Peptide Signaling in the Staphylococci

Matthew Thoendel, Jeffrey S. Kavanaugh, Caralyn E. Flack, and Alexander R. Horswill*

Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, United States

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* To whom correspondence should be addressed. Phone: 319 335 7783. Fax: 319 335 8228. E-mail: alex-horswill@uiowa.edu. Address: 540F EMRB, University of Iowa, Iowa City, Iowa 52242.

1. Introduction

Gram-negative and Gram-positive bacteria have evolved elaborate machinery to biosynthesize and respond to diverse small-molecule signals. As bacteria grow, these signals accumulate in the extracellular environment until a particular concentration is reached, usually at a specific cell density or "quorum", activating a regulatory cascade that controls some type of cellular process. This phenomenon is generally referred to as "quorum-sensing" and has been the subject of many excellent review articles.[1−3] The general paradigm is that Gram-negatives recognize small chemical compounds called *N*-acyl homoserine lactones that are membrane permeable and bind to a cytoplasmic receptor in order to exert a regulatory output. In contrast, the Gram-positives recognize peptides with diverse post-translational modifications using either a membrane-bound histidine kinase or cytoplasmic receptors.

Among the Gram-positives, the size and structure of these peptide signals vary widely depending on function and the

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Matthew "Magnet" Thoendel was born in David City, Nebraska. He received his B.Sc. in microbiology at the University of Iowa while studying acid sensing ion channels and *Pseudomonas aeruginosa* biofilms. He is currently earning his M.D./Ph.D. at the University of Iowa studying various aspects of *Staphylococcus aureus* physiology in Dr. Alexander Horswill's laboratory. While his work primarily focuses on the biosynthesis of signaling peptides, he also investigates other areas such as biofilm formation, stress response systems, and host−pathogen interactions. His background in microbiology has also lead to the interesting hobbies of homebrewing and charcuterie.

Jeffrey S. Kavanaugh received degrees in biochemistry from the University of Vermont (B.Sc., 1986) and the University of Iowa (Ph.D., 1992). As a graduate student in the laboratory of Dr. Arthur Arnone, he trained as an X-ray crystallographer and conducted high-resolution X-ray crystallographic analyses on mutant human hemoglobins. Upon completion of his graduate work, he remained in the Arnone lab as a research scientist to continue studies on hemoglobin's stereochemical mechanism with several internationally renowned researchers in the field of hemoglobin allosterism. In 2005, he joined Dr. Alexander Horswill's laboratory in the Microbiology Department at the University of Iowa, and he is involved in many aspects of the group's investigations on virulence regulation and biofilm formation by *Staphylococcus aureus*.

Caralyn E. Flack earned a degree in Physics from Bemidji State University (B.Sc., 2006). During her undergraduate studies, she participated in an astronomy research experience for undergraduates (REU) at the University of Wisconsin—Madison, studying the interstellar medium, and a physics REU at the University of California—Davis studying gravitational lens systems. Alhough she enjoys astronomy research, she also has a strong interest in pathogenic bacteria. She is currently a graduate student at the University of Iowa in Dr. Alexander Horswill's laboratory and is studying *Staphylococcus aureus* signal transduction.

Alexander R. Horswill was born in 1973 in Rochester, Minnesota. He studied bacteriology at the University of Wisconsin−Madison as an undergraduate and continued on as a graduate student in the laboratory of Dr. Jorge Escalante-Semerena. He completed his Ph.D. in 2001, researching short-chain fatty acid catabolism in *Salmonella*. For postdoctoral studies, he joined the group of Dr. Stephen J. Benkovic at Pennsylvania State University and studied enzyme engineering and peptide cyclization with inteins. In 2005, he joined the Microbiology Department at the University of Iowa as an Assistant Professor. His research program focuses on *Staphylococcus aureus*, with an emphasis on quorum-sensing mechanisms and inhibition, biofilm maturation and dispersal, and host−pathogen interactions.

producing bacterium and published examples of these regulatory mechanisms have become abundant. As notable examples, *Streptococcus pneumoniae* regulates competence with a 17-residue linear peptide.[4] *Bacillus subtilis* regulates sporulation and competence with a series of linear peptides,[5] one of which is post-translationally modified.[6] *Bacillus cereus* regulates the expression of virulence factors and *Enterococcus faecalis* controls plasmid-mediated conjugation with various linear peptides.[7,8] As this quick overview demonstrates, peptides are regulating an impressive array of cellular events, and this list continues to grow as additional systems are being discovered.

One of the more intriguing classes of peptide signals are the cyclic lactones and thiolactones. The first of these cyclic peptide signals was discovered in *Staphylococcus aureus* and is the focus of this review article. The peptide signal controls an autoactivation circuit and hence is referred to as an autoinducing peptide or "AIP". With the surge of studies on quorum-sensing and bacterial genome sequencing, it is now evident that the AIP scaffold and autoactivation circuitry is conserved among many Gram-positive bacteria.[9] Notably, all of the staphylococcal species make similar AIP structures,[10,11] and in recent studies, related signals have been identified in *Enterococcus faecalis*,[12−14] *Lactobacillus plantarum*,[15,16] *Listeria monocytogenes*,[17,18] *Clostridium perfringens*,[19,20] and *C. botulinum*.[21] Genome mining has revealed additional *agr*-like systems in other Gram-positives,[9] such as the outbreak *C. difficile 027* strain[22] and in some species of *Bacillus*.

In this review, we will focus on the accessory gene regulator or "*agr*" quorum-sensing system in *S. aureus* as a

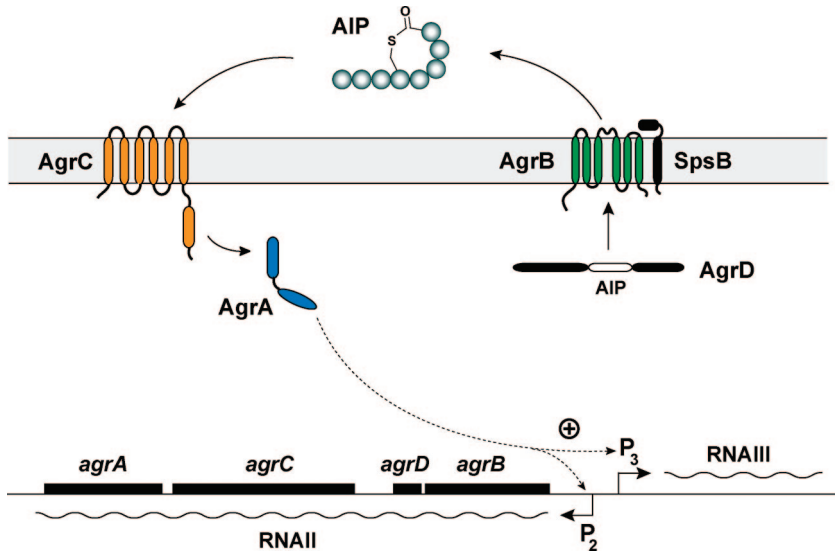

Figure 1. Schematic of the *agr* system in Staphylococci. The locus is composed of two divergent transcripts called RNAII and RNAIII, driven by the P2 and P3 promoters, respectively. The RNAII transcript is an operon of four genes, *agrBDCA*, which encodes the core machinery of the system. AgrD is the peptide precursor and is processed and exported through AgrB and SpsB action at the cytoplasmic membrane. SpsB is the house-keeping type I signal peptidase. At the threshold concentration, autoinducing peptide (AIP) binds to the AgrC receptor, a membrane-bound histidine kinase. AIP binding activates the AgrC kinase, resulting in phosphorylation of the AgrA response regulator and activation of the P2 and P3 promoters. AgrA also activates the PSMα and PSMβ promoters (not shown). This figure is an adaptation of the version reported by Thoendel et al.[66] Reprinted with permission from ref 66 Copyright 2009 The American Society for Biochemistry and Molecular Biology, Inc.

paradigm model. We will describe what is known about the function of each gene product in the *agr* locus and the mechanism of signal production. Signal sensing and output will be reviewed, along with the contribution of other regulatory inputs to *agr* function. We will also describe the current status of *agr* in biofilms and pathogenesis and outline the latest advances in *agr*-targeted therapies. Finally, the similarities and differences of the *agr* system in other Staphylococci will be described.

2. Overview of agr

S. aureus is a remarkable bacterial pathogen that is known for causing a diverse array of acute and chronic infections.[23,24] This pathogen secretes an impressive arsenal of virulence determinants to combat the host, including pore-forming toxins, tissue degrading enzymes, and immune evasion factors. It has been appreciated for many years that this secreted assortment of proteins is temporally regulated by growth phase.[25] Early studies on *S. aureus* mutants provided preliminary indications that a master regulator of virulence factor expression might exist. Mutants were isolated based on the loss of a single secreted factor (e.g., protease, α-toxin) and were subsequently found to have altered regulation of other proteins.[26−29] In follow-up analysis of the mutants, reduced levels of toxins and the proteolytic enzymes was apparent, while at the same time production of surface proteins, such as protein A, was increased.[27,29] The chromosomal region responsible for these changes was given various names including *exp*, *hla*, and finally *agr*.[26−28]

The *agr* genetic locus was mapped using transformation and linkage analysis and localized to a region between the *S. aureus purB* and *ilv-129* genes.[26,30] Sequencing the flanking regions of the transposon insertions identified the *agrA* gene, later found to encode a response regulator.[31] Early studies also indicated that *agr* temporal control was most pronounced at the postexponential and stationary phases of growth.[28] In a critical development, the spent media could activate the system independently of growth phase, indicating a secreted signal was modulating *agr* function.[32] By fractionating the spent media, a single active peak was isolated and mass spectrometry combined with peptide sequencing revealed the cyclic thiolactone AIP as the functional signal.[33,34] This pioneering work demonstrated that *agr* is a peptide quorum-sensing system and opened the doors to extensive follow-up analysis to examine the molecular and biochemical mechanism of the system, variation across clinical isolates, function in pathogenesis and biofilms, and parallel peptide systems in other Gram-positives. In this section, we will overview the basic molecular features of *agr* and virulon controlled by this system, as well as structure function studies on the AIP signal.

2.1. Molecular Arrangement of the *agr* Locus

The *agr* locus is located on the *S. aureus* chromosome and is considered to be part of the core genome and not a pathogenicity island. The locus is known to contain two divergent transcripts named RNAII and RNAIII.[35,36] The RNAII transcript is an operon of four genes, *agrBDCA*, that encode factors required to synthesize AIP and activate the regulatory cascade[35] (Figure 1). Briefly, AgrD is the precursor peptide of AIP, and AgrB is an integral membrane endopeptidase essential to biosynthesize AIP. AgrC and AgrA form a two-component pair where AgrC is the membrane histidine kinase and AgrA is a response regulator.[37] Upon binding of AIP, AgrC phosphorylates AgrA, which in turn activates the P2 and P3 promoters to autoactivate the *agr* system and upregulate RNAIII transcription. RNAIII is the major downstream effector of the *agr* system that post-transcriptionally regulates expression of virulence factors and the Rot transcriptional regulator.[38] AgrA can additionally activate transcription from two promoters for expression of phenol soluble modulins[39] (PSMs). In early *agr* studies, a third transcript called RNAI was identified that is controlled by the P1 promoter.[31] The P1 promoter is weak and RNAI

Table 1. The *agr* Regulon

gene	product	function	notes	ref
		Genes Upregulated by *agr*		
		Secreted Enzymes		
aur	aureolysin	metalloprotease	C[c]	287, 288
splA-F	Spl proteases	serine proteases	C	289
sspA	V8 protease	serine protease	C	287, 288
sspB	Ssp proteases	cysteine protease	C	287
scpA	staphopain	cysteine protease	C	287
sak	staphylokinase	plasminogen activator	C	28
lip	lipase	fatty acid breakdown	C	
geh	glycerol ester hydrolase	fatty acid breakdown	C	
ureA-G	urease	urea neutralization	C	
plc	PI-phospholipase C	phosphatidyl-inositol hydrolysis	C	290
fme	fatty acid modifying enzyme	bactericidal fatty acid neutralization	C	291
		Toxins		
hla	α-toxin	cytolysin, pore-forming	A[a]	28, 42, 128
hlb	*β*-hemolysin	cytolysin, sphingomyelinase	C	28
hlgBC	*γ*-hemolysin	cytolysin, two-component pore-forming	C	292
hld	*δ*-toxin	cytolysin, pore-forming	B[b]	28, 36
lukD/E	leukocidin	cytolysin, two-component pore-forming	C	292
lukS/F	Panton−Valentine leukocidin	cytolysin, two-component pore-forming	C	292
lukG/H	leukocidin	cytolysin, two-component pore-forming	C	
seb	enterotoxin B	superantigen	C	133, 293
sec	enterotoxin C	superantigen	C	294
sed	enterotoxin D	superantigen	C	295
tst	toxic shock syndrome toxin-1	superantigen	C	28
etaAB	exfoliative toxins	desmoglein cleavage (scalded-skin syndrome)	C	296, 297
		Immunomodulatory Peptides		
PSMα1−4	α PSMs	cytolysin, PMN chemotaxis, inflammatory	B	39
PSMβ1−2	*β* PSMs	inflammatory	B	39
		Regulators		
agrBDCA	*agr* regulator	quorum sensing	B	35
arcR	transcriptional regulator	arginine catabolism regulation	C	
		Regulatory RNAs		
RNAIII	RNAIII	gene regulation	B	36
rsaE	RsaE sRNA	gene regulation	C	46
		Surface Factors		
cap5	polysaccharide capsule type 5	antiphagocytic	C	298
cap8	polysaccharide capsule type 8	antiphagocytic	C	299
		Genes Downregulated by *agr*		
		Secreted Proteins		
ssl5,8	staphylococcal superantigen-like proteins 5 and 8	Ssl5 inhibits PMN adherence	C	300
		Surface Proteins		
fnbAB	fibronectin binding proteins A/B	fibrinogen and fibronectin adhesin	A	301
spa	protein A	antibody Fc-region binding	A	28, 115, 128
coa	coagulase	plasminogen to plasmin conversion	A	117, 302
SA1000	surface protein	fibrinogen and fibronectin adhesion	A	114
		Regulators		
rot	Rot transcription factor	gene regulation	A	40, 114

[a] A: Post-transcriptional regulation by RNAIII. [b] B: Direct regulation by the AgrA transcriptional regulator. [c] C: *agr* regulation has been demonstrated by microarray[39,41,43,303] or proteomic[304] studies.

transcript only encodes *agrA* and is not considered a significant player in *agr* function.

2.2. *agr* Regulon

The *agr* system regulates a diverse range of genes in *S. aureus* (see Table 1). Regulation can result in either increased or decreased expression and can occur at the level of transcription, mRNA stability, or translation initiation. This regulation can occur through direct AgrA binding, RNAIII positive action through binding to target mRNA, or indirectly through RNAIII-mediated inhibition of translation of the repressor of toxins (Rot). While the upregulation of secreted virulence factors and downregulation of surface proteins are the most well recognized effects of *agr* activation, many other genes involved in various metabolic pathways, transport, and at least one regulatory sRNA are also affected by this system.

2.2.1. Genes Regulated by RNAIII/Rot

RNAIII is the *agr*-induced regulatory RNA that is the primary effector of the system. RNAIII is capable of regulating gene expression at the post-transcriptional level by affecting mRNA stability and promoting or inhibiting mRNA translation (see section 6 for more information). Much of RNAIII's effect on gene expression, particularly at the transcriptional level, comes through its inhibition of the Rot transcriptional regulator.[40]

Secreted virulence factors are a well-recognized class of targets that are RNAIII regulated. These include toxins (α, β, δ, and bicomponent classes), proteases, lipases, enterotoxins, superantigens (TSST-1, SEB, SEC, SED), and urease, which have all been shown to be upregulated in a RNAIII-dependent manner.[41] In some cases, such as α-toxin, this upregulation is a direct result of RNAIII action, and in other cases, it is indirectly through Rot function[42,43] (see Table 1). Surface virulence factors responsible for functions such as adhesion (fibronectin binding proteins) and antibody binding (protein A) are well recognized to be downregulated by *agr* activation.[41] Additionally, many cellular functions, such as genes involved in nutrient transport and amino acid metabolism, are also downregulated by *agr* activation and Rot inhibition.[43]

2.2.2. Genes Regulated Directly by AgrA

AgrA was the first gene discovered in the *agr* operon and was recognized early on as a transcriptional regulator. AgrA was found to activate the *agr* P2 and P3 promoters, driving transcription of the *agrBDCA* quorum-sensing genes and the RNAIII regulatory RNA, respectively.[35,38] With the discovery of RNAIII as the major effector of *agr* regulation, AgrA was overlooked as a potential regulator of other genes outside of the core *agr* system. This oversight was likely due to the challenges of separating direct AgrA regulation and AgrA effects mediated through RNAIII. Further, searching for AgrA binding sites across the *S. aureus* genome failed to reveal other potential targets.[44]

This perception changed when Queck et al. published a report on direct regulation of genes by AgrA.[39] By searching for genes that were differentially regulated in the *agrA* mutant but not in the RNAIII mutant (both compared to wild-type strain MW2), they were able to identify genes regulated by AgrA independently of RNAIII. The most notable upregulated genes are the phenol-soluble modulins (PSMs). PSMs are short amphipathic peptides that have been shown to be important for pathogenesis as they have chemotactic, proinflammatory, and leukolytic activity.[45] *S. aureus* contains seven PSMs: four short (21−23 residues) α-type PSMs in one operon, two longer (44 residues) β-type PSMs in another operon, and δ-toxin encoded within RNAIII. The α and β PSM transcripts are directly upregulated by AgrA independently of RNAIII action. AgrA binding sites were identified within the α- and β-PSM promoters, and AgrA binding was confirmed using electrophoretic mobility assays (EMSAs),[39] demonstrating that AgrA activates at least four promoters: P2, P3, $P_{PSM\alpha}$, and $P_{PSM\beta}$.

Interestingly, AgrA was found to downregulate more genes than it upregulated (85 downregulated vs approximately 15 upregulated).[39] Given the large number of downregulated genes, it is unlikely that AgrA directly binds and regulates expression of each gene. It appears more probable that an additional regulator is impacted by AgrA and potential candidates were uncovered in the AgrA microarray. Or, alternatively, AgrA could affect metabolic pathways in a manner that leads to secondary changes in regulation. The downregulated genes fall under categories of carbohydrate metabolism (transport and utilization), amino acid metabolism (histidine degradation and arginine synthesis), and staphyloxanthin synthesis. To date, no studies have examined AgrA interaction with the promoters of these genes.

2.2.3. The sRNA RsaE is Upregulated by agr

A recent report regarding the presence of small noncoding RNAs (sRNAs) in *S. aureus* identified 11 candidates (RsaA−K) with potential to regulate gene expression.[46] One of these sRNAs, RsaE, was positively regulated by *agr*. RsaE is a 100-nucleotide RNA transcript located in an intergenic region (between SA0859 and SA0860). RsaE forms two hairpin structures, one of which contains a 5′-CCCC-3′ sequence in the loop portion similar to the 5′-UCCC-3′ motifs found in the 3′ functional hairpins of RNAIII. RsaE is well-conserved in staphylococcal species and other low G + C Gram-positive bacteria, including *Macrococcus*, *Geobacillus*, and numerous *Bacillus* species. RsaE regulates the expression of numerous gene targets likely in a mechanism similar to RNAIII with extended basepairing with the targeted mRNAs. In the case of three of RsaE's targets, *oppB*, *sucD*, and SA0873, the formation of the RsaE−mRNA complex prevents formation of the translation initiation complex as determined by toeprint analysis. In genome-wide transcriptome analysis, RsaE upregulated and downregulated approximately equal numbers of genes that fall under categories of (i) amino acid transport, (ii) nucleotide, lipid, and carbohydrate transport and metabolism, (iii) cell wall/membrane biogenesis, and (iv) inorganic ion transport. Exactly how RsaE is regulated by *agr* remains an open question.

2.3. AIP Signal

The *agr* system responds to the extracellular concentration of the AIP signal. The original identified AIP from strain RN6390 is an eight-residue peptide (YSTCDFIM) with the last five residues constrained as a thiolactone macrocycle between the cysteine side chain and the terminal carboxylate.[33,34] This peptide fragment is derived from the internal region of AgrD through a series of AgrB-dependent processing events (see section 3.3). Why Gram-positive bacteria like *S. aureus* have evolved to biosynthesize a cyclic peptide signal instead of adopting linear structures is not known. Cyclization does improve stability through resistance to proteolytic cleavage. Evidence has been reported that the AIPs are resistant to thermolysin, chymotrypsin, proteinase K, and V8 protease.[47] This metabolic resistance can extend functional lifetime of the signal in the host. In an animal model, the AIP lifetime has been reported at 3 h,[48] which is exceptionally long for a fast-growing bacterial pathogen. The other advantage of constraining the peptide structure through cyclization is the limitation on conformational entropy. ^{1}H NMR structural analysis of AIP demonstrates the signal adopts a constrained conformation, and the protons are solvent shielded.[47] These properties facilitate the molecular recognition and binding affinity for the receptor. Most of the reported binding constants are in the 10−30 nM range (EC_{50}) for AIP bound to the AgrC receptor,[49,50] making the system exquisitely sensitive to trace amounts of the signal. This remarkable sensitivity has advantages in particular environments, such as in the harsh locales of the host, where the *S. aureus* cells can still detect and respond to fleeting amounts of AIP in scenarios that do not conform to the classic quorum-sensing response.[51]

While the elucidation of the first AIP signal structure was fascinating, it was immediately apparent that diversity in the scaffold had evolved. Even across *S. aureus* strains, many important *agr* differences are known. These differences have

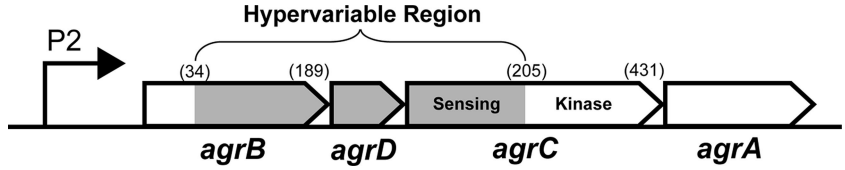

Figure 2. Hypervariable region of the *agrBDCA* operon. White areas represent highly conserved sections of genes. The gray regions mark where residue changes have occurred to establish different AIPs (AgrD), group-specific AIP processing (AgrB), and group-specific receptor recognition (AgrC). Numbers in parentheses indicate amino acid residue numbers marking the border of the variable regions.

been linked to hypervariable regions that range across the *agrBDCA* operon and encoded protein products[52] (Figure 2). In terms of operon order, the "hypervariable region" consists of the 3′ end of *agrB*, the *agrD* gene, and the 5′ end of *agrC*, where numerous basepair changes have occurred over time to establish the different *agr* groups. For instance, the first 34 *N*-terminal amino acid residues of the different AgrBs are completely conserved, while the rest of the protein shows substantial differences. Perhaps most notably, *agrD* gene variation results in divergent AIP structures.[33] Similarly, the *N*-terminus of AgrC shows considerable variation, where the AIP binding pocket is located, while the *C*-terminal kinase domain is highly conserved.

The unique features of the divergent *agrBDCA* operons in *S. aureus* isolates lead to the classification of four *agr* systems (referred to as *agr*-I, *agr*-II, *agr*-III, and *agr*-IV). Each *agr* system recognizes a different AIP structure, and to maintain grouping, these signals are termed AIP-I through AIP-IV. AIP-I, -II, and -III were identified first,[33] and a few years later, AIP-IV was independently reported by several laboratories.[50,53,54] The overall structure of the four *S. aureus* AIPs is similar. Each contains a five-residue thiolactone ring based on linkage of the cysteine sulfhydryl to the *C*-terminal carboxylate, whereas the *N*-terminus is variable with 2−4 residue extensions from the macrocycle. The molecular weight of these AIPs has been confirmed by mass spectrometry.[11,33,53] Figure 3 shows the residues in each of the four AIP structures.

2.4. *agr* Interference

Through a fascinating mechanism of chemical communication, these different AIP signals cross-inhibit each other's activity with surprising potency, a phenomenon called "*agr* interference".[33] The interference is mediated by three subclasses of cross-inhibitory groups: AIP-I/IV, AIP-II, and AIP-III. Because AIP-I and AIP-IV differ by only one amino acid and function interchangeably,[53] they are grouped together in the classification scheme, although this assignment has been controversial.[55] The potency of the cross-inhibition is impressive, as each of the three AIP groups bind to alternative AgrC receptors with affinity constants in the low nanomolar range.[49,56] The biological role of *agr* interference has remained elusive, but there are proposals that this phenomenon gives a competitive advantage to the producing strain.[57] How *S. aureus* has evolved this elaborate mechanism of cross-talk with *agr* group variation has been debated[58,59] and is beyond the scope of this review. For further insight on this intriguing topic, we refer the interested reader to the discussion by Novick and Geisinger and references therein.[59]

2.5. AIP Structure−Activity Relationships

Numerous studies have examined the essential structural features of the AIP signals. Methods for AIP chemical synthesis were developed and extensive structure−activity relationship (SAR) studies of the AIP scaffold were performed.[49,50,56,60,61] These studies uncovered critical properties of the AIP structure that are important for function. Notable findings are summarized below, and the reader is referred to excellent reviews for more information (see refs 47, 62):

(1) The presence of the macrocyclic ring is critical for AIP function and *agr* activation. Synthetic linear peptides do not activate *agr*, and hydrolysis of the thioester blocks function.[33]

(2) Removal of the *N*-terminal residues in AIPs eliminates *agr* activation but not inhibition. The AIP-I, AIP-II, or AIP-IV macrocyclic ring without the tail can serve as a universal inhibitor of the system.[49,61] These and other SAR studies have led to the proposal that the ring is the molecular "address" for receptor recognition and the tail is the "message" region needed for *agr* activation.

(3) The presence of the AIP thioester bond versus an ester is critical for *S. aureus agr* activation. This finding is based on the failure of a synthetic lactone to activate, which initially led to the hypothesis that acylation of

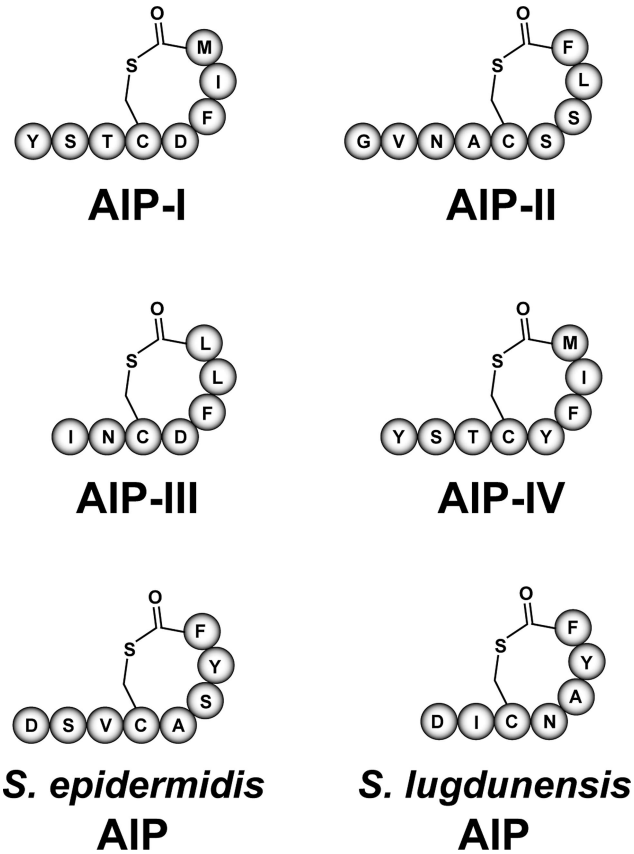

Figure 3. Structures of staphylococcal autoinducing peptides (AIPs). The four *S. aureus* AIPs representing *agr* types I−IV are shown. The most common *S. epidermidis* (type I) AIP and the predicted structure of the *S. lugdunensis* AIP are also shown.

the AgrC receptor is an important step in *agr* activation.[56] However, later studies demonstrated that AIP-binding interactions can be competed out with an antagonist, indicating reversible binding.[60] Further, the observation that a synthetic lactam will activate *agr*[50,56] and the subsequent identification of lactone-containing AIPs[63] both go against the acylation mechanism and provide a convincing demonstration that AIP binding to the AgrC receptor is reversible.

(4) The methionine side chain on AIP-I is labile. McDowell et al. initially reported that the methionine oxidizes to a sulfoxide under aerobic conditions, rendering the AIP-I nonfunctional in *agr* activation.[50] Neutrophils can exploit this labile residue and accelerate the deactivation of AIP-I using the oxidative burst.[64] However, the oxidized AIP-I is still effective at interference with the *agr*-II and III systems. Replacement of the labile methionine with isoleucine resulted in a functional AIP-I (M8I) in *agr* activation, but whether or not the molecule is more resistant to oxidative damage is unclear.[49] None of the other AIPs are reported to have a labile residue, although there is a methionine in a similar position in AIP-IV.

(5) Examples of mutations that convert an AIP structure into an activator are rare. In one of the only reported successes, an AIP-I D5N structure gained agonist activity for the *agr*-III system (EC_{50} = 360 nM).[49]

3. AIP Production

The section will outline the properties of AgrD and AgrB and summarize the current knowledge of the AIP biosynthetic mechanism.

3.1. AgrD

AgrD is a ribosomally produced propeptide that is the precursor to the final AIP signal (Figure 4). The AgrD peptide consists of 46 amino acids (when using *S. aureus agr*-I as the example) and can be divided into three general regions: (1) the *N*-terminal 24 residues form an amphipathic helix that is capable of targeting the peptide to the cell membrane,[65] (2) the middle section of AgrD encodes the final eight residue AIP-I molecule,[34] and (3) the *C*-terminal 14 residues appear to form a helix and tend to have numerous negatively charged residues.[66]

Among all staphylococcal species, each of these AgrD features is conserved[59,67] (Figure 5). The *N*-terminal helix can vary significantly in sequence with only a single glycine residue being conserved, however, the amphipathic properties are maintained. This helix is capable of targeting the AgrD propeptide to the cell membrane, presumably to associate with AgrB for processing.[65] Using AgrD-I as a model, truncation of the first 12 residues can be tolerated with some

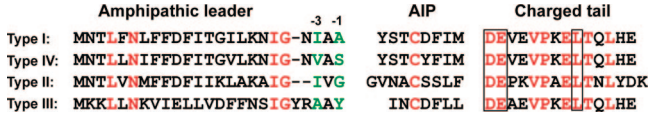

Figure 4. *S. aureus* AgrD sequences and domains. The four *S. aureus* AgrDs are aligned and separated into three domains that include an *N*-terminal amphipathic leader, AIP region, and *C*-terminal charged tail. Conserved residues across the four sequences are shown in red. Potential -1 and -3 signal peptidase cleavage sites are shown in green. Boxed residues in the *C*-terminal tail are essential for AgrB endopeptidase activity and AIP production.

AIP-I still being produced, but deletion of 14 residues prevents AIP-I synthesis. If the removed section is replaced with an artificial amphipathic helix, AIP-I production can be rescued, suggesting the function of this section is membrane targeting but not necessarily to mediate any specific interactions with AgrB. Replacement of the *N*-terminus with a classic Sec signal sequence prevented AIP-I production and the speculation here is that AgrB processing was bypassed.

The AIP-encoding section of AgrD shows considerable divergence across species (Figure 5), although some properties remain consistent. The only conserved residue is the cysteine required for formation of the thiolactone ring structure of AIP. Among staphylococcal strains, the exceptions to this rule are the *S. intermedius*, *S. pseudintermedius*, and *S. delphini* species, which have a serine in place of the cysteine and produce an AIP containing a lactone bond. In *S. aureus*, the AgrD cysteine is essential for making AIP but not necessary for AgrB cleavage of the *C*-terminal tail.[66]

The *C*-terminal tail is the most conserved portion of AgrD, especially the first nine residues,[52] and this tail is predicted to be negatively charged and adopt an α-helical conformation.[66] The presence of these nine residues is necessary for full AIP production.[66] Across the Staphylococci, aspartate and glutamate are conserved as the first two tail residues, but this conservation is not maintained in AgrDs from other Gram-positives (Figures 4 and 5). Mutations in either the aspartate or glutamate in *S. aureus* AgrD-I prevents AgrB cleavage activity and AIP production, as will mutation of the leucine at the ninth residue.[66] Proline residues are conserved in the third and sixth position of the tail, with the carboxy-proximal proline being absolutely conserved in every AgrD sequence (Figure 5).

3.2. AgrB

AgrB is a 22 kDa protein that localizes to the cell membrane[68] and is the primary enzyme for processing AgrD into the final AIP product. To support this statement, expression of only *agrB* and *agrD* is sufficient for AIP production in an *agr* deletion mutant.[34] Further, heterologous expression of *agrBD* in *Escherichia coli* or *B. subtilis* resulted in functional AIP,[66] demonstrating these are the only unique genes of the system required to make AIP. Multiple studies have demonstrated that AgrB has endopeptidase activity that can remove the AgrD *C*-terminal tail.[66,69,70] Two residues were identified as being essential for proteolytic activity, His-77 and Cys-84, suggesting that AgrB acts as a cysteine protease.[70] These two residues remain absolutely conserved in every AgrB-like protein, including those in nonstaphylococcal species, highlighting their importance in AgrB function. Additional mutations in well-conserved serine and histidine residues did not inhibit AIP production, further implicating His-77 and Cys-84 as forming the catalytic center.

In many respects, AgrB is the most unique feature of the staphylococcal *agr* system based on its lack of sequence similarity with other quorum-sensing proteins. AgrB does not share homology to cysteine proteases or other proteins in the database.[35] The only AgrB-like proteins that have been identified are those in related cyclic peptide signaling systems in other Gram-positive bacteria (Figure 6). This uniqueness makes AgrB a valuable genome mining tool to uncover new peptide systems.[9,71] When comparing the different AgrB sequences among *S. aureus agr* types, there is a significant

Figure 5. Sequence alignments of AgrD. Known AgrD sequences from various Gram-positive species are aligned and split into three sections that consist of an *N*-terminal leader, central AIP sequence (bolded), and charged *C*-terminal tail. Residues showing strong sequence conservation are colored red. AgrDs whose final AIP product has been confirmed are marked with an asterisk, and all others are depicted as eight residues by default, except for variants from within the same species with known structures. Species type numbers reflect variants as reported by Dufour et al.[52]

amount of variation between strains. Overall properties such as hydrophobic sections that make up transmembrane sections are conserved, although the sequences of these regions are diverse (a common feature of integral-membrane protein homologues). Interestingly, the *N*-terminal portion of AgrB is highly similar among staphylococcal species, with the first 34 residues being absolutely conserved among the four *S. aureus agr* types. This region is required for AgrB function; however, its role is currently unknown.[70]

One study defined the topology of AgrB within the cytoplasmic membrane.[69] Using alkaline phosphatase fusions, the approximate positions of transmembrane spanning regions were determined in *E. coli*. This method generated a topology map with six transmembrane regions, in which both the *N*- and *C*-termini are predicted to be inside the cell and a 27-residue loop located outside the cell. Topological studies indicate that the catalytic residues are located on the cytoplasmic face of the membrane.[70]

3.3. Mechanism of Processing

Since the original report of AIP production by AgrB and AgrD,[34] there have been only a limited number of studies examining the biosynthesis mechanism, especially in comparison to studies of *agr* regulation and role in virulence. This may reflect the difficult nature of performing biochemical studies on an integral membrane protein, combined with the fact that AgrB shows no homology to other characterized proteins. Nevertheless, there has been some progress made in understanding how AgrD is processed into AIP.

Zhang et al. were the first to demonstrate that AgrB acts as a protease to cleave AgrD.[69] Using epitope-tagged AgrD,

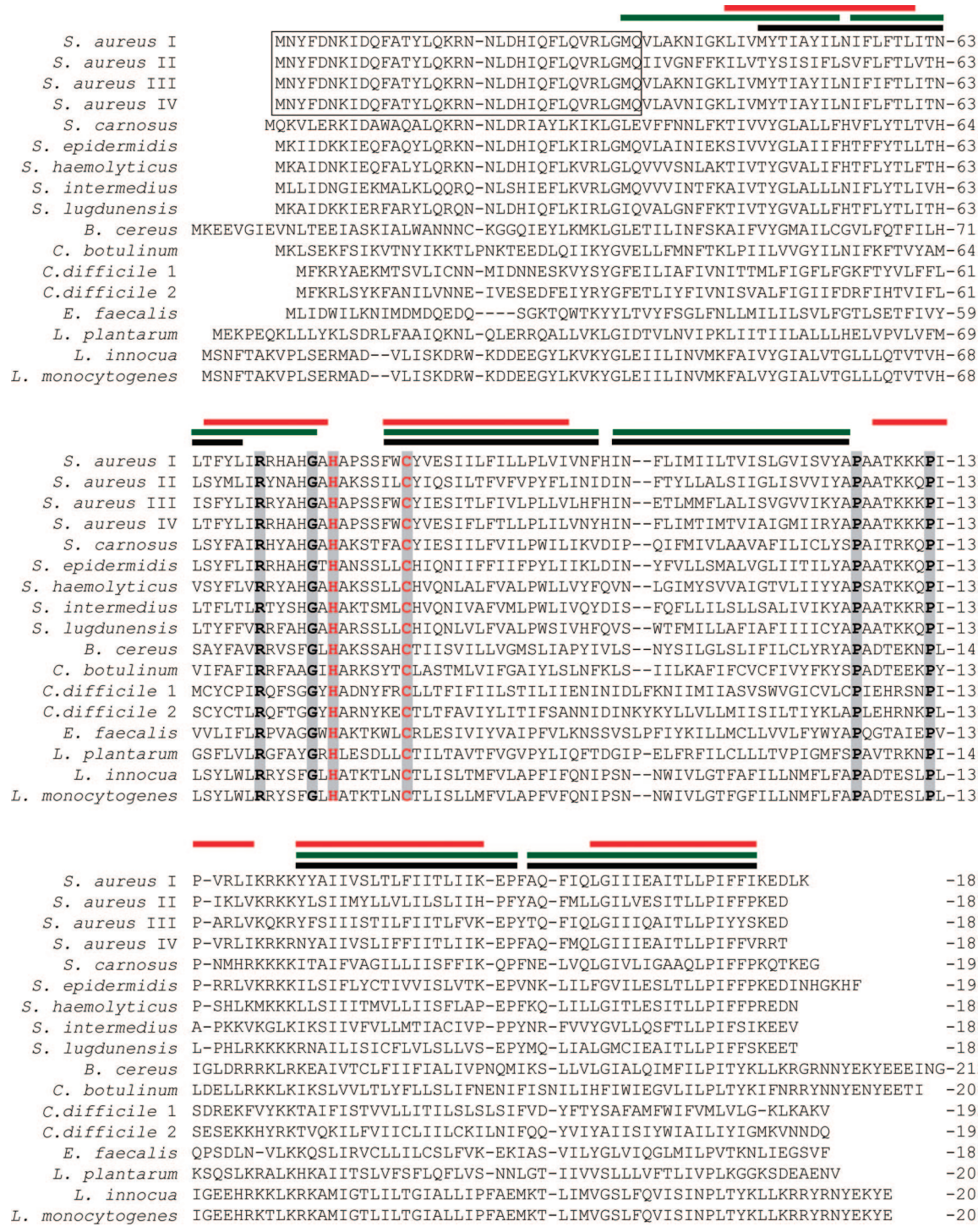

Figure 6. Sequence alignments of AgrB. AgrB sequences from selected Gram-positive species were aligned using ClustalW.[308] Conserved residues are marked with gray boxes, and the essential histidine and cysteine residues are colored red. The colored lines above the sequence represent transmembrane regions as reported by Zhang et al.[69] (red) or predicted using TOPCONs[309] (green and black bars represent the two most common predictions). The 34 *N*-terminal residues conserved among *S. aureus agr* types are boxed.

they demonstrated AgrB-dependent cleavage of AgrD via immunoblots. The predominant product seen is AgrD without the *C*-terminal tail, indicating that removal of this tail is an early step in AgrD processing. This cleavage activity is abolished by mutation of either the His-77 and Cys-84 catalytic residues.[70] Interestingly, AgrB activity is inhibited by a subset of protease inhibitors such as the serine protease inhibitors AEBSF, VFK-CMK, and TPCK. Other serine protease inhibitors (thrombin inhibitor, soybean trypsin inhibitor, and aprotinin) and cysteine protease inhibitors (E-64, E-64D, NCO-700, and Z-Phe-Gly-NHO-Bz) are not effective. The functional AgrB inhibitors (AEBSF, VFK-CMK, and TPCK) are known to block activity of some cysteine proteases, but the reason for lack of activity of the other serine and cysteine protease inhibitors against AgrB is unclear.

Beyond cleavage of the AgrD *C*-terminal tail, no studies have been reported for AIP thiolactone ring formation or transport across the membrane. A stable AgrB−AgrD structure is detectable, providing a clue to how the thiolactone bond might be formed[66] (see below). AIP can be produced by *E. coli* when only AgrB and AgrD are provided,

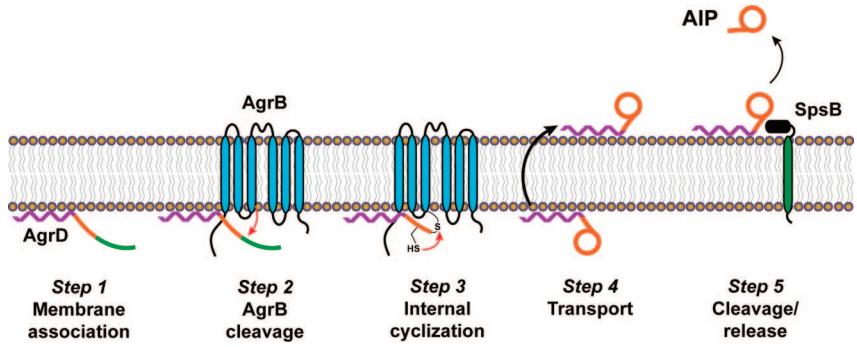

Figure 7. Model of AIP biosynthetic pathway. Step 1: AgrD is targeted to the cell membrane via its *N*-terminal amphipathic helix. Step 2: AgrB associates with AgrD and carries out a nucleophilic attack to remove the *C*-terminal domain of AgrD, resulting in formation of an AgrB−AgrD intermediate linked by a thioester bond. Step 3: the cysteine residue within the AIP-encoding portion of AgrD carries out a thioester exchange to form the thiolactone ring and release the AIP precursor from AgrB. Step 4: transport of the AIP precursor across the membrane, presumably by AgrB. Step 5: removal of the AgrD *N*-terminal domain by SpsB and release of the final AIP. This figure is an adaptation of the version reported by Thoendel et al.[66] Reprinted with permission from ref 66. Copyright 2009 The American Society for Biochemistry and Molecular Biology, Inc.

suggesting that AgrB is responsible for generating the thiolactone bond.[66] AgrB's role in transport of AIP across the membrane is less clear because other well-conserved transport mechanisms could mediate the process.

To make the final AIP molecule, the *N*-terminal leader must be removed from AgrD. Kavanaugh et al. investigated this process by purifying the activity capable of cleaving a fluorescein-labeled peptide mimicking the AgrD-I cleavage site.[72] The type I signal peptidase, SpsB, was identified as the enzyme capable of cleaving the AgrD *N*-terminal leader. Signal peptidases are membrane-bound, housekeeping proteases responsible for removing *N*-terminal signal peptides as proteins are being secreted through the Sec or Tat pathways.[73] In support of this proposal, signal-peptidase inhibitors were capable of preventing AIP production and *agr* activation in both *agr*-I and *agr*-II strains.[72] However, it remains untested whether signal peptidase is involved in AIP production in other staphylococcal strains. Signal peptidase cleavage sites typically contain small uncharged residues at the -1 and -3 positions of the cleavage site. *S. aureus* AgrD-I, -II, and -IV peptides fit this pattern, as do many other staphylococcal AgrDs, but some contain residues such as lysine or tyrosine at the predicted -1 position (Figures 4 and 5), which could disrupt signal peptidase activity. Further investigation is necessary to verify the universal requirement of signal peptidase in AIP biosynthesis.

Using all the experimental findings outlined above, a model for AIP biosynthesis has been proposed[66,71] (Figure 7). The first step is association of AgrD with the cytoplasmic membrane via its *N*-terminal leader. At the membrane, AgrB carries out a cysteine-dependent nucleophilic attack on AgrD, removing the *C*-terminal tail. This cleavage event results in the formation of a covalent intermediate in which AgrD and AgrB are linked through a thioester bond. To create the thiolactone ring, the AgrD internal cysteine carries out a thioester exchange with the bound AgrD−AgrB intermediate, removing the AIP precursor. At this point, the precursor is transported across the membrane, possibly by AgrB, and signal peptidase SpsB removes the *N*-terminal leader to release AIP into the extracellular environment.

Many questions about the mechanistic details of AIP biosynthesis remain to be answered: (1) direct evidence for AgrD binding to AgrB is unavailable, (2) the role of AgrB in AIP transport has been suggested but never demonstrated, (3) the oligomeric state of the AgrB protein is unknown and the current AgrB topology map has numerous buried charges and exposed hydrophobic loops,[69] suggesting the membrane spanning regions should be reexamined, (4) most biosynthesis experiments were performed on the *agr*-I system and the consistency of findings in more divergent systems is unknown, and finally (5) the mechanism of thiolactone bond formation has not been demonstrated.

3.4. Type-Specific Processing

Similar to the AgrD sequence variability of the different *agr* types, the AgrB proteins also evolved in concert with each class of AIP signal. This coevolution resulted in group specificity that enables each AgrB protein to process the AgrD propeptide of the same *agr* type. When considering the group specificity, it is important to note that AgrB catalyzes multiple steps in the AIP biosynthetic mechanism that should be analyzed independently. However, this type of analysis has not been performed to date, and instead specificity assessments have been made using AIP bioassays. In this case, neither AgrB-I or AgrB-III could use AgrD-II as a substrate for AIP production.[33] Similarly, AgrB-II could only process its cognate AgrD. Taken together, these findings highlight that group-specific processing events do exist, but the step in the AgrB mechanism mediating the specificity remains in question. Some leniency in the group specificity is apparent because the AgrD-I and III classes are similar enough to be functionally interchangeable.[33]

To define AgrB regions responsible for group specificity, chimeras were generated with different sections of AgrB-I and AgrB-II.[74] For AgrD-I to be processed, AgrB needs to contain a section encompassing the first transmembrane segment of type I sequence (residues 43−66), while the rest of the protein could be type II. In contrast, AgrD-II required one of two AgrB segments (residues 67−75 or 126−141) to be type II. These regions correspond to either the second and fourth transmembrane regions according to the published topology map[69] or two cytoplasmic sections based on computer prediction models (Figure 6). Both of these sections are fairly well conserved between types I and II, with six out of nine identical residues in the first segment and 12 out of 16 in the second, narrowing down possible critical residues in dictating specificity. Again, considering AIP production was measured as the output, it is unclear whether the specificity differences are due to AgrD peptide recognition,

cleavage, thiolactone ring formation, or transport of the peptide after processing.

4. AgrC

S. aureus AgrC is part of a growing family of peptide-inducible histidine protein kinases (HPKs) that regulate various group behaviors in Gram-positive bacteria by sensing the presence of inducer peptide pheromones (IPs) that are produced by the bacteria themselves. This family includes AgrC homologues in other Staphylococcal species,[52] *Clostridium perfringens*,[19,75] *Listeria monocytogenes*,[17] *Enterococcus faecalis*,[76] and *Lactobacillus plantarum*[16] that bind cyclic IPs, either lactones or thiolactones, and function as quorum-sensing systems that regulate bacterial virulence and/or adhesion. The family also includes HPKs that bind linear IPs and regulate the production of specific secreted factors, and thus we will refer to this greater class of peptide signals as "IP" in this section. Examples of such HPKs are PlnB,[77] SppK,[78] PlsK,[79] and SapK[80] that regulate production of bacteriocins in *Lactobacillus*, CbnK[81] that regulates peptide antibiotic production in *Carnobacterium*, as well as ComD[82] that regulates production of competence stimulating factor by *Streptococcus.* In this section, we have delved deeper into the unique features of AgrC-like HPKs with a hope of presenting a more insightful understanding of AgrC structure and function.

As their cognate IPs accumulate to a critical concentration, AgrC and other IP-responsive HPKs undergo ATP-dependent autophosphorylation in response to signal binding.[37] After autophosphorylation, they activate their respective response regulator (RR), AgrA in the case of AgrC, through phosphotransfer to an aspartic acid residue on the RR. The phosphorylated RRs in turn activate transcription by directly binding to regulatory DNA elements. In this way the IP-responsive HPK/RR pairs function as classic two-component systems. However, because the RRs of the IP-based quorum systems directly upregulate the expression of genes responsible for IP biosynthesis, the HPKs, RRs, and IPs collectively function as three-component systems that afford bacteria a means of measuring population density. Understanding the specificity of IP binding, and how structural changes associated with binding propagate through the membrane and into the cytoplasmic domain, is fundamental to understanding the HPK's signal transduction mechanism.

As outlined in this section, AgrC and the other IP-responsive HPKs are membrane-associated polytopic receptors that share significant structural homology, implying a shared stereochemical mechanism for transmembrane signal transduction. For several members of the family, it has been found that the determinants of IP binding specificity localize to similar regions of the *N*-terminal sensory domain, as do mutations that cause increased or constitutive activity (see section below). Together, these findings lend further support to the idea that the IP-responsive HPKs employ a common transmembrane signal transduction mechanism. There are likely to be differences between this mechanism and those employed by HPKs of the most common HPK subfamilies. Sequence analysis indicates the IP-responsive HPKs are missing a region of the cytoplasmic domain that is thought to play a role in modulating the relative levels of autokinase, phosphotransfer, and phosphatase activity. The allosteric regulation of AgrC enzymatic activity is achieved by modifying the distribution of AgrC among these various conformational states in an IP-dependent manner. The recent demonstration that AgrC is a dimer that undergoes symmetric signal transduction[83] implies that IP is bound with positive cooperativity. If this inference proves true, it has important implications for the efficiency and timing of virulence factor expression in vivo because positive cooperativity would allow a dramatic increase in virulence factor production to occur upon signal binding.

4.1. Structural Homology of AgrC Family Members

Comparison of the primary amino acid sequences of the IP-responsive HPKs, whether they are activated by cyclic or linear IPs, reveals a shared structural organization that is unique to the group.[84−86] Using primary amino acid sequences of AgrC from the four *S. aureus agr* groups,[53] the shared features of the IP-responsive HPK family are revealed using a COBALT[87] generated multiple sequence alignment (see Figure S1, Supporting Information). In this section, Figure S1 of the Supporting Information will be used to summarize the current knowledge of the structures of the IP-responsive HPKs.

Like all HPKs,[88] the IP-responsive HPKs are composed of the following structural features: (1) *N*-terminal sensor domain (residues 1−205 for AgrC-I) that contains membrane spanning α-helices (TMs, predicted using HMMTOP[89,90] and highlighted in gray in Figure S1 of the Supporting Information), (2) cytoplasmic domain (residues 206−430 for AgrC-I) consisting of a dimerization and histidine phosphotransfer (DHp) subdomain, (3) autophosphorylation site, a conserved histidine (H239 for AgrC-I) that is located within a region that has high propensity for coiled-coil formation (as predicted with COILS[91] and highlighted in blue in Figure S1 of the Supporting Information), and (4) *C*-terminal catalytic and ATP binding (CA) subdomain that contains the nucleotide binding site. It is generally accepted that both the DHp and CA subdomains are necessary for autokinase activity, whereas only the DHp subdomain is required for phosphotransfer and phosphatase activities.[88]

4.2. Structural Features of HPK_{10} Cytoplasmic Domains

When HPKs are assigned to subfamilies based on the presence or absence of conserved sequence elements (homology boxes) in their DHp and CA subdomains, all the IP-responsive HPKs belong to the HPK_{10} subfamily.[84−86] As noted by Grebe and Stock,[85] HPKs in this subfamily are characterized by CA domains that have several distinguishing features: they (1) lack discernible D-boxes, (2) have a single N-box asparagine (N339 in AgrC-I) instead of the more common two, (3) possess DHp domains whose H-boxes have a tyrosine (Y241 in AgrC-I) two residues downstream of the conserved phosphoaccepting histidine, and (4) are missing a proline that is located five residues *C*-terminal of the phosphoaccepting histidine in all HPKs of subfamilies one through four (i.e., in the majority of HPKs).

To gain insight into the structure of the cytoplasmic domain, a hypothetical AgrC-I structure was generated using the PHYRE server.[92] The AgrC-I amino acid sequence was threaded onto the crystal structure of the cytoplasmic domain of HK853 from *Thermotoga maritima*,[93] a member of the HPK of subfamily 1a, which is the only available structure of the intact cytoplasmic domain. Using the hypothetical structure, regions can be identified that are likely to have

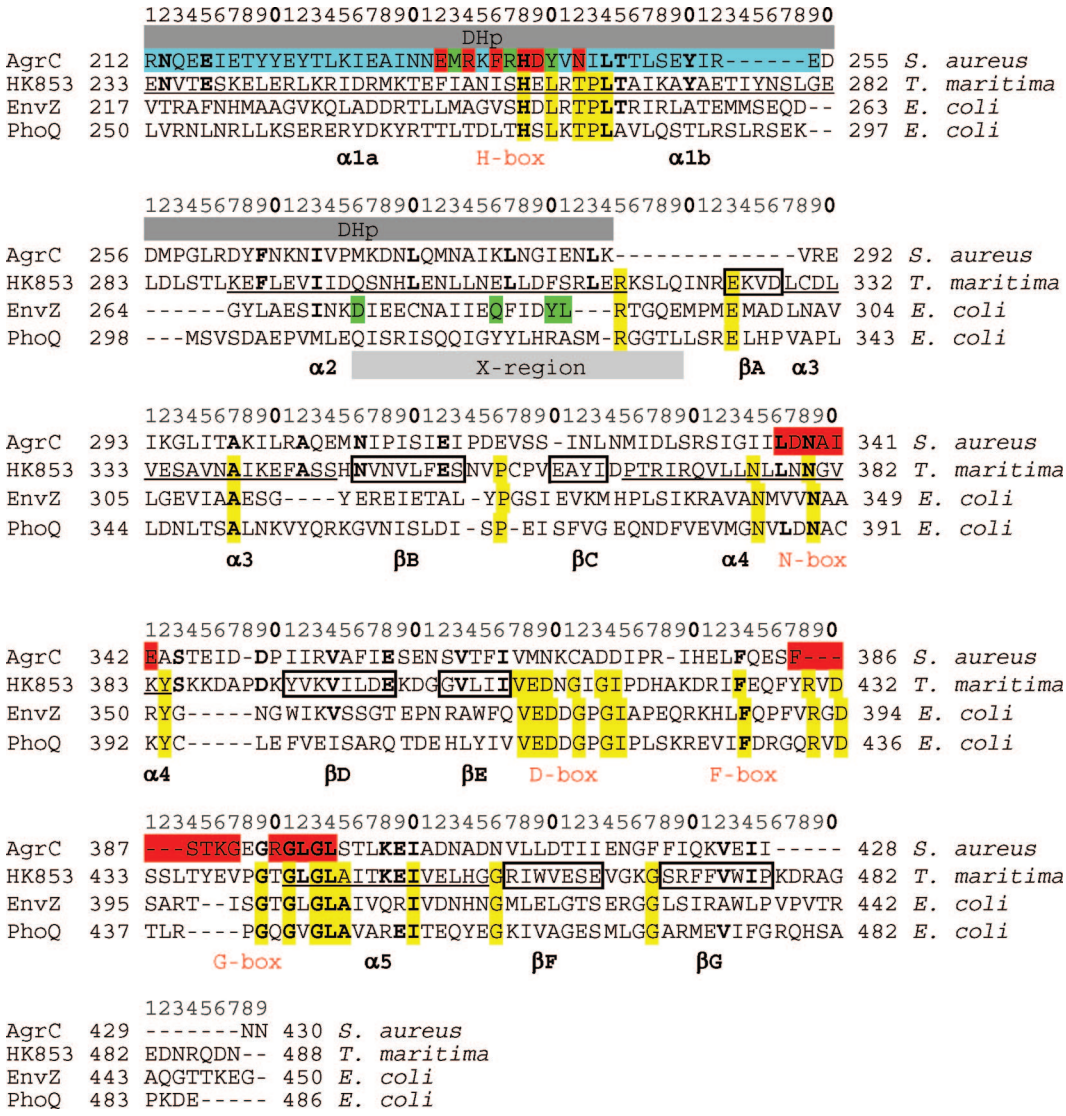

Figure 8. Alignments of cytoplasmic domains of HPKs. PHYRE[310] was used to align the sequence of AgrC-I, HK853 from *Thermotoga maritima*, and EnvZ and PhoQ from *E. coli*. Residues that are highly conserved in HPK10s are highlighted in red, and residues that are highly conserved in HK853, EnvZ, and PhoQ are highlighted in yellow. AgrC-I residues highlighted in blue have a high propensity for coiled-coil formation, and AgrC-I residues highlighted in green are positions where mutations result in constitutive activity. EnvZ residues highlighted in green are positions where mutations eliminate phosphatase activity without altering autokinase activity. Underlined HK853 residues are within α-helices and boxed HK853 residue are within *β*-strands. The locations of the DHp subdomain and the X-region are indicated. Protein and bacteria names as well as specific residue numbering are indicated in the margins, and a residue count line is provided at the top of each panel.

high structural similarity as well as others likely to have significant structural differences with potential functional implications. Figure 8 shows the sequence alignment of AgrC-I with HK853, and the cytoplasmic domains of *E. coli* HPKs EnvZ and PhoQ, which belong to HPK subfamilies 2b and 3a, respectively. While the overall identity between the cytoplasmic domains of AgrC-I and HK853 is a modest 18%, the relative spacing of the homology boxes is maintained and conserved residues are distributed throughout the sequence. There are several regions where multiple conserved residues are found within individual secondary structure elements of HK853 (α-helices are underlined and *β*-strands are enclosed in boxes). Collectively, these regions, which include the α5 helix, the *C*-terminal half of α3 through *β*B, and the *C*-terminal half of α4 through *β*E, form the nucleotide binding site and the core of the CA subdomain as seen in the ribbons drawing of the HK853 structure (Figure 9A). This suggests the portion of the AgrC-I CA subdomain not making direct contact with the DHp subdomain is likely to have a structure that is very similar to that of HK853 CA subdomain.

Given the putative coiled-coil (highlighted in blue in Figure S1 of the Supporting Information and Figure 8) and the spacing of conserved hydrophobic residues in the region corresponding to α2 of HK853, it is likely that the DHp subdomain of AgrC forms a helical-hairpin domain similar to those observed in the X-ray crystal structure of HK853[93] (Figure 9A) and the NMR structure of EnvZ.[94] It is also likely that AgrC dimerization involves formation of a four-helix bundle by the DHp subdomains as has been observed in HK853 (Figure 9B), EnvZ, and other HPKs.[88]

There are likely to be some structural differences between the AgrC1 and HK853 DHp subdomains due to the absence of the H-box proline (Pro265 in HK853) and a six-amino acid deletion corresponding to HK853 residues Thr275 through Leu280 (Figure 8). In HK853, and all HPKs of

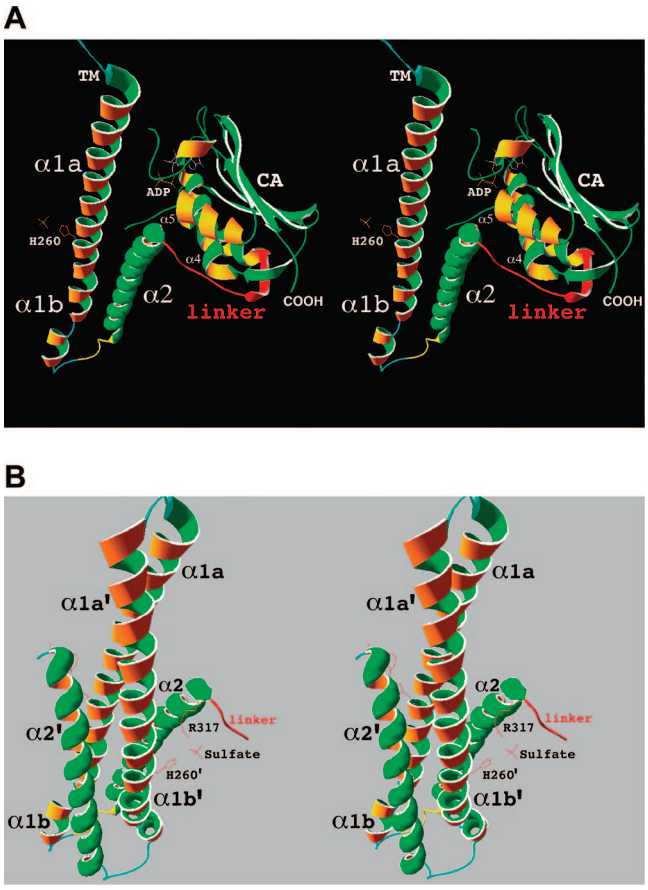

Figure 9. Structure of the cytoplasmic domain of HK853 from *Thermotoga maritima*. (A) Stereo diagram of HK853 drawn with Swiss-PDB Viewer[311] and PDB[312] entry 2C2A. Intersubdomain and βA residues that are predicted to be deleted in the cytoplasmic domains of HPK_{10}s are drawn in red. The side chain of phosphoaccepting His260 also is shown in red extending from α-helix β1a of the DHp subdomain helical-hairpin located at the far left of the figure. (B) Stereo diagram of the four-helix bundle formed by the helical-hairpins from the DHp subdomains of two HK853 monomers. The side chains of phosphoaccepting His260s and Arg317, as well as an ordered sulfate ion, are drawn in red.

subfamilies 1 through 4, the conserved H-box proline is located 5 residues, or approximately 1.5 helical turns, *C*-terminal of the phosphoaccepting histidine where it places a kink in the first α-helix of the DHp subdomain, such that the helix is split into two helices referred to as α1a and α1b (Figures 8 and 9A). Lacking this proline, the α1 helix of the AgrC-I and the other HPK_{10}s most likely will not be kinked. Also, the AgrC-I helical-hairpin is probably shorter than the HK853 helical-hairpin due to the six-amino acid deletion. Aligning each of the HPK_{10}s with HK853 using the PHYRE server reveals deletions ranging from 4 to 10 amino acids at this same position (data not shown), indicating that a shorter helical-hairpin is a feature that can be generalized to the entire HPK_{10} subfamily. These structural differences have the potential to affect phosphotransfer reactions between the HPK_{10}s and their response regulators. The recent X-ray crystal structure of the complex between HK853 and its response regulator, RR468,[95] shows that the four-helix bundle formed by the DHp subdomains is integral to the HK−RR interaction, with the α1 helix of DHp subdomain making extensive contacts with the α1 helix and β5−α5 loop of the RR (see Figure 1c of ref 95). In light of this, a detailed characterization of AgrC−AgrA interactions and their effect on the phosphotransfer reaction is a necessary part of a full understanding of AgrC's role in quorum-sensing.

It is important to note that the PHYRE produced alignment identifies a 13-amino acid deletion in AgrC-I corresponding to HK853 residues Agr317 through Leu329 (Figure 8). This deletion eliminates the intersubdomain linker and the first β-strand (βA) of the CA subdomain (drawn in red in Figure 9A). PHYRE alignments between the cytoplasmic domains of the other HPK_{10}s and the cytoplasmic domain of HK853 identify similar deletions of 11−20 amino acids at this position (data not shown). This region is present in other HPKs, such as EnvZ and PhoQ from *E. coli* (Figure 8), suggesting that the missing residues represent deletions in the HPK_{10}s as opposed to an insertion in HK853. The deleted residues constitute a significant portion of the interface between the DHp and CA subdomain (Figure 9A), and they appear to play in an important role in modulating the various activities of HK853 and EnvZ. On the basis of these differences with the HPK_{10}s, it is reasonable to expect their absence may have a substantial impact on the structures and activities of the HPK_{10}s.

Elimination of 11−20 amino acids from the intersubdomain linker region will restrict the conformational degrees of freedom available to the HPK_{10}s. Thus, the trans autophosphorylation observed for AgrC may be a consequence of AgrC's inability to access a conformation similar to that seen in the HK−RR complex of HK853.[83] Because there is no equivalent to Arg317 (of HK853; Arg389 of EnvZ) in the HPK_{10}s due to the deletion, and this arginine residue is required for phosphatase activity,[96] it is tempting to speculate that AgrC and other HPK_{10}s lack phosphatase activity. It is interesting to note that although the literature on AgrC and the other quorum-sensing HPKs is fairly extensive, there are no reports of these proteins having phosphatase activity. If AgrC does indeed lack phosphatase activity, this has important implications for the regulation of AgrA phosphorylation state by AgrC.

Beyond the differences noted above, the DHp domains of IP-responsive HPKs also are unique owing to the absence of a HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis proteins, and phosphatase) domain.[84,86] HAMP domains form a four-helix, parallel coiled-coil that is important for symmetric transmembrane signal transduction.[97,98] The domain is approximately 50 amino acids in length and normally links the coiled-coil region of the DHp domain to the last TM of the sensory domain,[93] which is shown as interactions between the *N*-terminal portions of α1and α1′ helices in Figure 9B. In the HPK_{10} subfamily, the last TM of the sensory domain (TM6) is essentially contiguous with the coiled-coil region of the DHp subdomain (illustrated in Figure S1 of the Supporting Information). If the structures depicted in Figure 9 were AgrC, the next turns of the α1 helices located at the top of the figures would be in the membrane. Importantly, the continuity between the DHp and TM6 implies that TM6 is also part of the dimerization domain in HPK_{10}s.

4.3. Atypical Topology of the HPK_{10} Sensory Domains

Whereas the prototypical sensory domain consists of a single, large extracytoplasmic loop flanked by two TMs,[88,99] the sensory domains of HPK_{10}s are predicted to be polytopic.[84,86] Published hydropathy analyses of AgrC-I,[37]

PlnB,[77] and ComD1[82] predict that the sensory domains of these proteins contain five to seven TMs with two to three intervening extracellular loops. The polytopic nature of the sensory domains has been confirmed by β-galactosidase (*lacZ*) and/or alkaline phosphatase (*phoA*) fusion analyses for *S. aureus* AgrC[37] and PlnB.[82] The AgrC-*phoA* fusions studies confirm that residues 33, 105, and 176 (black boxes in Figure S1 of the Supporting Information) are located on the extracellular side of the membrane, and residue 142 (red box in Figure S1 of the Supporting Information) is located on the cytoplasmic side of the membrane. Interestingly, the *phoA* and *lacZ* fusion analyses reveal topographical inconsistencies within the *N*-terminal halves of the AgrC and PlnB sensory domains (TM1 through TM3). Specifically, Ser33 reacts with extracellular nature in both AgrC-I and AgrC-II, whereas Asn35 of PlnB reacts with cytoplasmic nature, implying that the orientations of TM1 and TM2 are opposite in the two proteins. In PlnB, the reactivity of Gly64, located on the second predicted intervening loop, is consistent with an extracellular localization, which also supports an inside-to-outside orientation of TM2 in PlnB. In light of these discrepancies, it is important to note that topology prediction programs generate divergent models for the number and orientation of TMs, as Jensen et al. noted for AgrC sensory domain.[100] For example, MEMSTAT-SVM and MEMSTAT3[101] predict seven TMs for AgrC and PlnB instead of the six TMs predicted by HMMTOP[89] (Figure S1 of the Supporting Information). In both cases, the extra TM is the result of splitting TM2 into two TMs.

To obtain more accurate topographical description of the HPK_{10} sensory domains, we have analyzed all 16 sequences using TOPCONS,[102] a program that generates a consensus topology by averaging the topology predictions generated by five programs. Figure S2 of the Supporting Information shows the TOPCONS generated consensus residue topology predictions and plots of residue reliability value versus residue number for each of the 16 representative HPK_{10} sequences. The ambiguity in TM prediction noted above for the *N*-terminal portions of the AgrC-I and PlnB sensory domains can be generalized to all of the HPK_{10} sensory domains. The *N*-terminal third of nearly all the HPK_{10}s contain regions with low reliability values, indicating poor correlation between the topology predictions produced by the individual prediction programs. In contrast, the reliability values corresponding to the *C*-terminal two-thirds of each sensory domain are all ~0.8 or higher, indicating good correlation between the predictions produced by the individual prediction programs. Moreover, comparison of the bars indicating the TOPCONS residue topologies suggests that within this region the relative positions and orientations of the individual TMs show significant conservation between the various HPK_{10} sensory domains.

To obtain a quantitative measure of this conservation, we have created a consensus HPK_{10} topology plot (Figure 10) by counting the frequency with which each of the TOPCONS residue topologies occurs at each residue and plotting the frequencies versus residue number. The resulting consensus HPK_{10} topology plot identifies four highly conserved TMs (labeled TM3 through TM6) within the *C*-terminal two-thirds of the sequence that have the same orientation in all 16 HPK_{10}s. The plot also identified a fourth TM (labeled TM2), but the position of this helix is less conserved. The positions of these five TMs correspond well with the HMMTOP predictions (Figure S1 of the Supporting Information). In

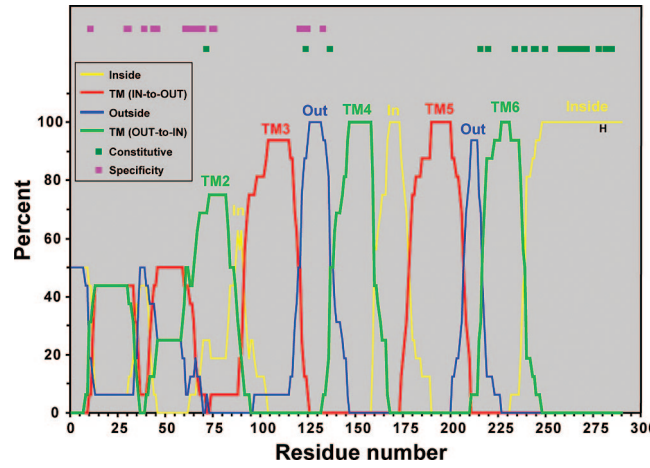

Figure 10. Consensus HPK_{10} topology plot. TOPCONS residue topologies were determined for each HPK_{10} family member (see Figure S2 of Supporting Information). The frequency of a particular topology at each residue is plotted as a percentage versus residue number. Labeling on the plot is as follows: outside, thin blue line; $TM_{OUT\text{-}to\text{-}IN}$, thick green line; inside, thin yellow line; $TM_{IN\text{-}to\text{-}OUT}$, thick red line). TM is an abbreviation for transmembrane. Across the top of the plot, boxes indicate residue mutations in HPKs that result in constitutive activity (dark green) or ligand specificity (magenta).

contrast, the region of the plot corresponding to TM1 does not show very high conservation between the 16 HPK_{10}s, with this region forming either one or two TMs in the various HPK_{10}s. The positions of mutations that confer ligand specificity and constitutive activity are identified in Figure 10. The ligand specificity determinants are localized within the less conserved *N*-terminal halves of AgrC[37,100,103,104] and other HPK_{10} sensory domains.[77,105−107] In contrast, mutations that result in constitutive activity localize within the more highly conserved *C*-terminal halves of AgrC[108] and other HPK_1 sensory domains.[77,78,107] This suggests that the *N*-terminal halves of the HPK_{10} sensory domains have evolved so they can respond to different ligands, while the *C*-terminal halves of the HPK_{10} sensory domains have maintained a structure that is important for efficient transmembrane signaling.

The HPK_{10} consensus topology plot (Figure 10) is also consistent with the bioinformatic analysis of the *agr* loci from 71 divergent staphylococcal strains reported by Dufour et al.[52] These authors further note that in three variant sequences, *S. xylosus*, *S. arlettae*, and *S. epidermidis 1*, the TM5s "include a perfect four-element leucine zipper motif" and speculate this helix may be involved in homodimerization (*S. epidermidis* leucines shown as bold, underlined, red letters in Figure S1 of the Supporting Information). In addition, Dufour et al. point out that the other 21 variants all have hydrophobic residues in the positions corresponding to the repeat leucines.[52] Comparison to the four *S. aureus* AgrC sequences shows that leucine is conserved at 11 of the 16 positions corresponding to the zipper motif and that the other positions contain either phenylalanine, isoleucine, or methionine. Interestingly, the first leucine of the repeat is predicted to be located within the cytoplasm, a prediction that was confirmed by *phoA* fusion analyses, as noted above. These observations suggest that TM5 helix extends into the cytoplasm where it participates in coiled-coil type interactions. If this hypothesis is correct, the cytoplasmic extension of TM5 is most likely making coiled-coil interactions with the *N*-terminal region of the α1 helix of the DHp subdomain

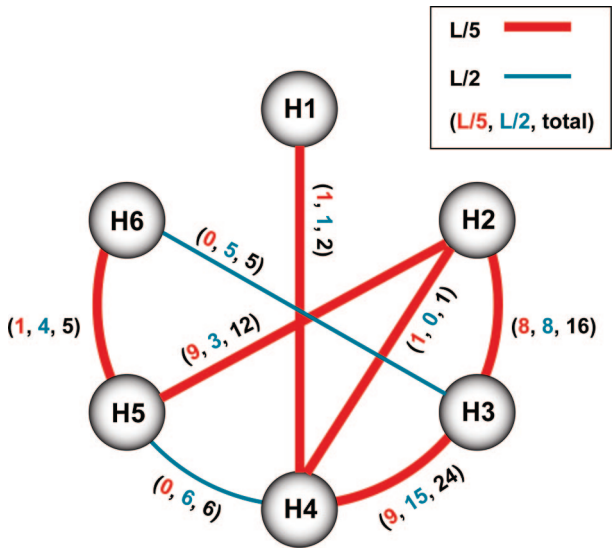

Figure 11. Helix−helix interaction map for AgrC-I. TMHit[313] generated transmembrane (TM) helices are indicated as circles and numbered according to AgrC-I topology.[37] Interactions between the helices are indicated by the lines (or arcs). The presence of a line/arc indicates that at least one predicted atomic interaction pair exists between the helices, and the absence of a line/arc means that the program predicts no contact pairs. An interaction pair is defined as a pair of residues that have at least one pair of atoms that are separated by less than the sum of their van der Waals radii plus 0.6 Å. Contact pairs can be predicted at a higher confidence level (called "L/5" and depicted by thick red lines/arcs) or at a lower confidence level (called "L/2" and depicted by thin blue lines). Next to each lines/arc are three numbers listed in parentheses, and these numbers are TMHit predicted interaction pairs in order of high confidence, low confidence, and total.

becauseTM6 is contiguous with the DHp domain in the HPK_{10}s and TM5 is predicted to form interhelix contacts with TM6 (see below). The continuity between the TM6 and the α1 helix of the DHp subdomain suggests there is a high likelihood that TM6 is part of the AgrC dimerization. This observation also raises the possibility that TM5 may contribute to the homodimer interface by participating in a four-helix bundle consisting of TM5, TM6, and TM5′ and TM6′ from the other AgrC subunit, consistent with the speculation of Dufour and colleagues.[52]

Because the AgrC mutational and chimera studies indicate that IPs interact with *N*-terminal portion of the HPK_{10} receptors, it is important to understand the linkage between the *N*-terminal and *C*-terminal regions of the HPK_{10} receptors, especially considering the *C*-terminal portion of the HPK_{10} receptors is responsible to transmembrane signaling. More specifically, it is important to identify the helix−helix contacts that occur between the TMs of the sensory domain as receptor activation is likely triggered by AIP-binding induced changes in these interactions.

In the absence of atomic resolution data about the three-dimensional structure of the AgrC sensory domain, we have used the program TMHit[109] to predict helix−helix contacts and generated a contact map that is shown in Figure 11. The analysis predicts extensive interactions for three different TM helical pairs in AgrC-I. These interactions include (1) TM2−TM3 with eight high confidence interactions, (2) TM2−TM5 with nine high confidence interactions, and (3) TM3−TM4 with nine high confidence interactions. When TMHit was tested against a data set consisting of TM helices from membrane proteins with known structures, predicted helix−helix contacts with eight high confidence interactions were identified with 82.4% accuracy, suggesting the predicted TM2−TM3, TM2−TM5, and TM3−TM4 interactions shown in Figure 11 likely exist in AgrC-I. Interestingly, the program predicts only limited interactions between TM5 and TM6. This observation suggests that the principle helix−helix interactions between TM5 and TM6 may be across the homodimer interface with the other AgrC monomer. Importantly, because the primary determinants of AIP-I binding specificity are located on the extracellular loop between TMs 3 and 4, and on TM2,[100,103,110] the extensive helix−helix contacts that TM2 is predicted to make with TM3 and TM5 provide a potential linkage between the IP binding site and the dimerization/trans-membrane signaling domain.

4.4. Ligand Specificity Determinants

There is fairly extensive literature concerning the location of ligand specificity determinants within the sensory domain of AgrC. Initial evidence that the AIP binding site is located within the *N*-terminal transmembrane domain came from studies in which maltose-binding protein (MBP) was fused to *C*-terminal portions of AgrC-I.[37] A MPB fusion protein containing only the cytoplasmic domain of AgrC (residue 199 through the *C*-terminus) spontaneously autophosphorylated, whereas a MPB fusion protein containing the portion of AgrC from residue 173 through the *C*-terminus autophosphorylated only in the presence of agr^+ supernatants or purified AIP. This finding indicated that the region between residues 173 and 199, which corresponds to the most *C*-terminal extracellular loop and TM of the sensory domain, contained at least some AIP binding determinants.

Using chimeric AgrCs that combined sensory domains of one *agr* type with DHp and CA subdomains of another *agr* type, it was possible to demonstrate that the AIP binding site is contained wholly within the *N*-terminal transmembrane domain.[60,61] More recent studies using a chimeric sensory domain consisting of the *N*-terminal half of AgrC-I and the *C*-terminal half of AgrC-IV were able to further localize the specificity determinants to the second extracellular loop (located between TM3 and TM4 in Figure 10).[104] Most recently, three research groups independently confirmed that extracellular loop 2 is a primary site of specificity determinants that differentiate between AIPs I and IV.[100,103,110] Within this loop, the sequences of AgrCs I and IV differ at only four positions (indicated in magenta text in Figure S1 of the Supporting Information), and all the groups were able to show that swapping these residues in several different combinations significantly alters the relative EC_{50}s for AIP-I and AIP-IV. Significantly, these results imply that when AIP binds to AgrC, the thiolactone ring interacts with extracellular loop 2 because the sequences of AIP-I and AIP-IV differ only at the first position in the ring (Asp in AIP-I and Tyr in AIP-IV). In additional site-directed studies, mutations in the region between residues 35−45[100] and 49−59[110] affected the degree to which AgrC receptors could activate, suggesting that the first extracellular loop may contribute to receptor activation by interacting with the *N*-terminal tail of AIP.

These findings are consistent with SAR investigations of AIPs that demonstrated the presence of the tail region is an absolute requirement for receptor activation. In particular, the finding that tailless AIP-I and AIP-I-D5A both inhibit AgrC-I activity suggests that receptor activation requires AIP-I to simultaneously bind both extracellular loops 1 and 2. Because Asn3 is a critical determinant of AIP-II activity,[50,56] and that AIP−II-N3A is an antagonist of AgrC-II,[49,61]

demonstrates that AIP-II must also bind extracellular loops 1 and 2 in order to activate its cognate receptor. Collectively, these findings imply that the structural rearrangements necessary for receptor activation are initiated when the tail of AIP binds to the first extracellular loop, triggering a displacement of TM2 that could be propagated to the DHp subdomain through alterations of TMs 5 and 6.

There is also substantial literature concerning the location of specificity determinants within the sensory domains of other HPK_{10}s that is consistent with localization to the first and second extracellular loops. Chimera studies utilizing SppK and PlnB,[77] similar to those conducted on AgrC-I/AgrC-IV sensory domains,[104] indicate that ligand specificity determinants for these receptors are localized within the *N*-terminal half of the sensory domains. Additionally, alanine scanning mutagenesis of PlnB identified two residues, Asp54 and Ser58 (magenta text in Figure S1 of the Supporting Information), that are required for IP activation. If PlnB were to adopt the consensus HPK_{10} structure, these two residues would be located on the first extracellular loop. In the case of *S. pneumoniae*,[106,107] there are two major pherotypes of competence-stimulating factor, CSP-1 and CSP-2, that cross activate the ComD1 and ComD2 receptors with EC_{50}s that are significantly higher than those of their cognate receptors.[105] There are only 12 amino acid differences between the sequences of ComD1 and ComD2, and they are all located within the first 80 residues of the sensory domain (see Figure S1 of the Supporting Information). The first five occur within the poorly defined *N*-terminus of the consensus HPK_{10} structure and may just reflect the low sequence and structural conservation of this region. The other seven differences cluster in the region between residues 47 and 59, which again would place them on the first extracellular loop of the consensus HPK_{10} structure.

4.5. Constitutive Mutations

To identify residues that increased HPK_{10} activity or made the receptor constitutive, error-prone PCR and assays were performed on AgrC-I,[108] SppK,[78] and ComD1.[107] In all three proteins, mutations with constitutive activity were identified at multiple sites (highlighted in green in Figure S1 of the Supporting Information and Figure 10). The constitutive mutants were found most frequently in the DHp subdomain, with a total of 3, 7, and 4 mutants mapping to the DHp subdomains of AgrC-I, SppK, and ComD1, respectively. These findings reflect the important role of the DHp subdomain in activation of the HK domain. Importantly, all of the constitutive AgrC-I mutants in the DHp subdomain were resistant to reverse agonism by AIP derivatives that function as antagonists of wild-type receptors, suggesting the structures of these receptors are locked in "on" conformations as a result of the mutations. In AgrC-I, five mutations (R180W, S183F, T197K, L205R, and L205H) were located on the flanks of TM6. Importantly, all of these mutants were sensitive to reverse agonsim, suggesting the AIP derivatives that function as antagonists can shift the structures of these mutants back to an inactive conformation. Interestingly, given the nature of these mutations, charged/polar → hydrophobic on the *N*-terminal end of TM6 and either polar → charged or nonpolar → charged on the *C*-terminal end of TM6, it has been proposed that these mutations will cause TM6 to be displaced in an extracellular to intracellular direction.[108] Similarly in SppK, two mutants, W213L and F218C, are found on the *C*-terminal side of TM6.[78] Two additional mutations were found in the second extracellular loop of SppK, I110V and T125A, but these both occur in a triple mutant in which the third mutation (Q237R) is located within the DHp subdomain.

4.6. Symmetric Transmembrane Signal Transduction

George and colleagues have recently reported that AgrC forms ligand-independent dimers that activate via trans-autophosphorylation upon interaction with AIP.[83] Co-immunoprecipitation was used to demonstrate the existence of ligand-independent dimers, and trans-autophosphorylation was demonstrated through a series of intermolecular complementation experiments. When AgrCs bearing mutations either in their G-box (G394A/G396A double mutant) or their H-box (H239Q) were expressed in the reporter strain, no activity was detected in response to AIP addition. To explain this result, in the case of the H-box mutant, $AgrC_{His}$, the site of histidine autophosphorylation has been deleted, and in the case the G-box mutant, $AgrC_{Kin}$, the nucleotide binding domain has been disrupted. However, when the $AgrC_{His}$ and $AgrC_{Kin}$ mutants were coexpressed in the reporter strain, dose-dependent activity was detected in response to AIP addition, with an EC_{50} of 40 nM that compared well to wild-type (EC_{50} = 10 nM). This activity is only possible if the mutant protomers complement each other's defect through trans-autophosphorylation within $AgrC_{His}/AgrC_{Kin}$ heterodimers.

Having demonstrated trans-autophosphorylation, George et al. further demonstrated that binding of AIP to a single sensory domain is sufficient for receptor activation and that receptor activation is symmetric.[83] Dose-dependent activity was detected when AIP-I was added to reporter strain in which full length $AgrC_{His}$ was coexpressed with $AgrC_{Kin}$ in which the AIP binding site was eliminated by deleting the first 135 amino acids of the sensory domains (extracellular loops 1 and 2). The activity was significantly lower than that of $AgrC_{His}/AgrC_{Kin}$ heterodimers (~1/3), which could have been due to weaker membrane association of the dimers containing the sensory domain deletion. To address this possibility, an analogous experiment was conducted using $AgrC_{Kin}$ containing mutations in the second extracellular loop of the sensory domain (T104V/S107A/S116I) that reduce the sensitivity to AIP-1 approximately 50-fold.[103] When this mutant, $AgrC_{Sensor,Kin}$, was coexpressed with $AgrC_{His}$, dose-dependent activation was observed. The activity was equivalent to that of $AgrC_{His}/AgrC_{Kin}$ heterodimers, confirming that binding of AIP to a single sensory domain is sufficient for receptor activation. Similar activation was observed when $AgrC_{Sensor,His}$ was coexpressed with $AgrC_{Kin}$, indicating that receptor activation is symmetric. In other words, binding AIP to a single sensory domain is capable of activating the catalytic domain of either protomer, which implies that AgrC dimers bind AIP with positive cooperativity. Alternatively, symmetric signaling could be due to AIP binding to a composite binding site made up regions from both sensory domains of the tetramer. To distinguish between these possible explanations, experiments were conducted with intermolecular complementation in which activation was accomplished by introducing a constitutive mutation rather than AIP addition. Introduction of a mutation into one protomer was found to activate the wild-type protomer in the absence of AIP, consistent with the idea that symmetric signaling is the result of ligand binding induced structural changes being propagated across the dimer interface. This

raises the possibility that AgrC binds AIP with positive cooperativity,[59] which may be advantageous for *S. aureus* survival in the host because it would allow for very rapid induction of virulence factor expression.

5. AgrA

AgrA has a sequence similarity to response regulators and is essential for activation of the *agr* P2 and P3 promoters.[35,38] Initial progress on AgrA characterization was slowed by technical challenges, but success in recent years has provided much insight on AgrA function. In this section, we briefly summarize the DNA-binding and structural studies that have been performed on this essential part of the *agr* regulatory system.

5.1. P2/P3 and PSM Promoter Binding

While initial analysis of AgrA indicated that it functioned as a transcriptional regulator,[35] skepticism over AgrA's role as a DNA-binding protein grew when experiments were unable to demonstrate AgrA binding to DNA probes representing the P2−P3 promoter region, likely due to purification challenges.[111] Koening et al. overcame this challenge and used purified AgrA in DNA-binding experiments to confirm that AgrA is indeed a transcriptional regulator.[44] EMSA experiments with the P2 promoter sequence indicated AgrA bound as a dimer with high affinity (K_d = 0.16 nM). This interaction occurred with 10-fold higher affinity compared to unphosphorylated AgrA (K_d = 3.8 nM). DNaseI footprinting assays of the P2−P3 promoter region demonstrated two regions of protection, both overlapping the AgrA binding site direct repeats in each of the promoters (Figure 12). AgrA binds to P2 with higher affinity than the P3 promoter (K_d 0.16 nM vs 1.7 nM). This difference can be attributed to two basepair changes in the downstream direct repeat of the P3 promoter (AC-to-CT difference). Mutation of these two basepairs to the idealized "AC" results in a P3 promoter with an even higher affinity for AgrA than P2. Interestingly, the P3 promoter also has an unusually long spacer of 20 bp, in contrast the normal 16−17 bp spacer length, and shortening of the P3 spacer by 3 bp elevates RNAIII levels.[111] The modified P3 promoter also bypasses the requirement for AgrA transcriptional activation, as demonstrated through RNAIII expression in an *agrA* mutant, presumably due to recognition by the housekeeping σ factor.

For many years, the possible role of AgrA interacting with other promoters besides the *agr* P2 and P3 went unaddressed. In 2008, Queck et al. used DNA microarrays to demonstrate that AgrA is capable of upregulating three chromosomal operons in addition to the *agr* P2 and P3 promoters.[39] These include the PSMα, PSMβ, and MW0370/0372 operons (see section 2.2.2). Closer examination of the promoters for these genes revealed potential AgrA binding sites. EMSAs and DnaseI footprinting experiments identified an AgrA protected region immediately downstream of the direct repeats in the *psm*α promoter at a site overlapping the -35 and -10 regions. Using the same approaches on the *psm*β promoter, AgrA protected regions were found overlapping the direct repeats. This study also identified other genes that were downregulated by AgrA independently of RNAIII, but whether AgrA inhibits transcription of these genes through promoter binding will require further investigation.

5.2. Structural Studies

S. aureus AgrA is the only member of the LytTR class of response regulator for which a structure has been determined.[112] Because of challenges with full-length protein expression, Sidote et al. purified the *C*-terminal domain (residues 137−238) and used this protein for structural studies. Using EMSAs, a 15 bp minimum DNA sequence was identified that maintained optimal AgrA−DNA interactions. This sequence contains the consensus 9 bp binding site in addition to 3 bp flanking this site on either side. The crystal structure was solved in cocomplex with the oligonucleotide duplex at 1.6 Å resolution and demonstrated that AgrA has a novel 10 β-stranded topology that includes a two-turn α helix. Interaction of AgrA with the oligonucleotide duplex results in a ∼38° bend that creates a concave DNA-binding surface, and due to this distortion, AgrA makes contacts with successive major grooves along a single face of the DNA. In the current model of binding to tandem repeats, AgrA interacts with the repeats as a dimer, but only the AgrA *N*-terminal regions form protein−protein interactions, while the *C*-terminal domains are spaced ∼10 Å apart. In the *C*-terminal region, only two residues, H169 and R233, make specific DNA contacts in the major grooves, while a third (N201) interacts with water in the minor groove.

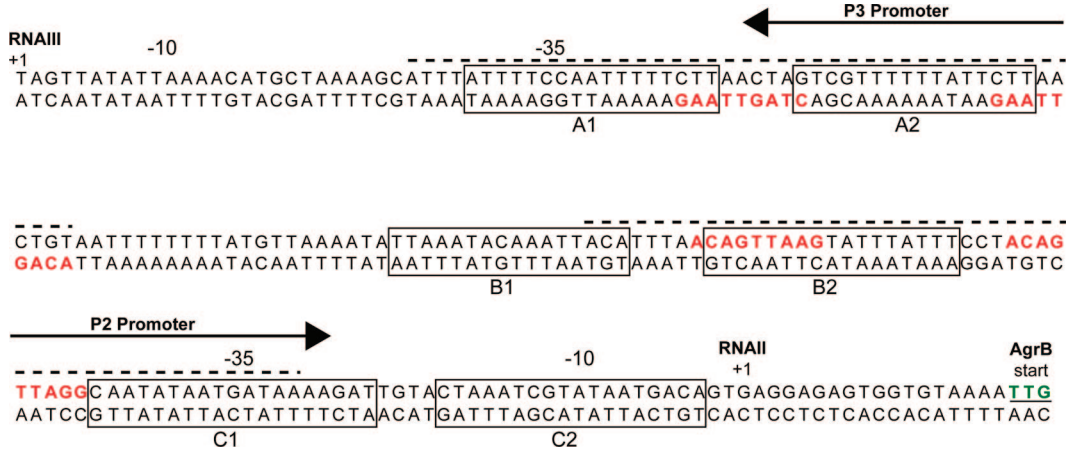

Figure 12. The *agr* P2−P3 intergenic region. RNAII and RNAIII transcriptional start sites are marked with a +1. Direct repeats recognized by AgrA are colored red, while areas protected from DNaseI cleavage by AgrA are indicated with a dashed line above the sequence. Boxes indicate SarA binding sites as reported by Rechtin et al.[143]

Surprisingly, these residues are not strictly conserved in LytTR family members, potentially due to the significant sequence variation among family members. Mutations of residues H169 and R233 to alanines increased K_d values 40−90-fold, indicating they are essential for AgrA DNA-binding affinity.[112] Unfortunately, the AgrA structure has not provided significant insight on the nonfunctional mutations that occur spontaneously in *S. aureus* strains at the AgrA *C*-terminus.[113] Sidote et al. suggest that the mutations could render AgrA unstable or result in the loss of other interactions or contacts with transcriptional machinery.

6. RNAIII

Following the discovery of the *agr* system, it was unclear how a single two-component regulator could have such a pleiotropic effect on gene expression, especially when some genes are being upregulated and others downregulated. The answer to this question came from mutations that occurred upstream of the *agr* P2 promoter. A second promoter, later named P3, was discovered close to the P2 promoter facing in the opposite direction.[36] Analysis of the transcript (named RNAIII) revealed a 0.5 kb mRNA containing a short gene, *hld*, which encodes the 26-amino-acid δ-toxin (also called δ-hemolysin). Surprisingly, when *hld* was deleted, the mutants showed the same phenotype as *agr* mutants, suggesting a regulatory role for δ-toxin or its transcript. When the RNAIII region was complemented under the control of an inducible promoter, it was able to rescue the phenotype of an *agr* null mutant.[38] This complementation occurred regardless of whether *hld* was present, suggesting that the RNAIII transcript itself was serving as the regulatory molecule. Following this critical observation, numerous studies have investigated RNAIII's function as an effector molecule of the *agr* system.[40,42,114−117] In addition to encoding δ-toxin, RNAIII is able to directly promote or inhibit translation of multiple virulence factors while also inhibiting the translation of a regulatory protein, Rot. The basic properties and mechanism of action of this extraordinary regulatory RNA will be outlined in this section.

6.1. Structure

At 514 nucleotides, *S. aureus* RNAIII is one of the largest regulatory RNAs found in prokaryotes. Structural studies using chemical and enzymatic probes combined with RNA-folding algorithms revealed a molecule consisting of 14 hairpins (Figure 13).[116] The folded molecule also contains three sites of long-range interaction in which distant nucleotides come together to form helices. These long-range interactions divide the RNA into three distinct domains. The 5′ domain is composed of the first 31 nucleotides and contains hairpin 1. The large central domain (nt 32−382) consists of hairpins 2−11 and contains multiple features. It encodes δ-toxin (nt 85−165), contains most of the region necessary for initiating α-toxin translation (highlighted green in Figure 13) while also possessing hairpin 7, which is involved in negative post-transcriptional regulation of genes such as *rot*. The 3′ domain (nt 383−514) makes up helices 12−14, which are primarily involved in negatively regulating translation of targeted genes.

In *S. aureus*, RNAIII is expressed during postexponential phase when cultured, consistent with its activation by *agr*.[36] While the P3 promoter is activated after P2, it is upregulated to a much higher extent.[118] The delayed activation can be attributed to the higher affinity of phosphorylated AgrA for the P2 compared to P3.[44] After transcription, RNAIII has a long half-life of up to 45 min.[115,119]

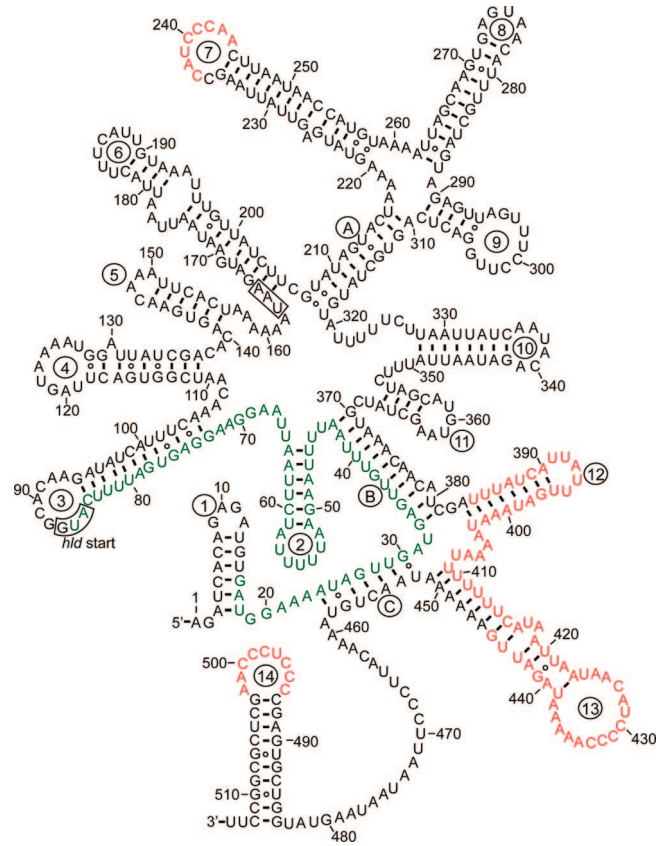

Figure 13. Secondary structure of *S. aureus* RNAIII. RNAIII hairpins are designated with circled numbers, while letters signify long-range interactions that establish general domains. *hld* start and stop codons are boxed. Nucleotides that basepair with and activate *hla* translation are colored green. Nucleotides demonstrated to inhibit translation of genes are red. This figure is an adaptation from Benito et al.[116] Reprinted with permission from ref 116 Copyright 2000 Cold Spring Harbor Laboratory Press.

When comparing RNAIII sequences of different staphylococcal species, the secondary structure is well conserved while the primary sequence is conserved only in the first 50 and last 150 nucleotides.[120] The size can vary significantly with some molecules being up to 700 nucleotides in length. Despite the difference in sequence, the function of the RNAIIIs are conserved as the molecules from various staphylococcal species are able to complement RNAIII-deficient *S. aureus*.

6.2. δ-Toxin

In addition to the RNAIII role as a regulatory RNA, within the transcript is the *hld* gene that encodes δ-toxin. The gene is located toward the 5′ end of the transcript at nucleotide positions 85−165 (*agr* Type I numbering). Most staphylococcal species contain a copy of *hld* in their RNAIII, however, *Staphylococcus lugdunensis* RNAIII does not carry the gene while *Staphylococcus warneri* contains two *hld* genes that differ by eight amino acids.[120,121] *S. aureus* δ-toxin is a 26-residue peptide that forms an amphipathic helix in solution.[122] It is capable of lysing cells by oligomerizing and inserting into targeted membranes to form pores or, at high enough concentrations, by acting as surfactant and disrupting the membrane and forming micelles (the reader is directed

to Verdon et al. for a comprehensive review of δ-toxin function[123]).

Translation of *hld* is typically delayed one hour after RNAIII is synthesized.[124] The mechanism behind the translation delay is unclear. The *hld* SD sequence is located at the base of hairpin 3 (Figure 13), suggesting that when this hairpin is present the ribosome initiation complex might have trouble recognizing the sequence. However, toeprint studies suggest that ribosomes are able to bind native RNAIII.[116] Additionally, the *hld* Shine−Dalgarno (SD) sequence and start codon are part of the RNAIII segment that basepairs with *hla* mRNA to promote α-toxin translation. Interestingly, a deletion of 155 nucleotides of RNAIII (including hairpins 11−14) at the 3′ end is able to relieve the *hld* translation delay.[124] This deletion prevents the long-range association that divides RNAIII into its three domains while also preventing negative regulation by RNAIII on Rot expression and other targets. It remains unclear whether the effect on *hld* translation is due to altered RNAIII structure versus changes in other gene expression that might influence translation.

6.3. Mechanism of Action

RNAIII can regulate protein expression by basepairing with the mRNA of the target gene. For translation to occur, the 30S ribosomal subunit must be able to recognize the ribosome binding site of the mRNA. This recognition occurs through the 16s rRNA basepairing with the SD sequence of the single-stranded mRNA. If the SD sequence is hidden by the formation of double-stranded RNA, either by forming a stem-loop structure or baseiring with another RNA, then translation initiation will be inhibited.

RNAIII is capable of promoting or inhibiting translation of various mRNAs by exploiting this mechanism. RNAIII can promote translation by basepairing and relieving a stem−loop structure that normally masks the SD sequence.[42] In contrast, multiple RNAIII stem loops (notably 7, 13, and 14) are capable of basepairing with target gene SD sequences, forming double-stranded mRNA structures unrecognizable by ribosomes.[40,114,115,117] To carry out this function, each of the three stem loops contains the sequence 5′-$UC_{(3-4)}$A-3′, which allows for direct basepairing with SD sequences.[40] Notably, this motif has been found in a variety of other small noncoding RNAs in *S. aureus* that are thought to act via a similar mechanism.[46] Through these interactions, RNAIII is capable of both positively and negatively regulating gene expression at the translational level.

Another mechanism of post-transcriptional regulation carried out by RNAIII is mediated through RNase III. RNase III is an endoribonuclease that carries out cleavage of double-stranded RNAs. RNase III specifically binds RNAIII is the absence of a target mRNA, enabling RNAIII to act as a shuttle to target the enzyme to different mRNAs.[115] By basepairing with the target mRNA, RNAIII acts to generate a cleavable substrate for RNase III. This cleavage leads to both the destabilization of the mRNAs as the half-lives are significantly shortened and, in some cases, results in the removal of the SD sequence to prevent ribosome binding of the mRNA.[115,117] This combination adds yet another level of post-transcriptional regulation for RNAIII to control gene expression.

Hfq is a well-conserved protein that is important for the function of many small regulatory RNAs in prokaryotes.[125] By binding to small RNAs, Hfq is able to both stabilize and promote the interaction with targeted substrates. Hfq is important in virulence for multiple bacteria, but its role in *S. aureus*, particularly in regard to RNAIII function, is less clear. In one study by Bohn et al., there was virtually no change in gene expression in an Hfq knockout.[126] However, a recent study by Liu et al. demonstrated decreased protease and nuclease expression while producing more pigment.[127] These mutants also displayed decreased cytotoxicity and virulence in a mouse peritonitis model. The reason for the discrepancies between the studies remains unclear. Hfq can be copurified with RNAIII and does bind in EMSA experiments.[115] However, multiple studies have shown that deletion of Hfq does not affect RNAIII stability or activity in regards to translational control or RNase III recruitment, suggesting that Hfq is not important for RNAIII function.[114,115,117]

6.4. Positive Regulation of α-Toxin

One of the first RNAIII functions identified was the positive regulation of *hla* gene translation into α-toxin (also called α-hemolysin).[42,128] α-Toxin is a secreted virulence factor that forms heptameric pores in cell membranes, resulting in lysis of the targeted cell. A post-transcriptional level of regulation for α-toxin was suggested when a RNAIII mutant was isolated that produced significant levels of *hla* mRNA with little α-toxin product.[42] In studies by Morfeldt et al., RNAIII was found to complex with *hla* transcript in vitro and in vivo using gel-shift experiments.[42] RNase protection studies indicate the 5′ end of RNAIII, including nucleotides 16−86, basepairs with the 5′ untranslated region of the *hla* mRNA (∼75% complementary). When no RNAIII is present, the 5′ region of *hla* mRNA forms a hairpin loop which masks the SD sequence in the stem, resulting in a confirmation that cannot be translated. RNAIII binding to the mRNA relieves this stem loop structure, allowing the ribosome to recognize the SD sequence and carry out translation (Figure 14). This was one of the first examples where a regulatory RNA was shown to promote translation of its targeted protein. This regulatory switch allows a second level of control over α-toxin production, in conjunction with the transcriptional control by other regulatory factors such as Rot, SaeRS, and SarA.[43,129,130]

6.5. Negative Regulation of Protein A

In contrast to its role in promoting *hla* translation, RNAIII inhibits the translation of multiple proteins. One of the first examples of inhibition came from the regulation studies on the translation of the *spa* gene into protein A. Protein A is a cell-wall anchored surface protein known for its ability to bind the Fc region of antibodies with high affinity. During lab culture, it is expressed during exponential growth, but protein A production is reduced during the transition to postexponential phase, suggesting a role for *agr* in down-regulation of the protein.

Early studies identified the 3′ region of RNAIII as being important for repression of *spa* transcription, although this was probably due in part to this domains role in regulating Rot (see below).[116,128] The recognition of RNAIII as a translational regulator of *hla*, along with its complementary sequence to *spa* mRNA, led to the hypothesis that RNAIII was inhibiting protein A expression at the level of translation.[115,131] To test this question, Huntzinger et al. generated translational fusions with the 5′ end of the *spa* mRNA fused in-frame with a β-galactosidase reporter. Using

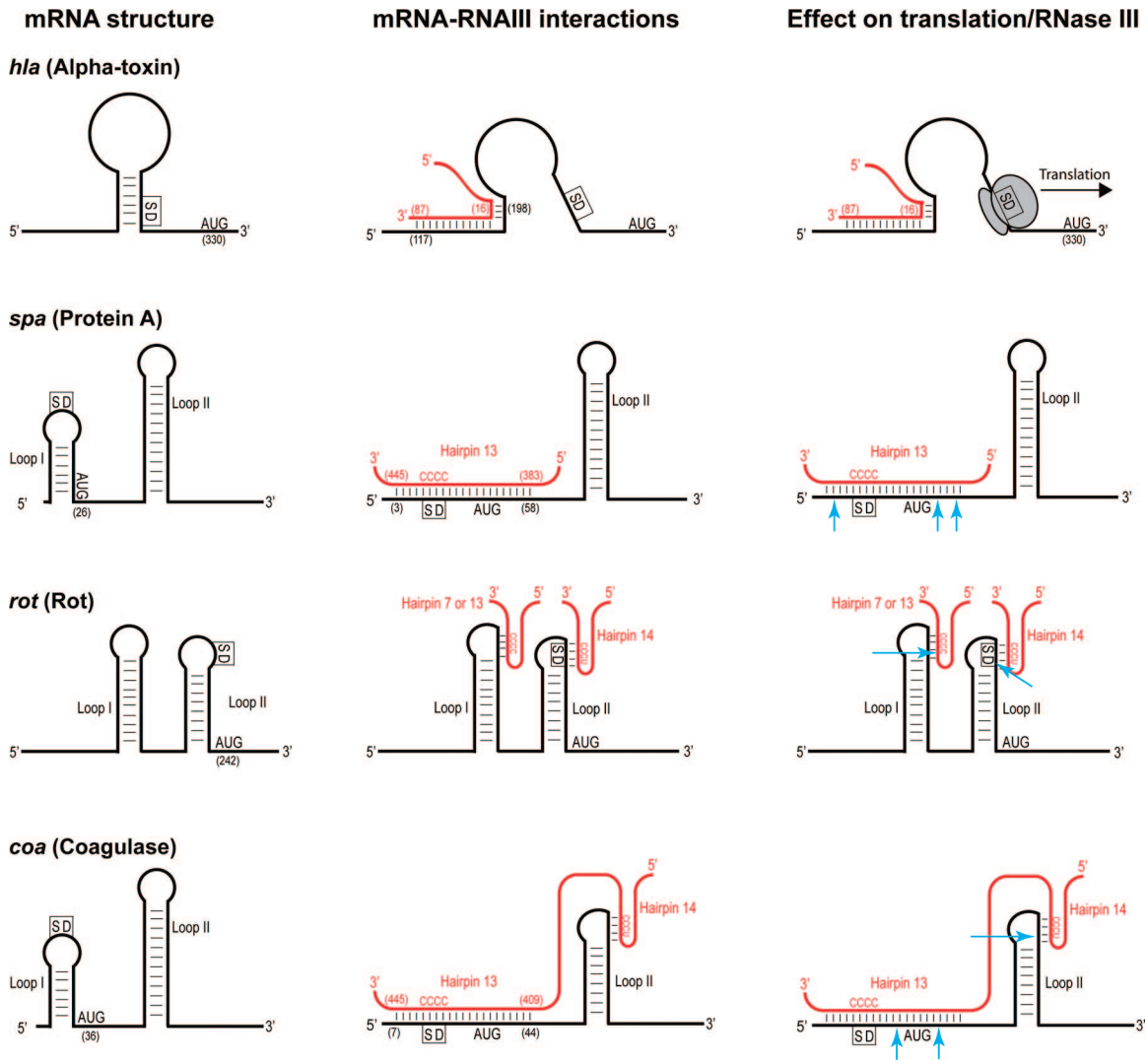

Figure 14. RNAIII mechanisms of post-transcriptional regulation on *hla*, *spa*, *rot*, and *coa*. RNAIII activates *hla* translation by disrupting the stem-loop and allowing ribosomes to access the Shine−Dalgarno (SD) sequence. RNAIII inhibits the translation of genes (*spa*, *rot*, and *coa*) by basepairing with the mRNA, blocking recognition of the SD sequence, and promoting RNase III cleavage. Targeted mRNAs are shown in black, while RNAIII sections are red. Numbers reflect nucleotide position from 5′ end of transcripts. Blue arrows indicate sites of cleavage by RNase III.

an *agr*-independent promoter, a significant decrease in reporter activity was apparent when RNAIII was expressed, indicating control at the post-transcriptional level.[115] The authors went on to show that stable RNAIII−*spa* complexes can be isolated and this complex formation is dependent on hairpin 13 of RNAIII. RNAIII binds to *spa* mRNA with an initial binding rate of 3×10^5 $M^{-1}s^{-1}$ and apparent K_d of 31×10^{-9} M, consistent with values obtained from other sRNA interactions. Translational inhibition occurs through the formation of an extended duplex of more than 30 base pairs between RNAIII and the *spa* mRNA, including the SD sequence and start codon (Figure 14). This leads to masking of the SD sequence and prevents formation of the transcription initiation complex.

RNAIII also represses protein A expression by altering the stability of the *spa* transcript. In the absence of RNAIII, the *spa* mRNA was found to have an unusually long half-life of 15 min.[115] The 5′ untranslated region contains a stem−loop structure in which the SD sequence is located in the external loop of the hairpin. Ribosomal binding and stalling at this structure is thought to help stabilize the transcript by interfering with initial degradation. When RNAIII binds the *spa* mRNA, it disrupts the 5′ hairpin through the formation of an extended duplex. This duplex serves as a substrate for RNase III, which cleaves the *spa* mRNA at two sites just downstream from the start codon. Cleavage at the first site also results in the basepaired RNAIII being cut, while the second site is in an internal loop and therefore does not result in RNAIII cleavage. The role of RNase III in *spa* regulation is further highlighted by the observation that full repression of *spa* expression cannot be achieved in an RNase III knockout. These findings support the idea that RNAIII works in concert with RNase III in order to suppress expression of targeted genes.

6.6. Negative Regulation of Rot

Perhaps RNAIII's greatest impact on gene expression is through its regulation of Rot. Rot is a global transcriptional regulator in the SarA family of regulators. It was originally identified when a Rot transposon mutant was able to restore hemolysis and protease production to an *agr* mutant.[132] Rot is best known for its ability to downregulate many secreted virulence factors including the α-, β-, and γ-toxins, extracellular proteases, urease, lipase, and enterotoxin B.[43,133] Rot also acts to upregulate the transcription of genes encoding

virulence factors, including protein A, coagulase, and clumping factor B.[43] Additionally, Rot is able to upregulate or downregulate a large number of other genes involved in various cellular processes, such as transport, cell-wall biosynthesis, and metabolism of amino acids and carbohydrates. Not surprisingly, there is considerable overlap in the genes identified as being *agr* regulated and those identified as Rot regulated.[41,43] Nevertheless, some differences are observed, highlighting the fact that *agr* gene regulation is not due simply to inhibition of Rot.

There are parallels between RNAIII regulation of Rot and protein A expression (Figure 14). The *rot* mRNA contains a large 5′ untranslated region that extends 293 base pairs upstream of the start codon.[134] This region contains multiple stem−loop structures, including loops I and II just upstream of the start codon in which the outer loop of loop II contains the *rot* SD sequence. Isolated RNAIII hairpins were tested for their ability to bind the *rot* loops and protect them from hydrolysis by RNase T1.[114] This revealed that RNAIII hairpin 14 bound Rot loop II with the highest affinity, while hairpins 7 and 13 bound best to loop I. Two of the three RNAIII hairpins are required for sufficient binding and inhibition of Rot translation, and no single hairpin appears to be required. This binding is mediated by the RNAIII 5′-UCCC-3′ motifs interacting with the 5′-UUGGGA-3′ sequences in loops I and II. Therefore rather than forming an extended duplex as with other mRNA targets, RNAIII instead inhibits Rot translation by forming two loop−loop interactions, with the interaction involving Rot loop II blocking the SD sequence.

RNAIII also promotes the degradation of *rot* mRNA in a mechanism involving RNase III. Translational fusions of Rot with β-galactosidase revealed that RNAIII is not able to fully inhibit the reporter when RNase III is knocked out, indicating a role for RNase III in Rot downregulation.[114] Multiple studies have indicated that the presence of RNAIII does not decrease the *rot* mRNA half-life, however, Northern blots indicated the presence of cleaved *rot* transcript when RNAIII and RNase III are both present.[40,114,134] The major cleavage site is located in the *rot* SD sequence and results in a nontranslatable transcript.[114] This cleavage is dependent on the presence of RNAIII hairpin 14, while a second cleavage event in the upstream loop I is mediated by hairpin 7. These interactions are thought to result in coaxial stacking between the loops, which provides a substrate that RNase III is able to recognize and cleave.

6.7. Regulation of Other Targets

To uncover additional genes regulated by RNAIII action, a bioinformatics search of the *S. aureus* N315 genome was carried out to identify mRNAs whose 5′ regions were predicted to basepair with hairpin 13 of RNAIII.[114] The mRNA predicted to bind RNAIII with the lowest free energy was SA1000, which encodes a fibrinogen binding protein. Similar to *rot*, SA1000 mRNA is predicted to form two stem-loops near the 5′ end, although the 5′ loop I of SA1000 mRNA contains the SD sequence and start codon. Using similar techniques as outlined above, hairpin 13 was found to interact with loop I via its SD sequence, followed by the rapid formation of a 40 bp extended duplex, including the SD sequence, which disrupts the loop I structure. This mechanism has the dual effect of inhibiting formation of the translation initiation complex and allowing RNase III to cleave the SA1000 mRNA at two sites on either side of the start codon. The result is an mRNA that cannot be translated and a decrease in SA1000 mRNA levels in the cell.

Another virulence factor that is directly downregulated by RNAIII is coagulase. Coagulase is a secreted protein that promotes the formation of clots by complexing with prothrombin to activate fibrin formation in plasma. Similar to SA1000, RNAIII hairpin 13 binds to a 5′ *coa* mRNA stem−loop containing the SD sequence (Figure 14), resulting in the formation of an imperfect duplex that consists of two basepaired regions flanking an internal bulge.[117] In addition to this interaction, RNAIII hairpin 7 binds to a third *coa* stem-loop approximately 50 nucleotides downstream of the start codon. This interaction is reminiscent of the RNAIII−*rot* mRNA complex in that the loop−loop interaction results in coaxial stacking rather than a long duplex formation and results in cleavage at the site of interaction. This seemingly extra loop−loop interaction is considered to be important both for specificity of RNAIII targeting and in destabilizing the *coa* mRNA by disrupting the stem−loop structure located within the coding sequence.

7. Regulation of agr

Many different environmental, host, and transcriptional regulators factors have been shown to impact *agr* system function. Because of the abundance of transcriptional regulators that have been identified with *agr* phenotypes, we have summarized these regulators in Table 2. Some of the best described examples of environmental, positive, and negative regulation are outlined in this section. Host factors also modulate *agr* function, and these interactions will be addressed later in section 10.

7.1. Environmental Cues

Following the discovery of *agr*, there was interest in how the system responded to various environmental or stresses. One of the earliest factors identified that modulates *agr* function is pH. Initial studies by Regassa and Betley examined how both high and low pH affected RNAIII expression.[135] In one study, the pH of the culture was maintained at intervals from 6.5 to 8.0 and RNAIII expression was monitored through Northern blots. Expression was optimal at pH 7.0 and then continually decreased until the media pH reached 8.0.

Similar *agr* inhibitory effects are observed when *S. aureus* is grown under conditions that generate acidic pH. When *S. aureus* is grown aerobically in media supplemented with

Table 2. Transcriptional Regulators That Modulate *agr* Function

regulator	effect on agr	ref
	Positive Regulators	
SarA	+	141, 142
SarU	+	174
SarZ	+	175, 305, 306
MgrA	+	148, 150
CcpA	+	140
ArlRS	+	173
	Negative Regulators	
SarX	−	177
SarT	−	176
Rsr	−	169
SrrAB	−	170, 171
σ B	−	158, 307
CodY	−	164, 168

sufficient metabolites to accumulate excess acetyl-CoA (e.g., glucose), the cells release acetate as an overflow byproduct to generate ATP through substrate level phosphorylation and recycle CoA.[136] The accumulation of acetate results in a drop in the extracellular pH of the local environment. As the primary metabolites are consumed, *S. aureus* transitions into postexponential phase growth and assimilates acetate as a carbon source, causing the extracellular pH to increase. As indicated above, low pH represses the *agr* system and buffering of culture media prevents this phenotype. The environment of certain colonization sites, such as the skin and vaginal mucosa, can have a local pH < 5,[137] which is low enough to inhibit *agr* activation and virulence factor expression.[138] Currently, molecular and biochemical mechanisms through which pH changes affect *agr* activation remain unclear, but they could have an important impact on *S. aureus* pathogenesis in the host.

While glucose supplementation can inhibit *agr* function, glucose also activates carbon catabolite repression, a regulatory system that modulates gene expression to adapt to preferred carbon source utilization. In low G + C Gram-positive bacteria, this process is mediated by three main proteins; the phosphotransferase HPr, a bifunctional HPr kinase-phophatase, and the transcriptional regulator CcpA.[139] In the presence of glucose, HPrK/P phosphorylates HPr, facilitating the formation of an Hpr−CcpA complex that can bind to catabolite-responsive elements (CREs) of promoter regions. Given the effects of glucose on virulence factor expression, CcpA's role in regulating *agr* was investigated. Interestingly, a *ccpA* knockout displayed a dramatic decrease in RNAIII expression in the presence or absence of glucose at a constant pH,[140] which suggests that CcpA activates *agr* in the presence of glucose. When examining the *agr* P2/P3 region, there is no obvious CRE sequence, implying that the regulatory effect of CcpA may be indirect and in need of further investigation.

7.2. Positive Regulation

7.2.1. SarA

Perhaps one of the best studied regulators of *agr* activation is the staphylococcal accessory regulator (*sarA*). SarA was originally identified as a transposon mutant that displayed altered exoprotein production.[130] It was evident early on that *sarA* mutants shared some characteristics of *agr* mutants such as decreased toxin production, but other changes were divergent, such as increased extracellular protease activity and decreased fibronectin-binding protein production. Molecular analysis confirmed that the *sarA* gene was indeed independent from the *agr* locus.[130,141]

Initial experiments on *sarA* mutants demonstrated that *agr* RNAII and RNAIII transcript production was diminished compared to wild-type strains.[142] SarA activates transcription in the *agr* locus by binding to the P2/P3 promoter region[142,143] (Figure 12). The identification of the SarA binding site has been the focus of multiple studies. Using DNase I footprinting assays with GST-tagged protein, Chien et al. identified a 29 bp protected region between the P2 and P3 promoters.[144] This protected region was later refined by aligning promoters of SarA regulated genes to a consensus 26 bp recognition site, which overlapped the original 29 bp site by 22bp.[145] Similarly, Rechtin et al. performed DNase I footprint studies, in this case using untagged SarA, and found that SarA protects three paired regions within the *agr* P2/P3 region: (1) boxes A1 and A2 overlap the -35 region and AgrA binding sites of P3, (2) B1 and B2 overlap the AgrA binding site of P2, and (3) C1 and C2 overlap the -10 and -35 regions of P2.[143] When compared with Chien's SarA binding site, there is overlap with the B1 box. The concept of multiple SarA binding sites in *agr* was further corroborated by Sterba et al. using a technique called systematic evolution of ligands by exponential enrichment (SELEX). Briefly, 98 bp double-stranded oiligonucleotides containing 50 bp of random sequence underwent selection for binding to SarA by EMSA.[146] By cloning and sequencing the enriched DNA fragments, a conserved 7 bp sequence (ATTTAT) was identified and deemed important for SarA binding. When compared to Rechtin's SarA boxes, six out of seven matches in this 7 bp sequence were found in boxes A2, B1, and twice in C1, lending further evidence that these indeed are SarA binding sites.

Crystal structures of SarA indicate that the protein is a winged-helix dimer. When complexed with DNA, SarA distorts the DNA helix to appear overwound, resulting in tighter packing of nucleotides.[147] The result is a shortening the spacing between the -35 and -10 elements of promoter. Interestingly, these elements in the P3 promoter are spaced further apart than in normal promoters (20 bp vs 16 bp), lending support to the hypothesis that SarA activates by overwinding the helix and tightening the -35 and -10 spacing for idealized σ factor recognition. However, this hypothesis is complicated by the proximity, and in some cases overlap, of AgrA binding sites to those for SarA.

7.2.2. MgrA

MgrA (also known as Rat) is a small transcriptional regulator related to the SarA family of regulators. MgrA regulates cell-wall turnover and activates the production of secreted toxins and proteases.[148,149] These parallels indicated an overlap with proteins induced during *agr* activation. In initial experiments, Ingavale et al. showed that a transposon insertion in *mgrA* caused a small decrease in *agr* RNAII and RNAIII levels.[148] Follow-up studies using an *mgrA* knockout demonstrated a complete loss of RNAIII production and no activation of an *agr*-P3 reporter fusion.[150] However, the same deletion in *S. aureus* strain Newman had a minor effect,[150] indicating strain-to-strain variation. Purified MgrA does shift *agr* promoter fragments in an EMSA, but the specificity of the MgrA interaction with the P3 promoter is in need of further investigation. Microarray analysis confirmed that MgrA activates toxin and protease production, along with many other genes,[151] but it failed to identify the *agr* genes or RNAIII as being differentially regulated, potentially due to the use of strain Newman.[150] Further analysis is necessary to clarify strain-to-strain discrepancies and define the MgrA and *agr* interconnection.

7.3. Negative Regulation

7.3.1. σ Factor B

σ B (SigB) is an alternative σ factor associated with the extracellular stress response in Gram-positive bacteria. In *S. aureus*, SigB is responsible for activating transcription of surface proteins and pigment production while downregulating production of secreted toxins and proteases.[152−156] Given the opposing regulatory output compared to *agr*, perhaps it is not surprising that SigB also downregulates *agr*.

SigB knockouts in a variety of backgrounds show increased RNAIII levels and stronger activation of P3 reporter fusions.[157,158] The molecular mechanism through which SigB inhibits RNAIII expression is unclear. There are no identifiable SigB binding sites within the *agr* locus and attempts to link the two global regulator systems through an unknown intermediate have been unsuccessful.[157,159] These observations point to a yet unidentified factor under SigB control that is responsible for the *agr* effect.

7.3.2. CodY

The link between cell metabolism and virulence factor production has been recognized for many pathogens. Similarly with *S. aureus*, the metabolic state can have dramatic effects on the expression of virulence genes.[160] One regulator responsible for mediating this interaction is CodY, a transcription factor found in low G + C Gram-positive bacteria that plays a role in adapting to metabolic stress and nutrient limitation. CodY also has been implicated in regulating virulence factors by sensing nutrient availability through binding cellular GTP and branched chain amino acids.[161,162] During nutrient limitation these pools become depleted, resulting in CodY being unable to bind DNA and relieve repression of target genes.[163]

The *agr* system is among the many genes regulated by CodY. A *codY* knockout elevated RNAII and RNAIII levels during exponential growth, indicating negative regulation when nutrients levels are high.[164] During stationary phase growth, nutrient pools are depleted and CodY does not have an effect. In *S. aureus*, the major CodY ligand is the amino acid isoleucine. Wild-type cells grown in the absence of isoleucine show elevated RNAIII expression, similar to the levels seen in a *codY* knockout, further suggesting a role for this protein in downregulating *agr* during exponential phase.[165]

The mechanism of *agr* repression through CodY has been investigated. Putative CodY binding sites have been determined in other Gram-positive species.[166,167] However, the closest match in the *agr* region is located near *hld* within the RNAIII transcript, potentially too far to have a direct affect on RNAII transcription. There is a second CodY binding site located within the RNAII transcript in the *agrC* coding sequence. Pull-down experiments using His_6-tagged CodY identified the *agrC* gene among the enriched fragments of DNA.[168] Two explanations of CodY repression on *agr* have been proposed. First, CodY binding could downregulate transcription from the P1 promoter, reducing levels of RNAI transcript that encode AgrA. Whether downregulation of P1 is significant enough to inhibit overall RNAII synthesis is unknown. Alternatively, CodY could act as a transcription roadblock within RNAII, preventing transcription of the 3′ end of *agrC* and *agrA*. CodY binding sites have been identified in the middle of other operons (*cap* and *ica*), and the effect on *agr* could be a parallel mechanism of action. While progress has been made, the CodY interconnection with *agr* is in need of further investigation to clarify these possible mechanisms.

7.3.3. Rsr

One of the most recently identified regulators of *agr* activity is Rsr (for repressor of *sarR*).[169] The *rsr* gene is located between the regulators *sarY* and *sarR*, however, the *rsr* gene is transcribed independently. Rsr is a 108-amino-acid protein that shares no homology with proteins outside of the Staphylococci. When *rsr* was deleted in multiple *S. aureus* backgrounds, an increase in SarR and the RNAII and RNAIII transcripts was observed, leading to an upregulation of the *agr* regulon.[169] Conversely, *rsr* overexpression inhibits RNAIII production. A deletion of *rsr* increased *S. aureus* virulence in a subcutaneous murine model, indicating the *agr* effect correlated in vivo. Rsr's effect on *agr* was independent of the SarR phenotype, as an increase in RNAIII was seen even in an *rsr sarR* double mutant. Considering the Rsr protein does not bind to the *agr* P2−P3 region, it remains unclear how loss of the protein increases RNAIII levels. Interestingly, the authors report that the *agr* mRNA sequence has features consistent with a small antisense RNA, leaving open the possibility that the RNA itself is regulating *agr* activation.[169]

7.4. Other Regulators That Affect *agr* Function

Another potential regulator of *agr* function in the staphylococcal respiratory response genes SrrAB. SrrA and SrrB form a two-component regulatory pair in which SrrB is membrane-bound histidine kinase and SrrA is a response regulator. SrrAB acts in response to anaerobic environments to regulate gene expression. In initial experiments, Yarwood et al. reported that *srrAB* mutants possessed an *agr* regulatory effect.[170] Follow-up studies demonstrated SrrA binding to the *agr* P2 and P3 promoters, as well as *tst* and *spa*, by EMSA, suggesting a direct regulation by SrrA on these promoters.[171] Overexpression of *srrAB* in various *S. aureus* strains made them less virulent in a rabbit endocarditis model, likely attributable in part to a repressed *agr* system. These findings are somewhat complicated by further studies using the clinical isolate MN8. In this strain, antisense suppression of *srrA* had no discernible effect on RNAIII levels[172] while other regulation was maintained. These conflicting findings on the Srr and *agr* interactions could be a consequence of strain-to-strain variation.

In addition to the examples described above, numerous other additional regulators have been characterized and described to affect *agr* function (Table 2). For the sake of brevity, a full description of these results will not be discussed. As examples, ArlRS,[173] SarU,[174] and SarZ[175] are reported to activate *agr*, while SarT[176] and SarX[177] downregulate *agr* function. In many cases, it remains undetermined whether these regulators directly affect *agr* transcription or if the mechanism is indirect through another factor.

8. Interconnection of agr and Biofilms

Biofilms are defined as communities of bacteria attached to a surface that are enclosed in an extracellular matrix. Biofilm development is especially important in the setting of a chronic infection where the multicellular aggregates exhibit increased resistance to antibiotics and host immune defenses. For more information, the reader is referred to numerous excellent reviews that describe the topic of biofilms in more detail.[178−182] In terms of *S. aureus* and other *Staphylococci*, biofilm formation has been implicated as a virulence mechanism in chronic infections that include endocarditis,[183,184] osteomyelitis,[185,186] and indwelling medical device infections.[187,188] The complexity of biofilm maturation and dispersal, along with the potential for the development of improved therapeutics, has led to a surge in interest in biofilm research over the past decade.

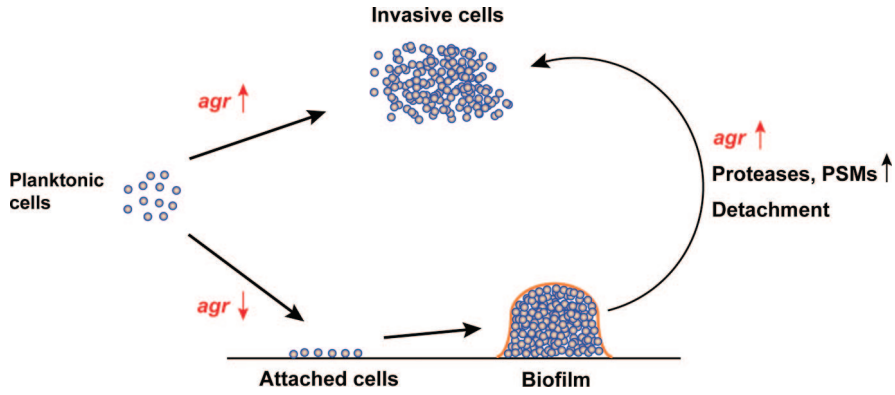

Figure 15. Schematic of the *S. aureus agr* system as a regulator of biofilm lifestyle. Under normal conditions with a functional *agr* system, *S. aureus* will secrete invasive factors and remain planktonic. When the *agr* system is inhibited or deactivated, *S. aureus* will preferentially adhere to surfaces and develop a biofilm if growth conditions permit. If the situation reverses in the biofilm and the *agr* system reactivates, the cells can detach in a protease and PSM-dependent manner and return to the planktonic state. It is important to note this schematic is based on the results of in vitro experiments and has not been confirmed in vivo.

8.1. Effect of *agr* Mutations on Biofilms

The interconnection of quorum-sensing and biofilm formation is a topic that has drawn considerable interest.[189−191] It only seems natural that a cell-density dependent mechanism would play an important role in controlling the development of a multicellular community, a topic that has been coined "sociomicrobiology".[192] Seminal early studies on *Pseudomonas aeruginosa* homoserine lactone signaling demonstrated an important link between the communication mechanism and biofilm maturation.[193] This finding and numerous follow-up studies led to the widely held belief that bacterial biofilms require quorum-sensing for maturation. Thus, it was somewhat surprising in preliminary studies by Vuong et al. that the *agr* system in *S. aureus* was not required to form a biofilm.[194] In fact, most clinical *S. aureus* isolates that could form a biofilm were *agr* defective, suggesting a functional *agr* system impeded biofilm maturation (Figure 15). This important discovery has been confirmed by other laboratories[138,195] and has been used as a way to assess *agr* function.[196] This finding has even fueled debate that *agr* is a poor target for therapeutic intervention due to the potential for promoting chronic infection.[197] However, *agr* mutation does not enhance biofilm formation under all conditions, especially in the presence of a biotic surface,[198−200] suggesting this is a topic that needs further investigation. Adding to the complexity of the interpretation is the tremendous variation in *agr* dynamics across *S. aureus* strains (see section 9.4).

8.2. Biofilm Dispersal with *agr* Activation

Yarwood et al. introduced an alternative model to explain the surprising role of *agr* in biofilms that sets *S. aureus* apart from other bacterial systems.[201] In the model, *agr* activation occurs in pockets of cells at the biofilm surface, and the cells detach from the community and disperse into the surrounding environment before the cycle repeats (Figure 15). Time-lapse flow cell studies using *agr*-dependent GFP reporters, coupled with flow sorting, were used to establish the concept.[201] This intriguing model was confirmed through addition of biosynthesized AIP to intact biofilms, resulting in *agr* activation across the entire biofilm and complete detachment.[138,202] The *agr*-mediated dispersal mechanism was conserved across multiple *S. aureus* strains and functions on diverse substratum chemistries.[138,203]

During normal biofilm development, bacterial cells undergo mechanical and active detachment processes in which they leave the biofilm and return to the planktonic state.[181] The discovery of *agr*-mediated dispersal demonstrates that *S. aureus* possesses a molecular mechanism to actively control biofilm detachment and seed new sites.[138,201] How is this complex multicellular structure disassembled to return the cells to a planktonic state? The logical conclusion is that the *agr* activation must induce regulatory changes that allow cells to detach from the extracellular matrix. The problem with this scenario is that the presence and relative contribution of matrix components to the *S. aureus* biofilm are controversial.

Early studies on *S. aureus* biofilms pointed to a key role in polysaccharide intercellular adhesin (PIA) as a matrix component.[204] However, the role of PIA in *S. aureus* biofilms is largely strain dependent,[138,195,205] with many recent clinical isolates developing PIA-independent biofilms.[203,206,207] In contrast, low-specificity proteases, such as proteinase K and trypsin, can disperse the biofilms of these PIA-independent *S. aureus* clinical isolates, leading to a recent surge in studies on protein-dependent biofilm formation.[158,198,208−211] Linking these observations back to *agr*-mediated biofilm dispersal, a well-known output of *agr* activation is the production of extracellular proteases. In *S. aureus*, at least 10 extracellular proteases are under *agr* control (see Table 1). Boles et al. demonstrated that the addition of a serine protease inhibitor abrogated AIP-mediated biofilm dispersal, and in parallel, genetic inactivation of the metalloprotease aureolysin (*aur*) and a serine protease cluster (*splABCDEF*) markedly reduced the dispersal mechanism.[138] While the possibility that extracellular proteases could be mediating dispersal is intriguing (Figure 15), the targets of the proteolytic action remain to be determined. As one potential target, studies have demonstrated that the surface-localized fibronectin-binding proteins (FnBPs) are important for biofilm formation,[208] and the *S. aureus* SspA (V8) protease is known to cleave these structures.[212]

Another class of *agr*-regulated proteins thought to be important in biofilm dispersal is the δ-toxin and PSM peptides. Given their amphipathic nature, PSMs possess surfactant-like properties, and surfactants play an important role in biofilm remodeling and dispersal in other bacterial species.[213−215] This same function has been proposed for *S. aureus*[194,216] (Figure 15). This proposal is based on the observation that purified δ-toxin has a negative impact on *S. aureus* biofilm formation in microtiter plates.[194] However, the addition of δ-toxin at the measured wild-type concentra-

tion of 1 μg/mL only had a minor effect on biofilm formation. Whether other *S. aureus* PSM peptides will display improved activity against biofilms, and whether gene knockouts of these peptides will affect biofilm maturation, remains to be determined. In summary, *agr*-mediated biofilm dispersal is a fascinating but complicated mechanism with many unanswered questions. Considering the dispersal of biofilms restores antibiotic susceptibility,[138,203] an improved understanding of the mechanism warrants further investigation for the development biofilm infection therapies.

9. Role of agr in S. aureus Pathogenesis

S. aureus is one of the most common causes of nosocomial and community-associated bacterial infections. This pathogen can cause a diverse spectrum of infections that can be acute, chronic, or toxin-mediated and affect most areas of the human body.[25,217] The rising prevalence of methicillin-resistant *S. aureus* (MRSA), and emergence of these strains in the community,[23] is amplifying the healthcare problem. Much of the acute disease manifestations have been attributed to the arsenal of secreted virulence factors induced by the *agr* system. Therefore, it is not surprising that the *agr* system plays an important role when examining *S. aureus* pathogenesis. In this section, we will define CA-MRSA and summarize the contribution of *agr* in ex vivo studies with phagocytes and in vivo animal model studies. We will also outline the role of *agr* in colonization and the challenges of strain variation.

9.1. Community-Associated MRSA

The epidemic waves of antimicrobial resistance in *S. aureus* are well documented and particularly concerning (reviewed in ref 23). In the late 1990s, the MRSA strains expanded from healthcare settings and began infecting otherwise healthy individuals in the community. These strains were coined "community-associated" MRSA (CA-MRSA) for their new properties and have become the most recent epidemic wave of resistance in *S. aureus*.[23,24] While the global spread has been extraordinary, perhaps more concerning is that these strains are hypervirulent. Of the CA-MRSA stains, the USA300 pulse field gel type has emerged as the most common.[218,219] While the exact reason for this expansion and hypervirulence of the isolates is unknown, it is apparent that USA300 strains have an *agr* type I system that functions at a very high basal rate. Evidence for this phenotype is clear when comparing RNAIII transcript levels to other strains.[220,221] The mechanism behind the enhanced *agr* dynamic range is not known, but it might explain the high level of toxins and extracellular enzymes produced by this strain.

9.2. Neutrophil and Macrophage Interactions

Polymorphonucleur leukocytes (PMNs or neutrophils) are professional phagocytes that are the first wave of defense at the site of infection.[222] The ability of *S. aureus* to evade phagocytosis or survive after engulfment is a critical determinant of virulence. Over time, the bacteria are capable of replicating within the neutrophils and inducing cell death, allowing the bacteria to escape and spread in the host. *S. aureus* employs an arsenal of virulence factors and strategies to avoid destruction by neutrophils (see refs 24, 222 for more information). The role of *agr* in these immune evasion mechanisms has been the focus of multiple studies. Shortly after phagocytosis, RNAIII expression is upregulated by *S. aureus* within the cells, even when there is only a single bacterium within a phagosome, suggesting that the *agr* activation can function in a confined intracellular space.[51] This activation occurs despite the presence of neutrophil-produced ROS capable of inactivating AIP-I through oxidation of the methionine in the macrocycle.[64] An *agr* mutant shows a reduced ability to lyse neutrophils, and this effect has been linked to the *agr* induced α-toxin and a group of low-molecular-weight toxins called PSMs. Individual knockouts of the *hla* gene encoding α-toxin[51] or the PSMα and PSMβ genes[223] results in reduced neutrophil lysis. The effect of eliminating the PSM toxins shows a more pronounced lysis defect compared to the α-toxin studies.[223] However, comparison of clinical isolates indicates there is considerable variation in neutrophil lysis phenotypes,[224] making study-to-study comparisons challenging.

Macrophages are another professional phagocyte that are capable of controlling *S. aureus* infections. Macrophages do exhibit longer lifespans than neutrophils, potentially serving as reservoirs for *S. aureus* dissemination within the host. Kubica et al. recently demonstrated intracellular survival of *S. aureus* for 5 days, following which the bacteria replicated and induced host cell lysis.[225] Isogenic strain studies demonstrated this mechanism was *agr*- dependent. In subsequent analysis of potential *agr* regulated factors, α-toxin and aureolysin mutants showed survival defects.[225] These studies highlight the important role of *agr* in *S. aureus* interactions with phagocytes and provide additional clues to how this system contributes to these initial responders at sites of infection.

9.3. Animal Models of Infection

Many animal model studies have compared *agr* mutants to isogenic parental strains to monitor changes in disease progression. Others have focused on the therapeutic value of inhibiting an otherwise functional *agr*-positive strain. Across numerous animal infection models, more often than not *agr* dysfunction is associated with a decrease in virulence. Some examples of these studies are presented.

9.3.1. Skin and Soft Tissue Infections

Skin and soft tissue infections are the most common type caused by *S. aureus*.[226,227] These range from minor inflammatory conditions to more invasive infection, and most of these cases are associated with the formation of abscesses, the hallmark of a *S. aureus* infection. The importance of the *agr* system in abscess formation has been demonstrated using mutants and small-molecule inhibitors of the system.[48,56,196,228,229] In time course studies using a murine skin infection model, activation of *agr* within an abscess can be observed with an *agr* P3-*lux* reporter within three hours of infection, followed by a decrease in reporter activity and eventual reporter reactivation 48 h later.[48] Abscess lesions of *agr* mutants are consistently smaller than wild-type and have reduced bacterial load.[229] The bulk of the phenotype is due to *agr*-dependent secreted virulence factors as demonstrated with studies on sterile supernatants from wild-type and *agr* mutant strains.[48] The role of *agr* in abscess formation has also been investigated using various inhibitors of the regulatory system. Interference of AIP signaling through the use of competing AIPs or AIP-sequestering antibodies decreased abscess formation.[48,56,196] These findings provide direct support for

the notion that *agr*-targeted therapies could be an option for the development of skin infection treatments.

9.3.2. Pneumonia

S. aureus is a common cause of community and healthcare pneumonia.[230] The role of *agr* in pneumonia has been investigated using infection models in which high bacterial loads are inoculated intranasally and followed for up to 72 h. Strains defective in *agr* displayed attenuated virulence in neonatal mice, showing both a decrease in the establishment of pneumonia and mortality.[231] These findings were corroborated using an adult mouse pneumonia model.[232,233] An α-toxin knockout was also unable to cause significant mortality, suggesting a critical role for this toxin during pneumonia progression.[232] Other *agr*-regulated factors, such as protein A and PVL, have also been implicated in pneumonia pathogenesis, and the altered levels of these proteins may contribute to the *agr* mutant phenotype.[234]

In addition to acute pneumonia infections, *S. aureus* is problematic for causing chronic lung infections in individuals with cystic fibrosis. Cystic fibrosis is a genetic disease in which a defective ion channel predisposes individuals to chronic lung infections by pathogens including *Haemophilus influenzae*, *P. aeruginosa*, and *S. aureus*.[235] In contrast to its role in pneumonia, the *agr* system is not essential for maintaining these chronic infections. Goerke et al. measured transcripts from cystic fibrosis sputum samples and found RNAIII to be expressed at low levels.[236] Interestingly, they also observed low expression of *spa*, a gene downregulated by *agr* activation, while α-toxin expression was variable depending on the sample. Even though RNAIII levels are low in this context, the downstream pattern of gene expression is somewhat similar to *agr*-activated cells. Some of these conflicting phenotypes could be due to small colony variants (SCVs) that often arise during chronic infections. SCVs accumulate various mutations that slow growth by limiting different metabolic pathways such as oxidative phosphorylation or thymidine synthesis.[237] SCVs show a gene expression pattern consisting of a downregulated *agr* system and decreased toxin and pigment production.[238] These adaptations make SCVs more resistant to killing by antibiotics and nonprofessional phagocytes, making it difficult to clear infections in these individuals. Taken together, the *agr* system is important for acute lung infections but is dispensable in the context of chronic persistence within the cystic fibrosis lung.

9.3.3. Infective Endocarditis and Osteomyelitis

S. aureus is the leading cause of infective endocarditis.[239] This disease is characterized by *S. aureus* growth on the cardiac valves or endothelium of the chambers, leading to the formation of vegetations consisting of bacteria, platelets, fibrin, and inflammatory cells. It is often associated with bacteremia, and treatment requires prolonged antibiotic therapy lasting weeks or in other cases surgery to replace the damaged valves. A rabbit model is often used to replicate infective endocarditis disease progression. When an *agr* mutant strain was compared to its wild-type parent in this model, there was a decrease in the frequency in which the *agr* mutant established endocarditis.[240] Vegetations formed by *agr* mutants contained fewer bacteria compared to the wild-type and led to reduced dissemination to the kidneys, a common secondary infection site.[241] All of these effects were dose-dependent, as higher inoculums diminished the difference between strains.[240,241] Deletion of *rot*, a downstream effector in the cascade, restored the virulence of the *agr* mutant in this model.[241] These findings indicate an important role for *agr*-regulated genes in establishing endocarditis.

Osteomyelitis is an infection of the bone caused by hematogenous seeding or direct infection from nearby tissue. *S. aureus* is the most common cause of osteomyelitis and often leads to chronic infections that require extended prophylaxis and sometimes surgery for treatment.[242] Virulence factors have been identified as being important for causing osteomyelitis such as the fibronectin binding proteins and collagen adhesin.[243] There is also evidence that *S. aureus* can persist in the osteoblasts responsible for generating the bone matrix, providing a protective niche from immune cells and antibiotics.[244] Similar to endocarditis, *agr* mutants displayed a decreased ability to establish osteomyelitis.[245,246] When infection did occur, less tissue damage was observed with the *agr* mutant. Future studies will be necessary to determine the relative contributions of *agr*-regulated proteins to the pathogenesis mechanisms of infective endocarditis and osteomyelitis.

9.4. Association with Diseases and Colonization

From an epidemiology perspective, one of the more intriguing observations is that the different *agr* classes show a correlation with disease. In the early stages, the correlations were rough assignments based on limited strain groups. The *agr*-I class was considered common and linked to many disease types, while *agr*-III and -IV isolates were rare and linked to toxic shock syndrome and exfoliative toxin related syndromes, respectively.[247] With the changing epidemiology, more comprehensive strain collections and the advent of rapid typing techniques have provided further insight on the correlations between *agr*, nasal carriage, and disease. In a small sampling of recent studies that used multilocus sequencing and other typing methods, the most common nasal carriage isolates fell into the categories called clonal complex 30 (CC30) or USA200 pulse field gel type (PFGE).[248,249] Interestingly, both the CC30 and USA200 strains are *agr*-III and possess the *tst* gene encoding toxic shock toxin. Looking at one collection of invasive MSSA bloodstream isolates, the most common group identified was CC8, which have the *agr*-I allele and is the same grouping as USA300.[249,250] In a large U.S. population-based study of invasive MRSA isolates, the USA100 (*agr*-II) group predominated at 53.6%, while USA300 (*agr*-I) was second at 31.6%.[251] It is important to note that this is only a sampling of studies, but some obvious trends are apparent, some that fit with older assignments and others that show divergence. Consistently, *agr*-I isolates are some of the most frequently linked to invasive disease, which parallels older observations.[247] Also consistent, the frequency of isolating an *agr*-IV strain continues to be a rare event.[249] What is emerging from recent samplings is that *agr*-II and -III classes are underappreciated. Previously, there was common belief that *agr*-III was relatively limited to toxic shock syndrome cases, but this group may be quite abundant in nasal carriage. Similarly, *agr*-II is often ignored as being rare, common in bovine mastitis and other animal infections,[252,253] but the incredible abundance of USA100 in invasive infections suggests *agr*-II function should be evaluated in these isolates.

9.5. *agr* Variation Across Strains

One *agr* phenotype that is often not appreciated is the variation in system dynamics across strains. As prominent examples, it is known that recent CC30 isolates, such as model strains UAMS-1 and MRSA252, have weak *agr* systems for reasons that are not clear.[254] This phenotype is not a general feature of *agr*-III alleles as USA400 strains possess a robust *agr* system.[220] Similarly, many historical *agr*-I isolates like COL have a muted output, while more recent, related isolates have systems that operate at a high level.[221] This strain-to-strain variation creates significant challenges for investigators in interpreting the *agr* contribution to experimental results. If a strain group is selected with a weak or defective *agr* system, little is learned through mutant analysis. Similarly, the use of strains with known chromosomal defects that distort RNAIII levels, such as NCTC8325 or RN6390, further complicates *agr* interpretations. Unfortunately in some early *agr* mutant studies, the strain classification and *agr* allele are unknown, making it hard to compare the effect of a knockout to more recent studies. As indicated above, the *agr* system is also hypersusceptible to mutation in many genetic backgrounds,[255] which has clouded the literature with misassignments of phenotypes. The recurring theme is that studies need to be performed in strains, preferably multiple strains in parallel, where there is adequate information to compare to the field in order to make accurate conclusions.

10. Mechanisms of agr Inhibition

The requirement for peptide signaling mechanisms in pathogenesis has raised considerable interest in quorum-quenching approaches. Similar to the Gram-negative acyl-homoserine lactones, nature has already evolved strategies to inhibit the *agr* system, and some of those mechanisms are outlined below. Antagonist discovery for the AgrC receptors has been and continues to be a fertile area for targeted approaches due to their extracellular exposure and lack of outer membrane barriers, diversifying the functional chemistry that can be employed in inhibitor design.[256] Other approaches, such as antibody sequestration of the signal and inhibition of the biosynthetic machinery,[196,257] are preliminary but gaining interest. The underlying complicating factor in *agr*-targeted therapy is the ongoing acute versus chronic dilemma. The general dogma is that *agr* inhibition tends to make *S. aureus* more adherent and "biofilm-like" and thus more recalcitrant to host defenses and antimicrobial therapy. While this concern has been raised,[197] it clearly is in need of further evaluation. Most of the in vitro enhancement of *agr* mutant biofilms appears limited to abiotic surfaces, and the difference is muted using a biotic surface.[198−200] Perhaps more importantly, *agr* mutants show defects in chronic biofilm infections in vivo, such as in osteomyelitis and infective endocarditis,[241,245] suggesting the biofilm enhancement phenotype could be restricted to abiotic surfaces or that the function of *agr*-dependent virulence factors is dominant in vivo. Nevertheless, the evidence that *agr* inhibition can attenuate the most severe invasive disease, and the hyperactive nature of *agr* in the outbreak MRSA strains, supports the continued efforts to identify effective *agr*-targeted therapies.

10.1. Host Factors That Inhibit *agr*

While the prospect of discovering quorum-quenching drugs is gaining popularity, not surprisingly, nature is a step ahead and mechanisms of inhibition already exist in the host. Low pH was one of the earliest identified environmental cues that inhibits *agr*.[135,137] Many common sites of *S. aureus* colonization, such as the skin and vaginal tract, are in the pH range of 4.2−6, and the *agr* system does not function at pH levels of 5.5 and below (see section 7.1). While blood is maintained at neutral pH, multiple host factors in blood can inhibit *agr* activation. Initially, Yarwood et al. identified that increasing concentrations of serum could quench the *agr* system in a clinical TSS isolate.[258] In a related study, Peterson et al. observed that lipoprotein-deficient serum failed to inhibit *agr* using *S. aureus* reporter strains. Through follow-up fractionation of lipoprotein components, apolipoprotein B (ApoB) was identified as the primary factor mediating the inhibition.[259,260] ApoB is an integral component of the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) particle, which circulate in high concentrations in blood. ApoB has two α-helical domains that anchor the protein in the lipoprotein micelle and a globular amino terminus that protrudes from the lipoprotein complex and can interact with soluble blood components. Considering ApoB could bind native AIP-I, but not linear, and this binding was inhibited by AIP-II and AIP-IV, Peterson et al. hypothesized that ApoB can sequester AIPs of all *S. aureus* classes.[259] Importantly, mice deficient in ApoB were more susceptible to wild-type *S. aureus* infection than an *agr* mutant, suggesting ApoB may have a host defense function against invasive bacterial infection.

Another abundant blood component, hemoglobin, is known to inhibit the *agr* system. Schlievert et al. found that human blood inhibited TSST-1 production, while simultaneously enhancing protein A levels, suggestive of an *agr* interaction.[261] In fractionation experiments, both the α and β chains of hemoglobin inhibited δ-toxin production, indicating repression of RNAIII. Whether the repression effect is due to direct or indirect action of the globin fragments is not clear, but Schlievert et al. speculate that the positive charge of the globin chains could be interacting with the negatively charged *S. aureus* phospholipids or surface-exposed residues on the AgrC membrane histidine kinase.

10.2. AgrC Receptor Antagonists

Structure−activity relationship studies provided critical details for the design of AgrC receptor antagonists (using numbering in Figure 16). Initially, the AIP-II scaffold drew considerable interest as an antagonist starting point, in part due to the interference activity of this AIP against the other three *agr* systems and the success of attenuating a murine skin abscess resulting from *agr*-I strain infection.[33,56] On the basis of the initial AIP−AgrC receptor interaction results,[56] an *N*-terminal truncation of AIP-II (**1**) was reasoned to have inhibitory properties.[61] The success was striking with universal inhibitory properties against all four *agr* types in the low nanomolar range. Site-directed mutagenesis also provided an important lead as alanine-scanning on AIP-I uncovered the D5A variant (**2**) as a potent inhibitor of *agr*-I (IC_{50} = 33 nM),[50] later shown to be a universal inhibitor.[49] These early clues lead to additional antagonist design approaches using all four AIP scaffolds.[49] Truncations of AIP-I (**3**) and AIP-IV (**4**) displayed impressive inhibitory

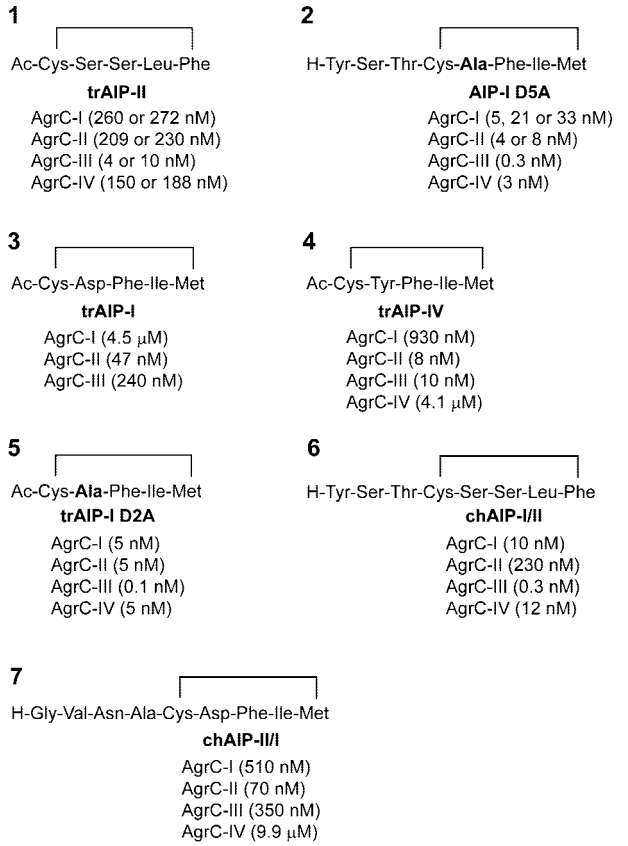

Figure 16. Global antagonists of *S. aureus* AgrC. Seven antagonists are shown based on SAR studies of the AIPs. IC_{50} values from *S. aureus* reporter strains for each AgrC type are shown underneath the structure.

properties, although trAIP-I still served as a weak *agr*-I activator. Combining the truncation and an aspartate to alanine mutation (**5**) resulted in an especially potent inhibitor with IC_{50}s of 0.1−5 nM for all four *agr* systems. The generation of chimeric AIPs (**6**,**7**) also created universal inhibitors, although not of the same potency of the truncation derivatives or AIP-I D5A.

10.3. Other Strategies of *agr* Inhibition

While targeting AgrC has been fruitful, the eventual challenge of this approach is the narrow application of a receptor antagonist toward one species of bacterial pathogen. Resistance also could be a complicating factor considering the hypermutability of the *agr* system and the likelihood that AgrC point mutations could reverse function of an inhibitor to an activator, as was observed in studies with AIP-I D5A and mutants of AgrC-IV.[100]

As a counter to this problem, researchers have stepped back further in the quorum-sensing mechanism and focused on the peptide signals themselves. The appeal of this strategy is that it resembles the host defense mechanisms where inactivating or sequestering the signal is the focal point. One promising therapeutic strategy was the preparation of monoclonal antibodies to the *S. aureus* AIP-IV signal.[196] The challenge of this approach is that AIPs are poor antigens due to their small size and labile nature. This challenge was averted by synthesizing a stable hapten of the AIP-IV, coupling to carrier proteins, and immunizing mice with the conjugate. Following screening, one of the Abs possessed high specificity for AIP-IV (K_D = 90 nM). In vitro quorum-quenching using prototype *agr*-IV strains, such as RN4850 and NRS168, was effective, with RNAIII production markedly reduced, α-toxin production eliminated by immunoblot and PARP cleavage assays, and protein A production increased, all signs of *agr* inhibition. Most importantly, murine skin abscess formation was inhibited and passive immunization against intraperitoneal infection was successful.[196]

Besides signal sequestration, there are several examples of small-molecule inhibition of cyclic peptide signal biosynthesis. For *agr*-I strains, linear peptide inhibitors of type I signal peptidase were identified and found to reduce AIP-I production and quench *agr* function.[72] Signal peptidase is an essential enzyme and also a promising antimicrobial target due to the extracellular location of the active site. One of the most notable examples of biosynthesis inhibition is the discovery of ambuic acid in the screening of natural product libraries for *E. faecalis fsr* signaling inhibitors through reduction in the secretion of gelatinase biosynthesis-activating pheromone (GBAP).[257] At 10 μM concentration, ambuic acid resulted in a 50% inhibition of gelatinase production, an output of the *fsr* system. Ambuic acid is a polyketide-derived epoxyquinone natural product made by *Pestalotiopsis* spp. and *Monochaetia* sp. fungi. The molecule contains a cyclohexenone moiety that has similarities to those in the antibiotic tetracycline and was found to have antifungal properties.[262] The ambuic acid mechanism of action was investigated, and the compound inhibits processing of the FsrB enzyme, an AgrB homologue, at levels of 100 μM or greater. The inhibition was evaluated using a tagged FsrD substrate and monitoring processing of the peptide precursor.[257] Perhaps most interestingly, Nakayama et al. evaluated the bioactivity of the compound against other Gram-positives with AgrB-like enzymes. Using signal production assays, the authors concluded that 100 μM ambuic acid could inhibit AIP biosynthesis in a *S. aureus agr*-I strain and *Listeria innocua*,[257] suggesting potential for general applicability toward quorum-quenching in Gram-positives.

11. *Staphylococcus epidermidis*

Staphylococcus epidermidis is an abundant human commensal found on the skin whose role as a human pathogen, until recently, has largely been underestimated. Though less of a problem in healthy individuals, *S. epidermidis* can cause severe disease in immunocompromised patients and has been identified as a common cause of nosocomial infections. These infections are typically associated with indwelling medical devices, including catheters and prosthetic implants, resulting in difficult to treat chronic infections.[263−265] The device infections are recalcitrant to host defenses and antimicrobial therapy due to the ability of *S. epidermidis* to form biofilms.[188] Currently, *S. epidermidis* and *S. aureus* rank as the most common causes of these types of infections on indwelling devices. The increasing use of indwelling devices, and the ubiquitous presence of *S. epidermidis* on the skin, has created a situation where *S. epidermidis* could be considered an "accidental" pathogen that is a consequence of our own medical progress.[188] Like other Staphylococci, *S. epidermidis* contains a functional *agr* quorum-sensing system[266] and many roles for this system have been reported. In this section, basics of the system, AIP structure and interference properties, and role in biofilms will be covered. The *agr* system has been linked to many other *S. epidermidis* phenotypes, such as production of the lantibiotic epider-

min,[267] physiological adaptation,[268,269] and exoenzyme production[269] that are beyond the scope of this review.

11.1. *S. epidermidis agr* System

The *S. epidermidis agr* system has many parallels to its *S. aureus* cousin with an *agrBDCA* operon encoding the core components, AIP signal, and RNAIII regulatory RNA that encodes δ-toxin.[266] Proteome analysis and expression profiling have shown that autoinduction of the *S. epidermidis agr* system occurs during late-exponential phase and results in the downregulation of cell-surface associated proteins and upregulation of secreted toxins and exoproteins.[268−270] There is speculation that deactivation of *agr* alters growth rate and metabolic profile,[268] but not every strain has a growth defect,[270] suggesting this issue is strain dependent and in need of clarification.

11.2. AIP Structure

On the basis of the similarities to the *S. aureus* paradigm, it was assumed that the *S. epidermidis* AIP structure is composed of a five-residue thiolactone ring with an *N*-terminal extension. A series of SAR studies were performed on the AIP to identify the preferred length of the *N*-terminal tail and other allowed modifications to the scaffold.[10,271] These studies were performed on strain Tu3298 (DSM 3095), which has an *agr* system that parallels the one found in the commonly used strain 1457 (AIP type I, see Figure 3). Although other classes (type II and III, see Figure 5) of *S. epidermidis agr* have been identified,[52] they are rarely the cause of human infections.[272]

To determine the preferred size of the *S. epidermidis* AIP (type I), synthetic AIPs were generated with *N*-terminal extensions of two, three, or four residues from the macrocycle and were tested using a biological reporter assay. On the basis of these experiments, an octapeptide "DSVCASYF" with a thioester linkage of the cysteine to the terminal carboxylate was the preferred inducer.[10] As anticipated, a linear version of the AIPs that lacked any chemical modification had no biological activity. Interestingly, replacement of the AIP thiolactone with a lactam or lactone also conferred no activity.[271] This result contrasts with *S. aureus*, where the AIP lactam derivatives are active at high concentrations.[50,60] The structure of the alternative *S. epidermidis* AIPs (class II and III) are predicted to be quite divergent in sequence[52] but have not been verified.

11.3. *agr* Interference

Using synthetic *S. epidermidis* AIP, it was observed that this signal could inhibit the *agr* system in *S. aureus* type I strains. At 100 nm AIP, *S. aureus* δ-toxin production was eliminated, while at 1 μM AIP, α-toxin and other exoprotein levels were reduced as protein A levels increased on the surface.[271] In studies with other *S. aureus agr* types, synthetic *S. epidermidis* AIP inhibited the type II and III systems, but surprisingly not the type IV system. In the converse experiment, only the *S. aureus* AIP-IV inhibited *S. epidermidis agr*, but the amount of inhibition was limited.[54] Considering that *agr* type IV system is most often found in *S. aureus* strains that cause skin infections, such as scalded skin syndrome,[53] it has been suggested that *S. epidermidis* and the type IV strains occupy a similar environmental niche and have evolved competition mechanisms.[54] In future evaluations of this niche competition proposal, it would be worthwhile to test the cross-species effects of *S. epidermidis* class II and III AIPs on *S. aureus agr* type IV strains.

11.4. *agr* Regulation of Biofilms

Analysis of the *S. epidermidis* genome indicates there are fewer toxins, degradative enzymes, and surface proteins compared to *S. aureus*.[273] This finding, along with the observations that *S. epidermidis* infections are less acute than *S. aureus*, and often associated with indwelling medical devices, has led to the conclusion that biofilm formation is the primary virulence mechanism.[187,188] *S. epidermidis* biofilms are a complex association of cells that exist within an extracellular matrix composed of polysaccharides, proteins, and DNA. Initial attachment to either host matrix proteins or an abiotic surface, such as a catheter or prosthetic implant, is mediated by a diverse range of surface adhesins and the autolysin Atl.[188,205] While both *S. aureus* and *S. epidermidis* express versions of Atl, some notable differences exist. *S. epidermidis* studies have shown that *atlE* transcription is growth-phase dependent and absent in an *agr* mutant.[274] This result contrasts with *S. aureus*, where *atl* expression is not under the control of the *agr* system.[194] Therefore, AtlE in *S. epidermidis* appears to play a role in initial attachment of this bacterium to synthetic polymers and is regulated by *agr*.

Multiple lines of evidence indicate that *S. epidermidis* biofilm detachment is controlled by *agr*. Similar to *S. aureus*, an *S. epidermidis agr* mutant forms thicker biofilms and the mutant has a detachment defect.[274,275] The *agr* activity also is limited to expression at the surface of the biofilm. It has been suggested that the detergent activity of PSMs mediate the detachment mechanism, while both PSMs and extracellular proteases have been linked to detachment in *S. aureus*.[138,194] Again, similar to *S. aureus*,[223] PSM production is strictly under *agr* control in *S. epidermidis*.[276] Taken together, *agr*-mediated biofilm detachment is a conserved mechanism across Staphylococci, but the specific *agr* regulated factors controlling the mechanism may be species dependent.

12. Other Staphylococci

The presence of loci similar to *agr* have been identified by PCR, Southern blot, and northern blot analysis in many other coagulase-negative Staphylococci,[52,120,277] but only a limited number of studies have been performed to validate the existence and function of these loci. The first species examined in some detail was *S. lugdunensis*, an emerging pathogen that was first described in 1988.[278] Unlike other coagulase-negatives, *S. lugdunensis* is known for causing severe infections, such as skin and soft tissue infections, brain abscesses, acute endocarditis, and bacteremia.[279,280] In the pregenomics era, *S. lugdunensis* was one of the first non-*aureus* Staphylococci to be investigated for the *agr* system. The *agr* locus was identified through Southern blot and sequencing, and it was found to contain an *agrBDCA* operon and RNAIII, although no δ-toxin was encoded in the RNAIII transcript,[281] despite the fact that *S. lugdunensis* produces a δ-toxin like activity.[282] Later studies demonstrated that the toxin activity was mediated by a three-gene cluster called SLUSH, an acronym for *S. lugdunensis* synergistic hemolysin.[283] Similar to the PSMs,[223,276] the expression of SLUSH is controlled by the *agr* system.[277] The RNAIII transcript was investigated and demonstrated to be functional through

heterologous complementation of a *S. aureus* RNAIII mutant.[121] In later studies, the *S. lugdunensis agrBD* genes were cloned and shown to make a functional AIP signal.[33] The AIP structure is proposed as a heptapeptide composed of the residues DICNAYF with the last five residues constrained as a thiolactone. Similar to the other Staphylococci, the linear form of the peptide does not activate *agr* in *S. lugdunensis*.[33] Despite the recent interest in this emerging pathogen, an *agr* mutant has not been constructed to assess phenotypes and the *agr* regulon remains unknown.

The only other staphylococcal *agr* system that has drawn significant attention is that of the *S. intermedius* group. The interest in the *S. intermedius* group stems from the fact that the AIP structure is a nonapeptide (RIPTSTGFF) with a lactone ring instead of the usual thiolactone in other staphylococcal AIPs.[11,52,63] Since this discovery, a total of four *agr* classes have been identified in the *S. intermedius* group, all containing the identical lactone macrocycle (STGFF) with variant *N*-terminal extensions.[284,285] This group includes *S. intermedius* as well as the more recently defined species *S. pseudintermedius* and *S. delphini*.[284] Thus, it is not surprising that isolates labeled as *S. intermedius* are sometimes mislabeled and belong to these other species. *S. pseudintermedius* is a member of the normal flora of dogs and a common cause of canine pyoderma.[284] Whether the lactone-containing AIP evolved in this group of Staphylococci due to the particular animal host environment or the commensal flora is not clear. The replacement of the AIP thioester bond with an ester reduces the reactivity of the carbonyl and should enhance the stability of the *S. intermedius* AIP signal.

In mechanism studies on *S. intermedius agr*, the AIP was shown to autoactivate RNAIII production in vitro, and the RNAIII transcript was demonstrated to encode a δ-toxin,[63] unlike the RNAIII of *S. lugdunensis*. Similar to *S. epidermidis*, the *S. intermedius* AIP inhibits all the *S. aureus agr* systems, with *S. aureus agr* type IV showing the most resistance.[63] For AIP biosynthesis, the *S. intermedius* AgrB will process its cognate AgrD, and interestingly also the serine-to-cysteine substitution, demonstrating this enzyme can produce both lactone and thiolactone-containing signals. In contrast, *S. aureus* AgrB cannot process an AgrD sequence with a serine substitution.[63] What features of *S. intermedius* AgrB have evolved to accommodate the less reactive serine nucleophile and ester formation are not clear. The AgrB residues or domains involved in AIP ring formation are unknown, but these *S. intermedius* changes must have evolved in conjunction with the lactone AIP to facilitate catalysis using a serine nucleophile.

13. Conclusions and Future Perspectives

The *agr* peptide quorum-sensing system is a fascinating mechanism of cell-to-cell communication that has drawn tremendous interest from staphylococcal researchers. The dramatic effects of *agr* on exoprotein expression, pathogenesis, and biofilm formation, coupled with the surprising variation that has evolved into the system, has made *agr* the focal point of hundreds of research papers. Perhaps more importantly, studies on other Gram-positives and genome mining has revealed that AIP-mediated communication is a conserved signaling mechanism.[9] In our current appreciation of evolutionary dynamics, all species of the genera *Staphylococcus*, *Enterococcus*, *Listeria*, and *Clostridium* appear to possess the machinery to make a cyclic peptide signal. While exceptions to this rule undoubtedly will be discovered, the conservation of *agr*-like systems across this group that includes some of the most clinically important bacterial pathogens highlights the emerging significance of cyclic peptide signaling. A variety of other Gram-positives also possess *agr* machinery, but notable exceptions include those of the genera *Streptococcus*, *Bacillus*, and *Mycoplasma*.[9]

Novick and Geisinger elegantly put forth several possibilities to explain the function of quorum-sensing in Staphylococci.[59] The suggested roles include (1) a bacterial response mechanism to host-specific signals, (2) autoactivation circuitry that is exponentially coupled to a regulatory output enabling exquisite control in variant growth conditions, and (3) a self-versus-nonself recognition mechanism for autocrine regulation in mixed populations. We agree with Novick and Geisinger's concerns regarding the default view that the *agr* system should conform with the textbook presentation of quorum-sensing as a cell-density dependent regulatory mechanism.[59] The recent work of Pang et al. demonstrates that *S. aureus* can activate *agr* in a human neutrophil phagosome[51] while extracellular *S. aureus* could not, suggesting that autoactivation can transcend the classical paradigm observed during standard in vitro culture. The confined space of the phagosome (1.2 femtoliters), each with ≥ 1 cocci, must facilitate the enhanced local concentration of AIP, allowing the signal to surpass the AgrC receptor threshold. The exponential coupling of this ligand−receptor interaction with the cooperative response of AgrC dimers enables *S. aureus* to differentially regulate accessory genes in this intracellular milieu.[51] This response is not limited to the neutrophil, as similar *agr* effects are seen with *S. aureus* confinement in synthetic lipid spheres.[286]

Going forward, there are still many *agr*-related questions remaining to be addressed. In terms of the basic signaling mechanism, numerous holes remain in our understanding of AIP biosynthesis in Staphylococci and this extends further into all Gram-positive *agr* systems. A focal point of open questions is the catalytic function of the AgrB enzyme. This integral membrane endopeptidase is one the most conserved and unique features of *agr*-like systems but has only been the target of limited study. Multiple studies have confirmed that AgrB catalyzes AgrD cleavage, and AgrB has been proposed to carry out thiolactone ring formation and AIP transport. However, almost no information is available on both the mechanism of ring formation and signal transport steps. Numerous other features of the enzyme remain poorly understood, such as the oligomeric state and the surprising concentration of charged resides in proposed transmembrane regions.[69] A three-dimensional structure of AgrB would answer many of these questions, but this endeavor could be challenging in light of difficulties in purifying integral membrane proteins.

Beyond the basic mechanism, the variation in *agr* dynamics that occurs across staphylococcal strains is another topic in need of investigation. The best example of this variation is the hyperactivity of the *agr* system in the outbreak USA300 CA-MRSA.[220,221] The mechanism behind the tremendous levels of RNAIII generated in these isolates versus other *S. aureus* remains to be determined. The difference could be at the level of AIP biosynthesis, AgrC−AgrA two-component signaling, P3 promoter activation, RNAIII stability, or possibly some other unexpected mechanism. The increased toxin and exoenzyme output due to this elevated *agr* system has been suggested as a possible reason for the

hypervirulence of USA300.[220,221,223] In the converse, the low RNAIII output in CC30 type isolates is also not understood.[254] With the high frequency of these isolates in nasal carriage,[249] the muted function of *agr* could have an underappreciated role in persistent carriage mechanisms.

With the surge in CA-MRSA infections over the past decade, the significance of the *agr* system has reemerged as a pathogenesis regulator. There is renewed interest in targeting this regulatory mechanism as a means of quenching acute infection. Currently, the bulk of quorum-quenching initiatives focus on development of AgrC antagonists, leaving room for the pioneering of innovative strategies to sequester the AIP signal, break down AIP, and inhibit biosynthesis machinery. Some initial successes indicate there is potential in these alternative quenching approaches.[196,257] For all the *agr* targeting strategies, structural studies on the AgrC receptor kinase and AgrB biosynthetic enzyme would aid our understanding of current antagonists and facilitate development of new leads. With the considerable interest in quorum-quenching approaches, it will also be important to reevaluate the possibility that *agr* inhibition will promote chronic infection.

In the current era of genomics, the uncovering of countless new cyclic peptide signaling systems across the Gram-positives highlights our current limited understanding of this emerging type of quorum-sensing. In most of these bacterial species, the *agr*-like systems have not been inactivated to assess the contribution to cellular processes or virulence of pathogenic strains. While the *S. aureus agr* system has served as the paradigm model for studies on signal production and two-component machinery, the outputs of related systems in other Gram-positives is likely to show tremendous variation that remains to be deciphered. With the increasing availability of improved genetic and molecular tools for less tractable organisms, there are opportunities for significant future discoveries in the area of peptide quorum-sensing.

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15. Supporting Information Available15

COBALT generated alignment of AgrC and representative AgrC homologues (HPK_{10}s); TOPCONS consensus topology predictions. This material is available free of charge via the Internet at http://pubs.acs.org.

16. References

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