS1-*6x-His-Leader-AIP-III-SSP* and Δ*agrD* Δ*mroQ* +pOS1-*6x-His-Leader-AIP-III*-SSP strains will help to better understand these data.

Several prior reports established a requirement for precise AIP-III N-terminal tail length for efficient AgrC-III activation (239,336). In these reports, the addition of as little as a single N-terminal tyrosine to AIP-III caused inactivation of AIP-III (239). Work from Zhao et al show that this species of AIP-III is generated following a reaction with recombinant AgrB-III, AgrD-III and MroQ, yet they identify correctly processed AIP in supernatant from Δ*mroQ* from MW2 (246). What remains unresolved is whether an AIP-III intermediate is able to activate AgrC-III and/or inhibit non-cognate systems.

A finding that Leader-AIP-III is sufficient for activation of AgrC-III and inhibition of non-cognate AgrCs would contrast the notion that AIP intermediates are inactive. It is possible that while Leader-AIP-III is active, any intermediate between Leader-AIP-III and AIP-III is inactive. This would explain previous data which show inactivation of AIP-III upon addition of an N-terminal tyrosine. Whether the AIP-III intermediate identified by Zhao et. al exhibits a similar inactivity is not known. Despite these remaining questions regarding the activity of miscellaneous AIP-III processing intermediates, my data show that Leader-AIP-III can inhibit AgrCs, suggesting a possible mechanism by which the Agr system in a Δ*mroQ* strain from MW2 remains functional.

Mass spectrometry analysis of supernatant from a Δ*mroQ* mutant from MW2 by Zhao et al revealed substantial amounts of mature AIP-III. These data contrast with my immunoblot analysis, where I do not see the presence of the Leader peptide in the

membrane fraction or supernatant of a Δ*mroQ* mutant from MW2. It is possible that there are limitations to my experimental analysis. Namely, there could be mature AIP-III present in the supernatant of a Δ*mroQ* mutant from MW2 that is not visible via my immunoblot analysis. However, reporter assays suggest that WT MW2 and a Δ*mroQ* mutant contain similar levels of inhibiting peptide. If the inhibitory peptide from a Δ*mroQ* mutant from MW2 was mature AIP-III, it would likely be visible via immunoblot, as I observe the presence of Leader peptide in the supernatant from WT MW2. Further, strains expressing Leader-AIP-III demonstrate an ability to inhibit non-cognate Agr systems.

While immunoblot analysis of these strains shows the presence of only Leader-AIP-III, I cannot be certain that mature AIP-III is not present as well. Another possibility is that bands observed via immunoblot analysis of strains from MW2 do not correspond to the intermediates I have predicted. For immunoblot analysis of strains from LAC (Agr I) and SA502A (Agr II), peptide controls for processing intermediates were included; however, Dr. Fang and I were unable to generate these controls for MW2 (Agr III). The only way to fully address this discrepancy would be to use mass spectrometry to analyze the AIP species present in the supernatant of WT MW2, Δ*mroQ*, Δ*agr*::*tet* + pOS1-6x-His-Leader-AIP-III-SSP and Δ*agr*::*tet* Δ*mroQ* + pOS1-6x-His-Leader-AIP-III-SSP strains.

MroQ Function is Conserved Across Strain Backgrounds

To interrogate if MroQ was sufficient for AIP-I-IV maturation in an isogenic strain background, I reconstituted Δ*agr*::*tet* and Δ*agr*::*tet* Δ*mroQ* mutants of LAC (Agr-I) with the entire Agr locus from each variant. I observed a loss of Agr system activation

in Δ*agr*::*tet* Δ*mroQ* mutant strains expressing the Agr-I, -II and -IV loci but not a Δ*agr*::*tet* Δ*mroQ* mutant expressing the Agr-III locus, supporting a requirement for MroQ-mediated maturation of AIP-I, -II and -IV, but not AIP-III (Figure 18). Additionally, reconstitution of a Δ*agr*::*tet* mutant from MW2 (Agr-III) with the Agr-I locus results in production of mature AIP-I, whereas reconstitution of a Δ*agr*::*tet* Δ*mroQ* mutant with Agr-I does not, suggesting that MroQ is active in Agr-III-containing strains (Figure 16A-C). The phenotypic similarities between isogenic mutants reconstituted with *agr-I-IV* and native strains containing *mroQ* mutations argues that MroQ function is conserved in all strain backgrounds and suggest that MroQ impacts the pathogenesis of strains harboring Agr-I, -II, and -IV, but not Agr-III, in a skin and soft tissue infection model. Furthermore, dispensability of MroQ for maturation of AIP-III in strain LAC suggests that whatever alternative protease(s) or factors facilitate processing of AIP-III must also exist in an Agr-I strain background.

MroQ maintains remarkable sequence conservation across strains of *S. aureus* that harbor each Agr allelic variant, with 100% amino acid identity across Agr-I, -II, and -III-containing strains and 95% identity in Agr-IV-containing strains. The reduced conservation of MroQ from Agr-IV strains represents 12 of 247 amino acids. It remains plausible that MroQ-mediated maturation of AgrD-IV is impacted by these amino acid differences, however, my isogenic reconstitution studies indicate MroQ from a Type I strain can promote maturation of AIP-IV (Figure 18). The catalytic residues E141, E142, and H180, remain conserved in the sequence of MroQ from Agr-IV-containing strains (244). Further, 3D modeling revealed a predicted structure identical to that of the MroQ from Agr-I, -II, and -III-containing strains, potentially

indicating this limited divergence in sequence identity is not critical for enzymatic activity or structural integrity.

The Role of MroQ During Infection

My work highlights the impact of MroQ on the pathogenesis of Agr-I (LAC), Agr-II (SA502A), and Agr-III (MW2) strains. Infection of mice with a Δ*mroQ* mutant of strain LAC has substantially reduced abscess pathology compared to animals infected with the parental WT strain (Figure 17A-B). This contrasts with what is seen for animals infected with a Δ*mroQ* mutant from SA502A (Agr Type II) and MW2 (Agr Type III), which exhibited pathology similar to animals infected with WT SA502A or MW2 (Figure 17C-F). These data cement a requirement for MroQ in the severity of Agr-mediated skin pathology for a Type I Agr system, but not Type II or Type III. This observation suggests that despite decreased Agr system activation of an SA502A Δ*mroQ* mutant in vitro, the strain has restored Agr activity in vivo, possibly due to host protease-mediated maturation of AIP-II or host-mediated induction of *S. aureus* proteases capable of facilitating AIP-II maturation. Animals infected with SA502A strains have substantially larger abscess formation compared to those infected with LAC or MW2, suggesting that this strain is more virulent in a skin and soft tissue infection model. It is possible that the abscess formation seen in a Δ*mroQ* mutant from SA502A is a consequence of bacterial dosage. Because SA502A seems to be more virulent in a skin and soft tissue infection model, it may require a lower initial dose of bacteria. In this condition, differences in abscess pathology between animals infected with WT SA502A and Δ*mroQ* mutant strains may be observed. This notion is supported by infection data from isogenic mutants complemented with *agr-II,* where animals infected with Δ*agr*::tet Δ*mroQ* +

pJC1111-*agr-II* have reduced abscess pathology compared to animals infected with Δ*agr*::*tet* + pJC1111- *agr-II* (Figure 19A). Of note, while I observed similar modest reductions in CFU among animals infected with Δ*mroQ* and Δ*agr* mutants from the LAC strain background (244,245), this was not true for all strains and infection conditions, where CFU were largely unchanged. Given the differences in abscess size and severity between animals infected with LAC, SA502A or MW2, it is reasonable to assume these strains do not have the same level of virulence in skin and soft tissue infection model. As such, it is possible that bacterial dosage used in this infection was not ideal for observing differences in CFU. Despite similar levels of bacteria, I saw significant differences in abscess pathology between WT and Δ*agr*::*tet* mutant strains, irrespective of background. These data speak to the contributions of Agr to abscess pathology stem primarily from the well-established role for Hla and PSMs in promoting tissue damage during skin infection, though impacts on bacterial burden are less defined (337–340).

MroQ and AIP Release

How and if MroQ contributes to AIP release is unknown. My data demonstrate that peptide processing intermediates accumulate at the *S. aureus* membrane and are not appreciably released from the cell in Δ*mroQ* mutant strains. Translocation of AIP must occur before or after the final N-terminal processing step and MroQ could play a direct or indirect role in this process. Though the translocation-first model has been favored, the lack of an ATP-binding cassette in either AgrB or MroQ does not support a role for active transport by either protein (231,243,246). Another possible mechanism of release could involve the insertion of the N-terminal leader of AgrD into the membrane

in a way that positions the peptide for processing by an AgrB – MroQ complex, with subsequent passive diffusion of AIP outside of the cell.

Alongside Dr. Liwei Fang, I have initiated experiments to better understand the mechanisms of AIP maturation and release and the contributions of AgrB and MroQ to this process. Amongst these is work to interrogate the physical relationship between MroQ and AgrB and its impact on the release of mature AIP. In these experiments, I attempted to ask if MroQ and AgrB-I interact via co-immunoprecipitation using a FLAG-tagged MroQ and a His-tagged AgrB. Because I had already generated a Δ*mroQ* strain from LAC expressing pJC1111-FLAG-*mroQ*, I first tried to introduce nucleotides that encode a *6x-His* upstream of *agrB* in this background via allelic exchange. However, I had difficulties when tagging AgrB, as efforts to add a N-terminal His tag rendered AgrB non-functional. Because *agrB* overlaps *agrD,* I was unable to use allelic exchange to add nucleotides that encode *for 6x-His* downstream of *agrB.* Rather, I generated a plasmid which expresses *agrB-His* under a constitutive promoter (pOS1-P_{sarA}-sod_{RBS}-*agrB-6x-His*). I found that this expression of *agrB-6x-His* toxic to *S. aureus.* This toxicity is likely due to the methods of expression of AgrB rather than the C-terminal tag, as previous work has successfully generated a functional AgrB-His (241). That work also expresses *agrB-6x-His* under the control of a constitutive promoter on a high copy plasmid, though these differ from the ones I used. It is possible that the toxicity I observed upon introduction of pOS1-P_{sarA}-sod_{RBS}-*agrB-6x-His* into *S. aureus* is a consequence of the plasmid or promoter chosen. Future efforts will focus on generating a Δ*agrB* + pJC1111-P_{HELP}-*agrB-6x-His* from LAC then introducing nucleotides that encode for a FLAG tag upstream of *mroQ* in this background via allelic exchange.

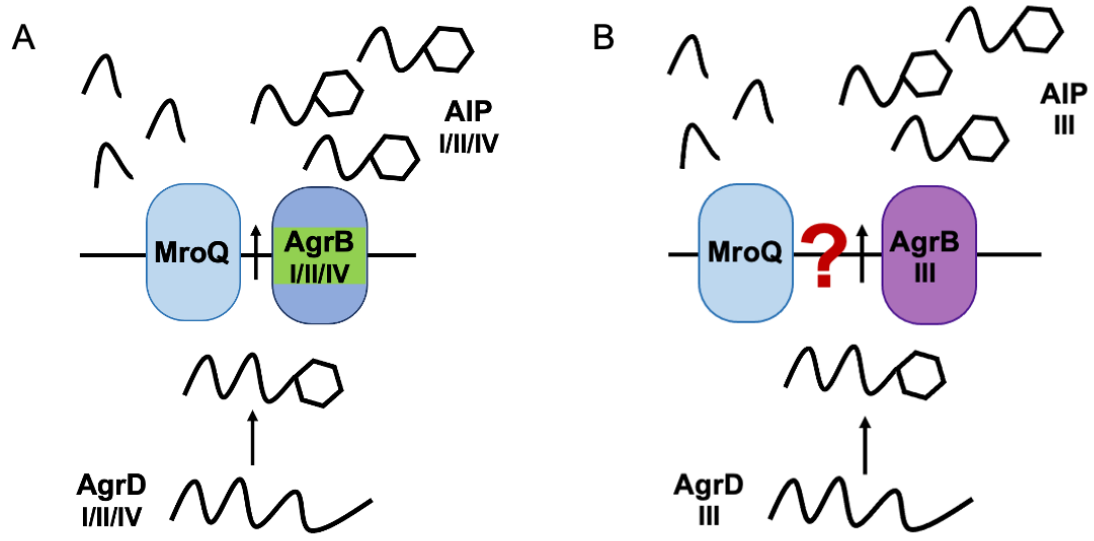

Figure 23. A Revised Model for AgrD Processing. Modification of AgrD occurs over a series of steps. AgrB cleaves the C-terminus, causing formation of a thiolactone ring. For strains harboring Agr-I, -II, and -IV, MroQ cleaves the N-terminus, and the mature AIP and N-terminal leader peptide intermediate are released into the supernatant. The mediators of N-terminal cleavage of AgrD-III are still unclear, though my data support a possible role for MroQ in this process. Whether AIP maturation occurs through a concerted effort by an MroQ – AgrB complex is yet to be determined.

Though I haven't yet been able to examine the physical relationship between AgrB and MroQ via co-immunoprecipitation, work from the dissertation of Stephanie Marroquin showed an interaction between AgrB and MroQ via bacterial two-hybrid (341). While these data are compelling and suggests AgrB and MroQ interact, our efforts to express MroQ in *E. coli* have resulted in several substitutions within the protein. As such, this bacterial two-hybrid data should be confirmed using an analysis where MroQ is expressed in *S. aureus.* Confirmation of a physical relationship between AgrB and MroQ would provide insight into the mechanisms of AIP maturation and release (Figure 23).

Dr. Fang has continued work to better understand the process of AIP production. The current understanding of AIP maturation is that AgrD is post-translationally modified through a series of events that begins with cleavage at the C-terminus by AgrB and subsequent thiolactone ring formation (Figures 8 and 23). Most often, this cleavage event is believed to occur first because AgrD peptides without a thiolactone ring are targeted for degredation. However, recent work from Dr. Fang has demonstrated that MroQ is able to cleave an AgrD processing intermediate that does not contain a thiolactone ring. This suggests that initial cleavage by AgrB at the C-terminus may not occur first or that this cleavage could occur in any order, even simultaneously with MroQ-mediated processing at the N-terminus (Figure 23). A concerted effort such as this would likely require a physical relationship between AgrB, MroQ, and AgrD. Further, while my immunoblot analysis implies a requirement for MroQ in AIP release, Dr. Fang has observed that AgrB is also required for this event. Together, these data support a model where AgrB and MroQ may contribute to AIP maturation as a complex, with cleavage by each protease at their respective terminus in close succession before release of the resulting peptide products (Figure 23). How these proteins contribute to release and whether release is more dependent on one or the other is still unclear.

MroQ Homologues in Other Gram-Positive Bacteria

Defining a conserved region or regions that confer MroQ specificity for Agr peptide maturation could give insight into MroQ function in other AIP-containing Gram-positive bacteria. Notable bacteria with Agr systems include *Clostridioides difficle, Enterococcus faecalis, Staphylococcus epidermidis*, and *Listeria monocytogenes* (34,211,280,282,288,342,343). Studies from Olson et al. revealed a requirement for

Agr in *S. epidermidis* skin infection models, highlighting the relevance of this system across multiple species (291). In *S. epidermidis* and *L. monocytogenes*, AIP maturation is believed to occur in a manner similar to that of *S. aureus* (214,291). While AgrB has been implicated in this process, whether other proteases facilitate AIP maturation in these bacteria is less clear (211,214,288,291,344,345).

I have identified MroQ homologues in both *S. epidermidis* (63% amino acid identity), *L. monocytogenes* (37% amino acid identity), and *E. faecalis* (38% amino acid identity). Given the ability of MroQ to exert a conserved function across widely divergent *S. aureus* AgrD sequences, it is certainly feasible that this function could at least be maintained in *S. epidermidis, E. faecalis,* and/or *L. monocytogenes.* Conservation of MroQ function across species would provide tremendous insight into peptide processing in Gram-positives.

In addition to sequence similarity, the MroQ homologues from *S. epidermidis* (*SERP_RSO2700*) and *L. monocytogenes* (*lmo2070*) are also similarly arranged in their respective genome with respect to the *agr* locus and *groES. SERP_RSO7500* is four genes upstream of the *S. epidermidis agr* locus and just downstream of *groES* (Figure 21B). Though I was unable to generate a Δ*SERP_RSO7500* strain from *S. epidermidis,* this arrangement and the close relation of *S. epidermidis* and *S. aureus* Agr systems suggest that *SERP_RSO2700* could serve as a protease which facilitates AIP maturation in *S. epidermidis. lmo2070* is not located near the *L. monocytogenes agr* system in the genome, but is downstream of *groES*, like its staphylococcal counterparts. Further, *lmo2070* is directly upstream of a small peptide (*lmo2071*) similar in size to AgrD (Figure 21B). Though *lmo2070* is not near the *agr* locus, its homology to MroQ still

suggests a possible proteolytic function. Whether this is toward the *L. monocytogenes* AgrD, *lmo2071,* or both is still unclear.

Despite multiple attempts, I have yet to generate a Δ*lmo2070* strain from *L. monocytogenes.* Initial trials lead to bacterial death upon shift to the 40˚C nonpermissive temperature in the presence of chloramphenicol, suggesting either an experimental issue is occurring or that *lmo2070* is essential. To identify if initial trials failed due to experimental error, I obtained a pKSV7 plasmid construct from Dr. Nancy Freitag that has been previously used to delete a known transcription factor in *L. monocytogenes.* Again, I observed bacterial death upon shift to the 40˚C nonpermissive temperature in the presence of chloramphenicol. This result suggests that an experimental error is preventing generation of a Δ*lmo2070* mutant. Given that mutagenesis attempts are failing at the step which promotes integration of pKSV7 into the *L. monocytogenes* chromosome, it is likely that an issue lies in the competent *L. monocytogenes* cells being used. Future attempts to generate dlmo2070 mutants should begin with preparation of a new stock of competent *L. monocytogenes* cells.

Summary

Altogether, this work reinforces the prevailing model that MroQ mediates AIP processing and release in *S. aureus.* Further, this work demonstrates a conservation of function on a range of peptide precursors, a remarkable phenomenon considering the divergent peptide sequence. Additionally, my data indicate that AIP-III may be unique in its ability to be active without full maturation, a phenomenon not previously appreciated in *S. aureus* Agr systems. Work from myself and Dr. Fang has allowed for the asking of more specific questions about AIP maturation and release and the

contributions of MroQ and AgrB to this process. Overall, my studies add to the rapidly evolving knowledge of MroQ and its role in Agr system activation. This work on MroQ in also expands our understanding of the myriad roles of Type II CAAX protease family proteins in *S. aureus.* More broadly, the presence of MroQ homologues in other Gram-positive bacteria could reveal a group of bacterial Type II CAAX proteases with a defined function in peptide maturation, a finding which would contribute to the growing knowledge of the role of this protein family in bacteria.

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