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Regulation of virulence in *Staphylococcus aureus*: molecular mechanisms and remaining puzzles

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Summary

The *agr* locus encodes a quorum sensing (QS) circuit required for the virulence of a spectrum of gram-positive pathogens and is, therefore, regarded as an important target for the development of chemotherapeutics. In recent years, many of the biochemical events in the *Staphylococcus aureus agr* circuit have been reconstituted and subject to quantitative analysis *in vitro*. This work, in conjunction with structural studies on several key players in the signaling circuit, has furnished mechanistic insights into the regulation and evolution of the *agr* quorum sensing system. Herein, we review this progress and discuss the remaining open questions in the area. We also highlight advances in the discovery of small-molecule *agr* modulators and how the newly available biochemical and structural information might be leveraged for the design of next generation therapeutics targeting the *agr* system.

Introduction

Staphylococcus aureus (*S. aureus*) is a commensal symbiont and an opportunistic pathogen. Once *S. aureus* invades host tissues, it causes both acute and chronic illnesses such as bacteremia, sepsis, endocarditis and toxic shock syndrome (Lowy, 1998). To establish and sustain its infection, this bacterium deploys a diverse arsenal of virulence factors, depending on its growth phase. During the lag and early exponential phases, *S. aureus* produces cell wall-associated factors that facilitate tissue attachment and evasion of the host immune system, allowing the bacteria to accumulate and form a biofilm (Kong et al., 2006) (Figure 1A, left panel). Once the bacterial population reaches the late exponential phase, it begins to secrete a spectrum of exoproteins, including proteases, hemolysins and super-antigens, and at the same time down-regulates the cell wall-associated factors, leading to dispersion of the biofilm and the spread of the infection (Dinges et al., 2000) (Figure 1A, right panel). This population density-dependent behavior essentially delineates two stages of the *S. aureus* life cycle, i.e., an adhesion phase and an invasion phase. The timing and expeditious transition

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between these two phases occurs through an intercellular communication process called quorum sensing (QS), in which the bacterium produces a diffusive molecule, termed the autoinducer (AI), as an indicator of the local population density. Detection of the AI is central to the decision making process that ultimately controls gene expression programs (Waters and Bassler, 2005).

The chromosomal locus responsible for QS in *S. aureus* is named *agr* (*a*ccessory *g*ene *r*egulator). The locus encodes a signaling circuit that both produces and senses the AI, a small peptide named the AIP (for *a*uto*i*nducing *p*eptide), featuring a unique thiolactone linkage between the C-terminal carbonyl and the sulfur atom in a cysteine side-chain (Novick and Geisinger, 2008) (Figure 1B). Importantly, it has been known since the late 1990s that inhibition of *agr*-mediated signaling attenuates the spread of *S. aureus* infections in animal models, thus qualifying *agr* as a potential drug target (Mayville et al., 1999). These early discoveries have fueled interdisciplinary efforts to understand the underlying mechanisms of *agr*-mediated signaling. Accordingly, all of the protein components encoded by the *agr* locus have been extensively studied in whole cell-based systems, primarily employing mutagenesis and sequence swapping approaches. Recent years, however, have seen the emergence of *in vitro* reconstitution systems for studying most biochemical events in which these proteins participate. These studies have collectively addressed, or provided a promising starting point to address, many long-standing mechanistic questions regarding the regulation and evolution of the system. In this review, we integrate the results of both in-cell and *in vitro* studies to provide an up-to-date mechanistic description of the *S. aureus* QS circuit. We also discuss on-going efforts to identify agents that interfere with *S. aureus* QS as a potential route to treating infections and highlight opportunities in this area presented by recent biochemical breakthroughs.

Basic architecture of the *agr* autoinuction circuit

Analogous to other QS systems, production and sensing of the AIP in *S. aureus* are mutually enhancing, leading to a positive-feedback autoinduction circuit (Novick and Geisinger, 2008) (Figure 1B). In the *agr* locus, the P2 operon encodes a polycistronic messenger RNA (mRNA), termed RNAII, containing four *o*pen *r*eading *f*rames (ORFs), from which four Agr proteins involved in the autoinduction circuit are translated (Novick et al., 1995). AgrD, the precursor of the AIP, is first proteolytically processed by a membrane-bound peptidase, AgrB, to generate a thiolactone intermediate. This intermediate is then exported across the membrane and subject to a second cleavage to release the mature AIP pheromone into the extracellular space. The AIP is the activating signal of the sensing pathway, which is detected by a classic *t*wo-*c*omponent signaling *s*ystem (TCS) consisting of the membrane-bound *r*eceptor *h*istidine *k*inase (RHK), AgrC, and the response regulator (RR), AgrA. The AIP interacts with AgrC to activate a phospho-relay cascade that leads to the phosphorylation AgrA. Upon phosphorylation, AgrA binds to the P2 promoter, up-regulates the transcription of RNAII and thereby the production of all four Agr proteins, conferring positive feedback to the AIP synthesis.

Regulation of virulence-factor production is accomplished through the AgrA-dependent P3 operon, located back-to-back with P2, that encodes RNAIII, the mRNA for δ-toxin (an

exoprotein) and a pleiotropic regulatory factor (Novick et al., 1993) (Figure 1B). RNAIII primarily functions through base-pairing to the 5'-ends of virulence-factor mRNAs, suppressing the synthesis of proteins required for the adhesion phase of the life cycle, while de-repressing those involved in the invasion phase. A full discussion of the targets and mechanisms of action of RNAIII is beyond the scope of this review, and readers are referred to a recent account of this topic (Fechter et al., 2014). It is worth noting, however, that RNAIII-independent *agr* effectors have also been identified in recent years, with the most prominent example being *p*henol-*s*oluble *m*odulins (PSMs) that facilitate the bacterium's immune evasion (Queck et al., 2008).

Intraspecies variation of *agr*

One of the most intriguing features of the *agr* locus is its polymorphism within a single species. Within five years of the first *agr* locus being cloned in *S. aureus*, four allelic variants were reported (Jarraud et al., 2000; Ji et al., 1997). The variable region spans half the length of RNAII, covering coding region of the main body of AgrB, the entirety of AgrD and the sensor domain of AgrC (Figure 1B, marked with a blue, double-headed arrow). This setting allows each *agr* variant to specifically produce, and mediate autoinduction in response to, its own AIP. In *S. aureus* strains carrying different *agr* variants, the vast majority of conserved, structural genes (excluding mobile genetic elements) are predominantly identical, suggesting that the variation occurs at a sub-species level. Strains harboring each *agr* allele are therefore classified as a pherotype or a specificity subgroup.

While *S. aureus* strains from all four subgroups are capable of qualitatively similar autoinduction when cultured alone, the effect of AIPs on the induction of a heterologous *agr* system is, in most cases, strongly inhibitory (Ji et al., 1997; Lyon et al., 2002; Mayville et al., 1999) (Figure 1C). The only exception lies between the two most closely related groups, I and IV – AIPs from these groups have 7 identical residues out of 8 positions (Figure 1C). Clinical isolates of *S. aureus* from one infection site rarely exhibit variegation in the *agr* locus, primarily because an *agr*-heterologous cell population cannot achieve cooperative autoinduction to support the fitness of all participant subgroups (Traber et al., 2008).

A long-standing puzzle of *agr* polymorphism concerns the evolutionary advantage offered by individual *agr* alleles. A correlation has been observed between *agr* variants and infection types (Traber et al., 2008). For instance, group-III strains are overrepresented in menstrual toxic shock syndromes, while the exfoliatin-producing strains causing scalded skin syndrome are predominantly group-IV. In an insightful study performed by Geisinger et al., all four *agr* alleles were introduced, one at a time, into an *agr*-null background strain through chromosomal insertion at an identical site (Geisinger et al., 2012). Comparison of these alleles on an isogenic background revealed major differences in the temporal control of autoinduction: induction was achieved earliest with group-I and group-IV and latest with group-III alleles. This observation argues for a model in which each *agr* variant has a different schedule for autoinduction. Conceivably, such differences in induction timing might underlie, at least in part, correlations between subgroup type and the site of infection. This idea merits further investigation as does the detailed mechanism underlying the differential autoinduction timing and dynamics mediated by the different *agr* groups.

Biochemistry of *agr* autoinduction

Several steps in the *agr* autoinduction circuit have been investigated using well-defined reconstitution systems. These studies have provided a number of mechanistic insights, but have also pointed to the involvement of other, as yet uncharacterized, molecular players in the autoinduction circuit. In this section, we focus primarily on the biochemical mechanisms that this circuit harnesses to achieve timing, specific and restricted autoinduction.

Translation of Agr proteins

Although the coding sequence of all four Agr proteins are co-transcribed in the polycistronic RNAII, this transcript is exquisitely designed to finely coordinate their translation. For instance, the *r*ibosomal *b*inding *s*equences (RBSs) and initiating codons governing the translation of AgrD and AgrA are more favorable for translation efficiency than their counterparts in the AgrB and AgrC ORFs (Novick et al., 1995). This arrangement likely accounts for the desired stoichiometry between the AgrB-AgrD and AgrC-AgrA enzyme-substrate pairs. In addition, since the synthesis of AgrB and AgrC is coupled to co-translational insertion into the cell membrane, the intervening AgrD ORF presumably localizes in close proximity of the bilayer (Libby et al., 2012) (Figure 2). Because the 46-aa AgrD is highly hydrophobic and likely unstructured unless associated with the lipid bilayer (*vide infra*), it might be an important protective measure to synthesize AgrD close to the cell membrane to prevent its aggregation and degradation.

Formation of the AIP thiolactone

One of the most fascinating features of the *agr* system is the thiolactone structure within the AIP, closing the 16-atom macrocycle. The thiolactone is generated by a single proteolysis reaction involving AgrB and AgrD (Qiu et al., 2005; Thoendel and Horswill, 2009). The substrate, AgrD, contains the mature AIP sequence sandwiched between an N-terminal leader peptide and a C-terminal recognition sequence (Figures 1B and 3A). AgrB-catalyzed proteolysis clips off the recognition sequence as a linear peptide and concomitantly installs the thiolactone within the remaining N-fragment, herein referred to as the thiolactone intermediate. The recognition sequence is enriched in acidic residues and highly conserved in staphylococcal AgrDs. The significance of the sequence conservation is not yet fully understood (Thoendel and Horswill, 2009). The N-terminal 18 residues of the leader form an amphipathic helix that anchors AgrD to the cell membrane, putatively by lateral association (Zhang et al., 2004) (Figures 1B and 3A). This form of localization is required for AgrD processing, as the substitution of its N-terminal region with an artificial amphipathic helical sequence, but not a hydrophobic trans-membrane domain, is tolerated (Zhang et al., 2004). In all staphylococcal AgrDs, the amphipathic sequence is followed by an “IG” motif that putatively act as a helicity breaker to facilitate the proteolytic release of the mature AIP from the thiolactone intermediate (Kavanaugh et al., 2007).

AgrB, the peptidase responsible for proteolysis of AgrD, is a multi-pass membrane protein. Two residues invariable in all known AgrB homologs, one cysteine and the other histidine, have been identified as the catalytic diad (Qiu et al., 2005) (Figure 3B). An elegant peptidyl-transfer mechanism has been proposed for this reaction (Thoendel and Horswill, 2009), in

which AgrB first attacks the scissile bond using its active-site cysteine to form an acyl-enzyme thioester intermediate with the concomitant release of the linear AgrD C-fragment (Figure 3B). The intermediate is then resolved by transferring the peptidyl group of the AgrD N-fragment to the side-chain thiol group of its internal cysteine residue (C28), which leads to the formation of the thiolactone macrocycle. This 'proteolytic cyclization' process is unusual from a thermodynamic perspective in that it results in the net conversion of a stable peptide bond into a high-energy thioester bond. Reconstitution of this reaction, employing highly purified AgrD peptides and liposome-incorporated AgrB, has confirmed that the thiolactone N-fragment is indeed a kinetically favorable product (Wang et al., 2015). Intriguingly, the proteolytic cyclization exhibited a dynamic equilibrium behavior in the *in vitro* system. The equilibrium constant determined therein revealed that, in order to maintain a sufficient intracellular pool of the thiolactone intermediate to support the rapid production of AIP, *S. aureus* cells have to efficiently degrade the C-terminal cleavage fragment of AgrD, limiting its half-life to the order of 10 seconds (Wang et al., 2015) (Figure 3C). In other words, the bacterium follows Le Châtelier's principle, harnessing the favorable free energy from the hydrolytic degradation of one cleavage fragment to power the installation of a high-energy thiolactone motif in the other. In line with this notion, over-production of the AgrD C-fragment in *S. aureus* causes a decrease of AIP production. Because of its thermodynamic contribution, degradation of the AgrD C-fragment should be added as an essential step to the AIP production pathway.

Translocation and maturation of the AIP

After being processed by AgrB, the thiolactone intermediate undergoes a second proteolysis event to release the freely diffusive AIP pheromone from the membrane-anchoring N-terminal leader peptide. An active or facilitated translocation event is required for the successful secretion of the AIP due to its presumed lack of membrane permeability. Intriguingly, the *agr* locus does not encode designated proteins to account for these steps. Consequently, AgrB had been surmised to also export and/or cleave the thiolactone intermediate (Zhang et al., 2002). Biochemical studies have since ruled out a role for AgrB in the second proteolysis step (Qiu et al., 2005; Wang et al., 2015) and, while an involvement in AgrD translocation has not been formally ruled out, the absence of an ATP-binding cassette within the enzyme would make such a translocation activity quite extraordinary.

In principle, peptide translocation could occur either before or after the second proteolysis event, although a few lines of evidence favor the former scenario. In 2007, Kavanaugh and coworkers showed that the general signal peptidase, SpsB, cleaves a heptapeptide mimicking the leader peptide-AIP junction of AgrD-I at the expected site (Kavanaugh et al., 2007). Because the catalytic domain of SpsB localizes in the extracellular space, the authors argued in favor of the translocation-first model. It should be noted that only indirect evidence is provided in this study for the ability of SpsB to cleave the native thiolactone intermediate. More recently, large quantities of the AgrD leader peptide have been detected in the *S. aureus* extracellular matrix (Gonzalez et al., 2014; Schwartz et al., 2014). This result also supports the translocation-first model, as the alternative, proteolysis-first model would entail separate translocation of both leader and AIP fragments, which would be substantially less

economical. The transporter responsible for AIP production, regardless of the substrate being exported, is yet to be identified.

The second proteolysis of AgrD sets the length of the exocyclic tail region within the AIP. Intriguingly, depending upon the *S. aureus agr* group, the AIP tail can vary between 2-4 amino acids in length (see Figure 1.4), with even larger variation found in other gram-positive species (Olson et al., 2014; Sturme et al., 2005). Nonetheless, this proteolysis event is remarkably specific within a given *agr* group – the growth media of over a dozen AIP-producing gram-positive strains have been subjected to mass-spectrometric analysis and in no case has a single *agr* locus been found to produce chemically heterogeneous AIPs (Gray et al., 2013). The sequence contexts of the scissile bonds are so diverged among their AgrD precursors that it would be nothing short of shocking if the orthologs of a single protease family carried out the AIP-releasing function in all species.

How might such variation within AIP length have arisen? Here we present a model that reconciles the absence of a designated AIP-releasing protease to both the homogeneity of the cleavage site within each native AIP, as well as the tail-length variation among AIPs from closely-related the *agr* loci. The AgrD-thiolactone intermediate consists of two structurally rigid and putatively protease-resistant elements, i.e., an amphipathic α-helix and a thiolactone macrocycle, flanking an 8-aa linker starting from the conserved, helicity-breaking glycine and ending at the ring-forming cysteine (Figures 4A and 4B). Because all known AIP-releasing sites are located within this linker, we postulate that this region exhibits considerable conformational flexibility so that each peptide bond is potentially cleavable if exposed to a protease that recognizes the relevant sequence context. As a consequence, the cleavage site is dictated by a competition among all proteases with access to this linker (Figures 4A and 4B). In wild-type *agr* systems, the exquisite specificity of the AIP-releasing proteolysis would come from the selective pressure imposed by the need for highly efficient conversion of AgrD to the native AIP pheromone. The protease of choice thereby overwhelms all other competitor proteases in terms of both efficiency and specificity (Figures 4A and 4B). Conceivably, however, sequence changes occurring within or adjacent to the AgrD linker region through random mutagenesis or DNA recombination may loosen the proteolysis specificity (Figure 4C). The mutant strain may thus produce a series of AIPs with variable tail lengths (Figure 4C), which significantly increases the likelihood for a new, activating AIP-AgrC pair to emerge through a second mutational event affecting the sensor domain sequence of AgrC (Figure 4D). Importantly, because autoinduction is by nature a collective behavior of bacterial populations, selection pressure against the AgrD mutant would be low if it stays within its parental population (Schuster et al., 2013). This may allow persistence of the mutant and also facilitate the co-evolution between AgrD and AgrC. Once bacteria containing this nascent AIP-AgrC pair are isolated from a parental population, they would be again selected for AIP-production efficiency, leading to the co-evolution between the new AgrD-protease pair and hence, the re-establishment of AIP-releasing specificity (from Figure 4D to 4B). We imagine that this model should be experimentally testable through a combination of AgrD mutagenesis and peptidomics. Ultimately, identification of the AIP-releasing proteases in all *S. aureus* subgroups will provide a key starting point to investigate the evolutionary trajectory of these *agr* variants.

Activation of the AgrCA TCS

By sequence homology, AgrC and AgrA form a TCS. AgrC adopts the modular architecture commonly seen for RHKs, consisting of an N-terminal, membrane-integrated sensor module that detects AIP and a C-terminal histidine kinase (HK) module that carries out enzymatic functions (Figure 5A) (Lina et al., 1998). These two modules are connected via a short, α-helical interdomain linker (Wang et al., 2014). The HK domain contains two subdomains (Gao and Stock, 2009). Proximal to the sensor is the *d*imerization and *h*istidine *p*hosphorylation (DHp) subdomain, which folds into an α-helical hairpin and dimerizes through the formation of a four-helix bundle. Consequently, AgrC, as do most RHKs known to date, forms an obligate dimer. The distal, C-terminal subdomain is the so called *c*atalytic and *A*TP-binding (CA) subdomain. In an AgrC homodimer, the CA subdomain of one subunit binds to the ATP and catalyzes the *in trans* phosphorylation at a histidine residue on the DHp subdomain of the opposite subunit (Cisar et al., 2009) (Figure 5A). Upon phosphorylation, AgrC transfers the phosphoryl group to AgrA to turn on its activity as a transcription factor.

Although conforming to the fundamental roles of RHKs, AgrC also possesses some distinct sequence features and is classified as a member of the “HPK10” competence kinase subfamily of RHKs (Grebe and Stock, 1999). A recent survey of the protein database expanded this subfamily to more than 300 non-redundant sequences that are exclusively from low-GC gram-positive bacteria (*Firmicutes*) (Wang et al., 2014). In all HPK10 sequences, an Asn residue substitutes for the conserved “G1-box” Asp, which normally hydrogen-bonds to the N-6 amino group on the adenine base of the nucleotide. A recent crystal structure of the AgrC CA domain indicates that this Asn residue indeed takes the place of the canonical Asp (Srivastava et al., 2014). Therefore, the substitution is likely responsible for the exceptionally weak affinity between full-length AgrC and ATP: the $K_{\mathbf{m}}$ is about 2 mM (Wang et al., 2014) (Figure 5B). This property renders the kinase activity of AgrC, and perhaps all HPK10-subfamily members, strongly dependent upon the cellular ATP level, which reflects the energy condition of the bacterium (Figure 5B). In particular, when energy starvation drives down the cellular ATP level, the AgrC kinase activity will be diminished even in the presence of AIP activators. This mechanism may account, in part, for the down-regulation of *agr* autoinduction in *Staphylococcus* and competence induction in *Streptococcus* (both mediated by HPK10 subfamily members) in the stationary growth phase (Claverys et al., 2006; Wright et al., 2005).

Until recently, all mechanistic studies on the AgrCA TCS employed cell-based assays, which, while informative, precluded detailed biophysical and biochemical characterizations (Novick and Geisinger, 2008; Thoendel et al., 2011). The successful reconstitution of the AgrCA TCS thus stands as an exciting breakthrough in this field (Wang et al., 2014). Key to this achievement was the use nanoscale lipid bilayer discs, or nanodiscs (Ritchie et al., 2009), to afford an active preparation of full-length AgrC. This new system has already led to some remarkable insights. For instance, maximal activation of AgrC-I only occurs when the receptor is embedded in a highly anionic lipid bilayer that approximates the native lipid composition of the *S. aureus* cell membrane. Within this membrane environment, AIP-I non-cooperatively binds to the receptor in a 2:2 stoichiometry, while the inhibitor, AIP-II,

competes for both sites where AIP-I binds. Dissociation constants (K_D) of both ligands were found to be in the mid-nanomolar range. Interestingly, experiments employing this reconstitution system ruled out a previous model in which the AIP binding results in acylation of AgrC *via* opening the chemically labile thiolactone (Mayville et al., 1999). AgrC-I possesses a baseline level of autokinase activity, which is strongly activated when bound to the agonist, AIP-I. Interestingly, engagement with the AIP-II inhibitor leads to reduction in baseline AgrC activity, indicative of inverse agonism, while the AIP-III inhibitor has no effect on basal AgrC activity, consistent with neutral antagonism (Geisinger et al., 2009). Kinetic studies on the reconstituted AgrCA TCS indicate that autophosphorylation, rather than phosphoryl transfer, is the rate-determining step. Unlike most RHKs that have been characterized *in vitro*, AgrC-I lacks phosphatase activity on AgrA in all ligand states tested. Perhaps not coincidently, AgrA features one of the fastest rates for spontaneous chemical dephosphorylation ($t_{1/2}$ = 3.9 min at 37 °C) among transcription-factor RRs (Thomas et al., 2008). Thus, a “kinase-off, phosphatase-on” ligand state of AgrC may be dispensable for the rapid inactivation of AgrA, potentially allowing shutdown of the *agr* signaling in response to, for instance, energy starvation even in the presence of activator AIPs.

Perhaps the most intriguing finding from the reconstituted *agr* TCS is the signaling plasticity of AgrC-I (Wang et al., 2014): the RHK exhibited four distinct levels of autokinase activity when bound to AIP-I, -II, -III and a non-native partial activator, the truncated AIP-I (Lyon et al., 2002). This plasticity contradicts the generally accepted two-state model of RHK autokinase activation (Wang et al., 2013). Employing a chimeric-protein strategy in which the entire sensor domain was replaced by a stable coiled-coil motif, rotational perturbations were systematically introduced to the α-helical interdomain linkers preceding the AgrC-I HK domain. Autokinase analysis of these chimera proteins revealed that the kinase activity of the AgrC-I HK domain changes gradually with the magnitude of twisting movement applied to the linkers (Figure 5C). This result is in stark contrast with a previous report arguing for a model in which HK domains are inactive unless subjected to highly specific conformational inputs (Moglich et al., 2009). We note that HK domains exhibiting gradual input-response properties, when recombined with non-cognate sensor domains during the course of evolution, should enjoy a better chance of generating a signaling-competent new RHK (Capra and Laub, 2012). Furthermore, full-length AgrC-I appears to harness the signaling plasticity of its HK domain: cysteine-specific crosslinking data is consistent with a model in which AIP-I or AIP-II binding rotates the interdomain linker in different directions and thus confers activation or inhibition to the kinase activity (Figures 5C and 5D). These findings provide the first view on molecular motions triggered by ligand binding on a membrane-bound RHK.

AgrA phosphorylation and transcription activation

Acting as the phospho-receiver in the TCS as well as a transcription activator, AgrA consists of two domains, each assuming one of its two functions. The N-terminal *rec*eiver domain is shared across all RR proteins and dimerizes upon phosphorylation at its conserved Asp residue (Gao and Stock, 2009). The C-terminal *D*NA *b*inding *d*omain (DBD) belongs to the LytTR protein family and binds to the consensus DNA elements located in the P2 and P3

promoter region (Nikolskaya and Galperin, 2002). In the crystal structure of the AgrA DBD in complex with a cognate, 16-nucleotide (nt) DNA fragment, the DBD is enriched in β-strands and harnesses residues on its inter-strand loops for the interaction with DNA (Sidote et al., 2008). This unique binding interaction causes bending of the DNA double helix by 38°. Oxidative stress inactivates DNA binding by inducing disulfide-bond formation within the DBD between two cysteine residues at positions 199 and 228 (Sun et al., 2012).

Autoinduction of *agr* depends on a different temporal pattern of RNAII and RNAIII transcription. Tight repression of the P3 promoter is needed to avoid premature RNAIII-mediated mRNA degradation prior to autoinduction. At the same time, a reasonable expression level of RNAII is required to prime the autoinduction circuit. In congruence with this idea, experiments have confirmed that the baseline level of RNAII transcription is higher, while its activation is less dramatic, compared to that of RNAIII (Reynolds and Wigneshweraraj, 2011). How could the same pool of AgrA possibly exercise differential regulation over two operons? The answer lies within their promoter sequences. Both P2 and P3 promoter regions contain, in the orientation of transcription, two AgrA-binding elements followed by the -35 and -10 boxes required for RNA polymerase (RNAP) recognition. In spite of the similar architecture, the P2 recognition sequence provide stronger affinity to AgrA than that in P3 (Koenig et al., 2004). Thus, the promoter occupancy of P2 would be higher than that of P3 in the case where there is limited availability of phosphorylated AgrA, i.e. prior to full autoinduction. Moreover, the spacer between the -35 and -10 boxes measures 18 nts in the P2 promoter and 20 nts in P3, both deviating from the optimal 17-nt length for RNAP binding (Reynolds and Wigneshweraraj, 2011). Strikingly, shortening the spacer in P3 to the optimal length dramatically enhances the baseline transcription activity both *in vitro* and *in vivo* (Morfeldt et al., 1995; Reynolds and Wigneshweraraj, 2011). In light of this observation, the DNA-bending effect of AgrA binding, as well as the dimerization induced by AgrA phosphorylation, has been postulated to rearrange the -35 and -10 boxes back to the optimal conformation for engagement of RNAP (Reynolds and Wigneshweraraj, 2011). This model provides an attractive explanation for the more substantial up-regulation of RNAIII production during autoinduction.

Reagent development for the manipulation of *agr*

S. aureus requires *agr* not for survival but for virulence. Interfering with the autoinduction, or quorum quenching (QQ), should therefore be effective in combating infection whilst, in principle, reducing the likelihood of resistance development versus classic, bactericidal or bacteristatic, antibiotics (Cegelski et al., 2008). It has long been known that QQ agents are effective in containing the spread of *S. aureus* in mouse models of infection (Mayville et al., 1999). However, the utility of this therapeutic strategy in treating an existing infection is still very much an open question (Otto, 2004). It is noteworthy that silencing of the *agr* system is known to strengthen the *S. aureus* biofilm and the QQ strategy might therefore contribute to the maintenance of chronic infection (Kong et al., 2006). By extension, activation of the *agr* system might represent a more attractive strategy in tackling chronic *S. aureus* infections: not only does it disperse the biofilm, the constant induction of *agr*-regulated genes also takes a toll on the fitness of the bacterium, perhaps rendering them more susceptible to classic antibiotics. Practical application of this strategy would entail a global activator with the

ability to turn on the autoinduction of all four *S. aureus* subgroups. This reagent could potentially be a cocktail of "clean" activators each of which activates one or more *agr* variants without substantially affecting the rest. Unfortunately, the efficacy of *agr* activators in animal models has not been tested to date, nor has any clean *agr* activator ever been developed. Indeed, nearly all the medicinal chemistry efforts in this area to date have focused on the development of QQ agents, although based on the above discussion, the identification of global *agr* activators clearly merits investigation.

Structural-activity relationship (SAR) analysis of AIP-AgrC interactions

Native AIPs provide a rich source of information for the design of QQ agents: once the structural elements required for binding are dissected from those needed for receptor activation, selective perturbation of the later should yield competitive inhibitors of the AgrC receptor. To this end, extensive SAR studies have been performed on AIP-I, -II and –III and have reached consensus on several important points. First of all, the 5-aa, 16-membered macrocycle is of utmost importance for binding (Figure 6A) (Mayville et al., 1999; McDowell et al., 2001). To date, no linear peptide has ever shown any activity on a native AgrC. Expanding or contracting the size of the macrocycle is also deleterious to AIP activity (Johnson et al., 2015). Secondly, hydrophobic residues at the C-terminal end of the peptide, two in AIP-II and three in each of the other AIPs, are necessary (but not sufficient, see below) for tight binding to AgrC (Figure 6A). Alanine point mutations at these positions cause severe loss of potency (Mayville et al., 1999; McDowell et al., 2001; Tal-Gan et al., 2013b). Last, but not least, the agonist activity of an AIP is highly sensitive to structural modification. Hotspots required for AgrC activation locate to the exocyclic tail and the second residue within the macrocycle (Figure 6A). Modification at these sites can convert an AIP into a *global agr* inhibitor (Johnson et al., 2015; Lyon et al., 2002; McDowell et al., 2001; Tal-Gan et al., 2013b), although in some cases a more complex pharmacology can result (Johnson et al., 2015).

Recently, solution structures of all four wild-type AIPs, as well as a series of well-characterized analogs, have become available (Tal-Gan et al., 2015; Tal-Gan et al., 2013a). These structures have revealed some interesting correlations with the activities of these peptides. For instance, in all tight-binding AIP-I, -III and –IV analogs, the three C-terminal hydrophobic side chains form a distinct hydrophobic surface (Figure 6B, top panel). In contrast, truncated versions of AIP-I and AIP-III lacking the exocyclic tail, no longer maintain this surface due to their dramatically different macrocycle conformations (Figure 6B, bottom panel). Perhaps not coincidentally, both truncated peptides suffer a severe loss of potency despite the fact that their hydrophobic triad remains intact.

AgrC-targeting agents

Target-specific AgrC inhibitor design has been predominantly limited to engineering of the parent AIP scaffold. To date, AIP-I, -II and -III have all been successfully converted into QQ reagents capable of inhibiting the autoinduction of all four *S. aureus* subgroups (Figure 7A) (Lyon et al., 2000; Lyon et al., 2002; Tal-Gan et al., 2013b). Despite this success, the AIP backbone remains peptidic in nature and hence suffers such drawbacks as high immunogenicity and lack of stability *in vivo*. In an attempt to address this problem,

modification of single residues in AIP-III through the substitution of amino acids with the corresponding peptoid or N-methyl mimics has generated a few global QQs (George et al., 2008; Tal-Gan et al., 2014). Full conversion of an AIP global inhibitor into a peptido-mimetic has, however, yet to be achieved.

Aside from synthetic peptides derived from the native AIP scaffold, a few secondary metabolites from other microbes have been shown to inhibit *S. aureus* autoinduction. It should be pointed out that the mechanism of action of these compounds is yet to be rigorously determined. Nonetheless, some of these inhibitors share astonishing structural similarity to the native AIP architecture, despite their disparate origin. For instance, solonamides, cochinmicin and avellanin, identified from marine bacteria, actinomycetes and sponges, respectively, each possesses a 16-membered macrocycle and are therefore believed to function through competitive inhibition of AgrC (Figure 7B) (Desouky et al., 2015; Igarashi et al., 2015; Mansson et al., 2011). Two other natural products, 3-oxo-C12-HSL and 4-hydroxy-2-heptylquinoline N-oxide (HQNO) originating from *Pseudomonas aeruginosa*, are capable of quenching *S. aureus* autoinduction with a low-micromolar IC_{50} (Figure 7C) (Gordon et al., 2013). In particular, the HSL may act as an allosteric inhibitor of AgrC at lower concentrations as was inferred from its functional interaction with AIPs in cell-based assays (Murray et al., 2014). Given the similar, amphiphilic structure of these two compounds and the sensitivity of AgrC activation to lipid composition (Wang et al., 2014), it is tempting to speculate that they function through interacting with the cell membrane.

The recently available nanodisc-reconstitution system of AgrC and the solution NMR structures of AIPs potentially open new avenues for the screening or design of nonpeptidic AgrC ligands. Nanodiscs are particularly amenable to small-molecule library screening employing affinity-based approaches, which have given rise to potent ligands of several G-protein coupled receptors (Annis et al., 2007). Solution structures of AIPs and the configurations of the structural determinants for receptor binding observed therein, conceivably could be harnessed as the template to search for small-molecule AgrC ligands employing chemoinformatic strategies (Kolb et al., 2009).

AgrA-targeting reagents

In contrast to AgrC, which has four variants in *S. aureus*, AgrA has uniform sequence in all four subgroups, potentially making it a better therapeutic target. Analysis of the crystal structure of AgrA LytTR domain in the absence of bound DNA suggests that targeting a small molecule to an exposed hydrophobic cleft might disrupt the AgrA-DNA interaction (Leonard et al., 2012). As a proof of principle, Leonard et al. screened a focused small molecule library and identified a few hits with low-millimolar affinity for AgrA (Leonard et al., 2012). Screening of a much larger library led to the discovery of S*taphylococcus* a*ureus* *vi*rulence *in*hibitor (savirin), which blocks *S. aureus* autoinduction in the mid to low micromolar range (Figure 7D) (Sully et al., 2014). Biochemical studies indicate that the small molecule blocks the AgrA association with DNA. This compound robustly inhibits autoinduction phenotypes of *S. aureus* cultures and attenuates the lesion size in the classic murine abscess model. Importantly, resistance did not emerge upon extensive passage of *S.*

aureus in the presence of savirin. Thus, savirin is viewed as a promising lead compound for further medicinal chemistry studies.

Conclusions and Outlook

The *agr* locus plays a key role in the onset of *S. aureus* pathogenicity. This regulon has been the focus of intense study for well over two decades, making it one of the best-understood QS circuits in any bacterium. Indeed, all biochemical events directly involving *agr*-encoded proteins have now been reconstituted *in vitro,* allowing the associated processes to be carefully scrutinized. These investigations have revealed much about the inner workings of the QS circuit, but at the same time have exposed several hitherto unknown features of the process that await further biochemical characterization. First and foremost, it has become increasingly evident that *agr*-encoded proteins cannot account for all the steps in the core autoinduction circuit. Key protein participants remain to be identified in the translocation of the AgrD-thiolactone intermediate, the AIP-releasing proteolysis (at least in some subgroups), and the degradation of the AgrD-C fragment. Encoded apart from the *agr* locus, these proteins may well carry out other essential functions. Therefore, their identification and characterization will shed light on the driving forces, and restraints, governing the evolution of a hyper-variable system on a predominantly uniform genetic background. In addition, the biochemical properties of the AgrCA TCS suggest it may be responsive to spurious changes in cellular redox potential, ATP levels or lipid composition (Sun et al., 2012; Wang et al., 2014). These regulatory connections still await further examination *in vivo*. Once confirmed, they may be applicable to a wide range of Gram-positive QS systems as the underlying biochemical properties are dependent upon conserved sequence features.

The past decade has seen a rapid growth in the number of reagents that modulate *agr*. The vast majority of these tools are *agr* inhibitors and, perhaps as a consequence, all pharmacological studies performed on animal models involve, to our best knowledge, the administration of QQ agents. By contrast, the development of clean and/or global *agr* activators remains a persistent challenge for the field. Only when these reagents become available can the therapeutic value of *S. aureus* biofilm dispersion be explored in the context of chronic infections. Critically, medicinal chemistry efforts in this area are hampered by the absence of high-resolution structural information on how the AIP is recognized by the AgrC sensor domain. The challenges here can hardly be overstated – to date, there is no high-resolution structure available for any membrane-embedded RHK sensor domain. Nonetheless, advances in membrane protein X-ray crystallography and in single particle cryo-EM approaches offer some hope for the future, especially given the availability of numerous AgrC orthologs, including those from thermophilic species, for screening purposes (Wang et al., 2014).

As a result of the knowledge gaps listed above, we believe that the potential of targeting the *agr* circuit as an anti-infective modality is far from being fully explored. We imagine that interdisciplinary efforts will be needed to address these outstanding problems. Given the widespread occurrence of *agr*-like TCS systems in gram-positive bacteria, the concepts and tools that emerge form such efforts are likely to have wide-spread implications for our understanding of the complex behavior of bacterial pathogens.

Acknowledgements

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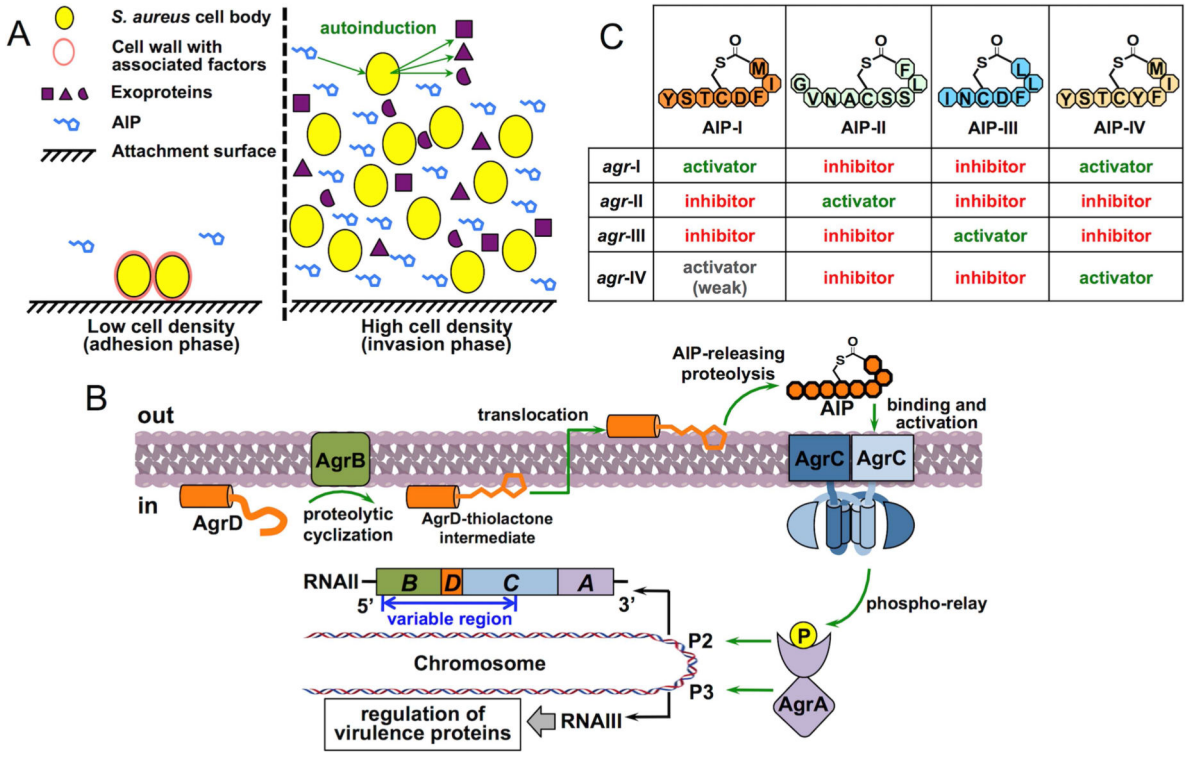

Figure 1. Role of the *agr* QS circuit in virulence regulation in *S. aureus*

(A) Two phases of the *S. aureus* life cycle featuring distinct patterns of virulence protein production. At high cell density, AIP accumulates in the extracellular environment and triggers the *agr* QS circuit leading to decreased production of cell wall-associated factors and a simultaneous increase in exoprotein production. (B) Schematic of the *agr* autoinduction circuit. (C) Structure and efficacy of AIPs from all four *S. aureus* subgroups.

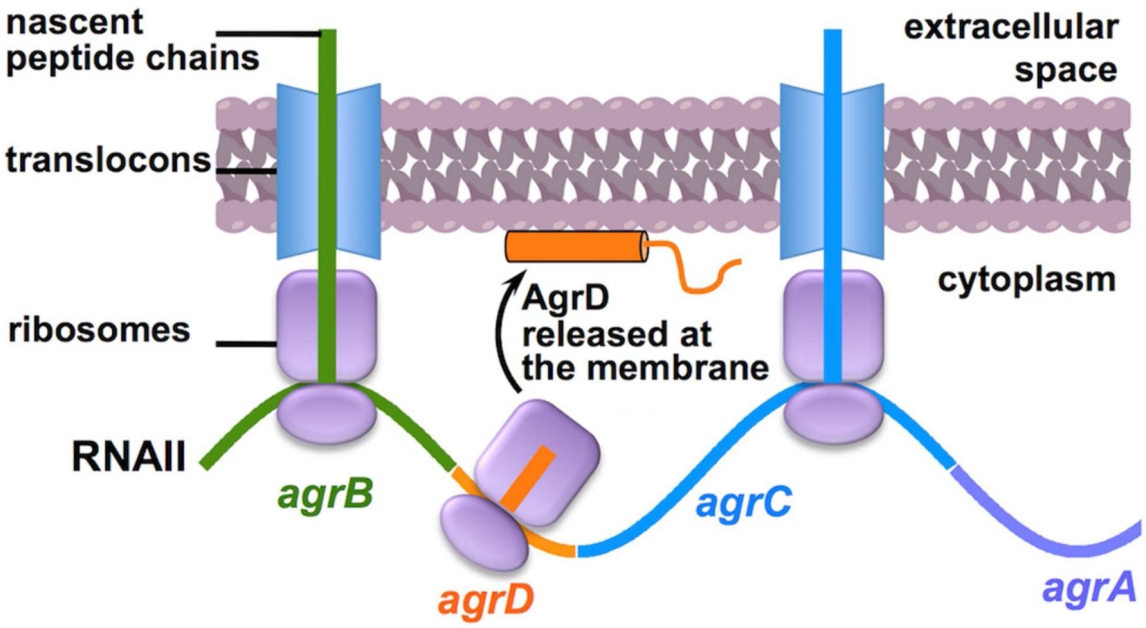

Figure 2. Membrane localization of the RNAII polysome

Schematic shows the site of AgrD synthesis relative to the cell membrane as a consequence of the co-translational insertion of the flanking RNAII-encoded membrane proteins (AgrB and AgrC).

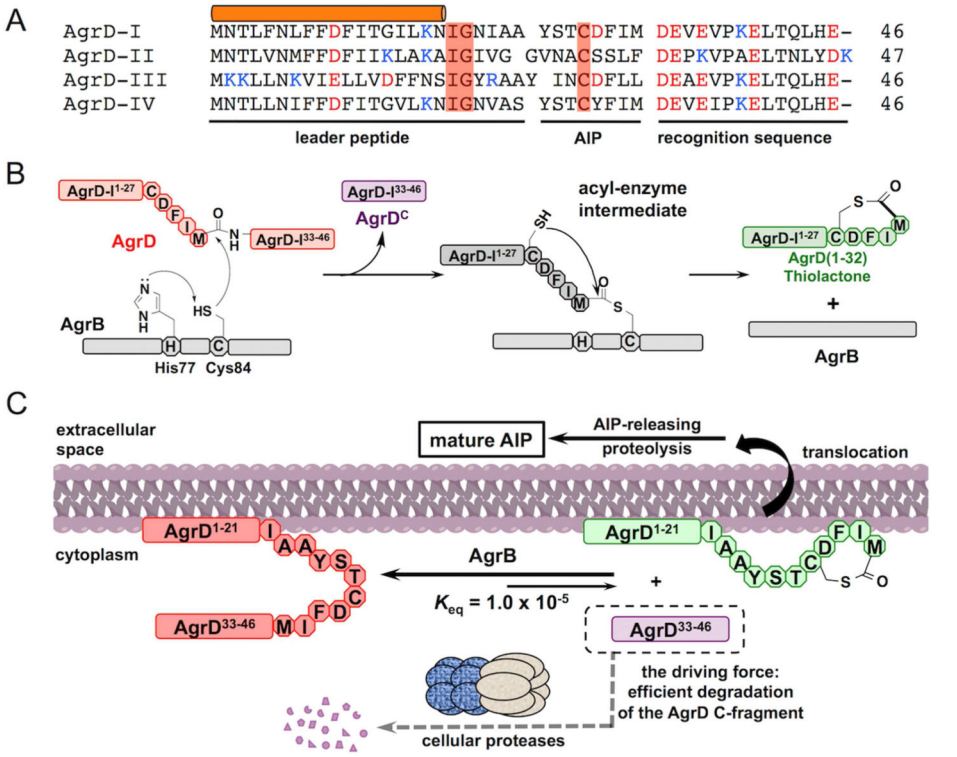

Figure 3. Formation of the AIP thiolactone

(A) Sequence alignment of the four AgrD variants from *S. aureus*: acidic and basic residues are highlighted in red and blue, respectively. Residues playing explicit roles in AgrD processing are highlighted with red boxes. The cylinder indicates the amphipathic α-helical sequence. (B and C) Schematics showing (B) the catalytic mechanism and (C) the thermodynamic driving force of the AgrB-mediated proteolytic cyclization of AgrD, exemplified by the formation of the group-I AgrD thiolactone intermediate.

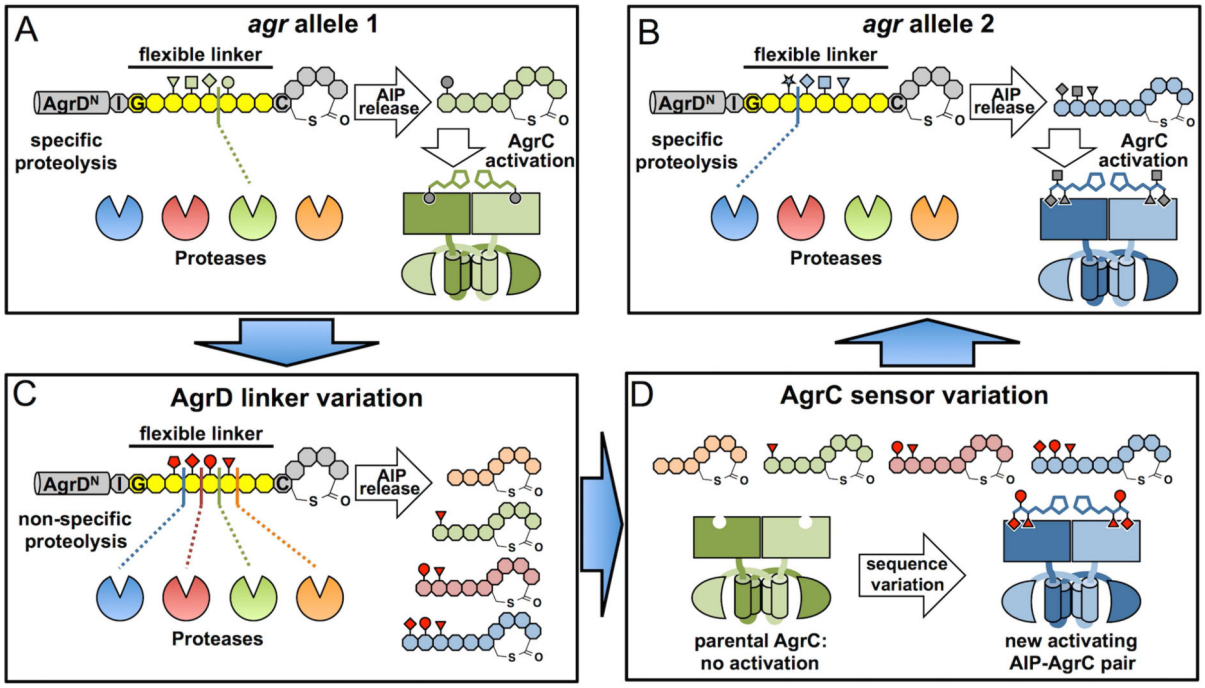

Figure 4. A Hypothetical trajectory for hyper-variation of *agr* alleles

(A and B) Specific AIP-releasing proteolysis and AIP-AgrC recognition in two hypothetical, wild-type *agr* variants from the same bacterial species. Amino-acid residues in the AgrD thiolactone intermediate are shown in octagons, with the proteolysis-susceptible linker highlighted in yellow. AIP, AgrC, the AIP-releasing protease and side-chains of four linker residues in the thiolactone intermediate are colored for group specificity (green in panel A, blue in panel B). (C) Loosened AIP-releasing specificity after sequence variation occurs within the AgrD linker region depicted in (A). Side-chains of four linker residues subject to changes are highlighted in red. (D) Further sequence variation in the AgrC sensor domain gives rise to a new, activating AgrC-AIP pair (blue). This nascent, functional *agr* allele would then be selected for releasing specificity of the new AIP to become the wild-type allele depicted in (B).

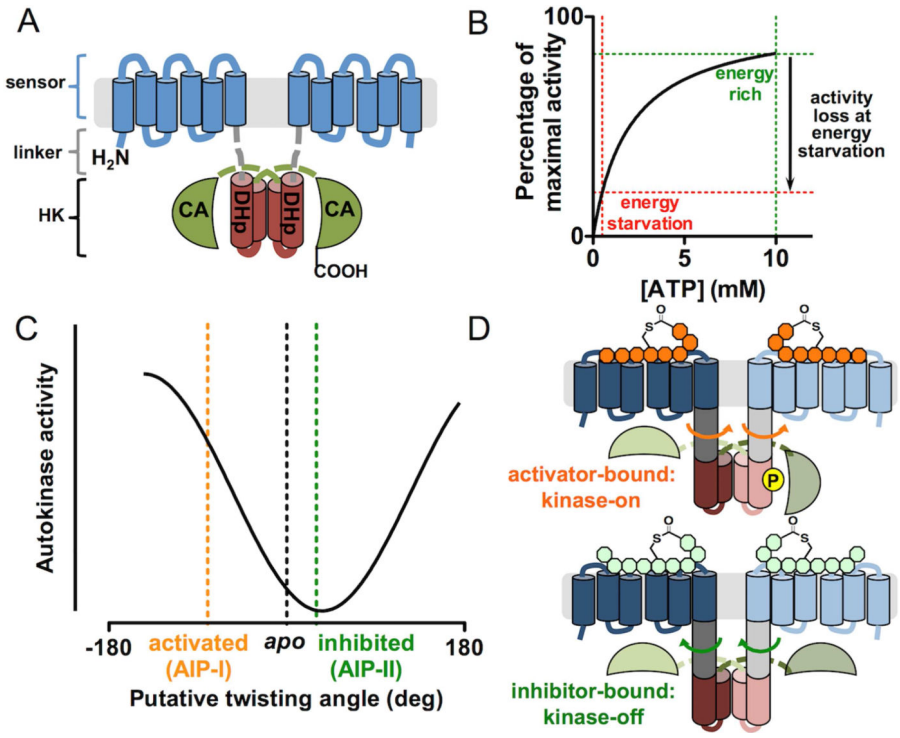

Figure 5. Activation of the AgrC-AgrA TCS

(A) Domain architecture of AgrC-I. The protein is shown as a homodimer with the sensor, DHp and CA modules colored in blue, brown and green, respectively. The interdomain linker is depicted as gray dashed lines. (B) A Michaelis-Menten plot of AgrC based on a K_m value of 2 mM (Wang et al., 2014). The loss of kinase activity at energy starvation ([ATP] = 0.5 mM) relative to energy-rich conditions ([ATP] = 10 mM) is highlighted. Cellular ATP levels are inferred from measurements performed in *E. coli* (Tran and Unden, 1998). (C) The gradual responsiveness of the autokinase activity of the AgrC HK domain to rotational movements imposed at the interdomain linker pair. Conformational inputs in full-length AgrC-I under native ligand states are marked with dashed lines. (D) Schematic showing the opposite direction of linker rotation triggered upon the binding of an activator (top) or an inhibitor (bottom) AIP.

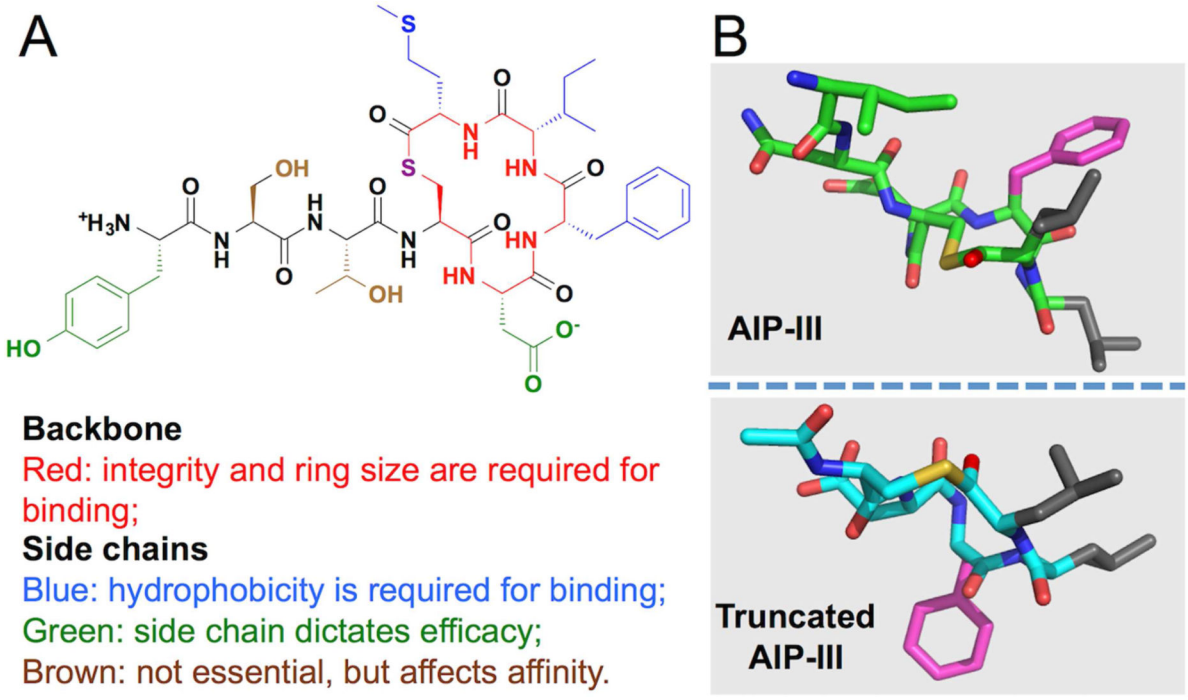

Figure 6. SAR analysis of AIPs

(A) Summary of SAR studies of *S. aureus* AIPs, exemplified by AIP-I. (B) Comparison of the AIP-III solution structure to a less potent ligand, the truncated AIP-III. Side chain of Phe5 (in AIP-III numbering) is highlighted in magenta, and Leu6 and Leu7 in gray.

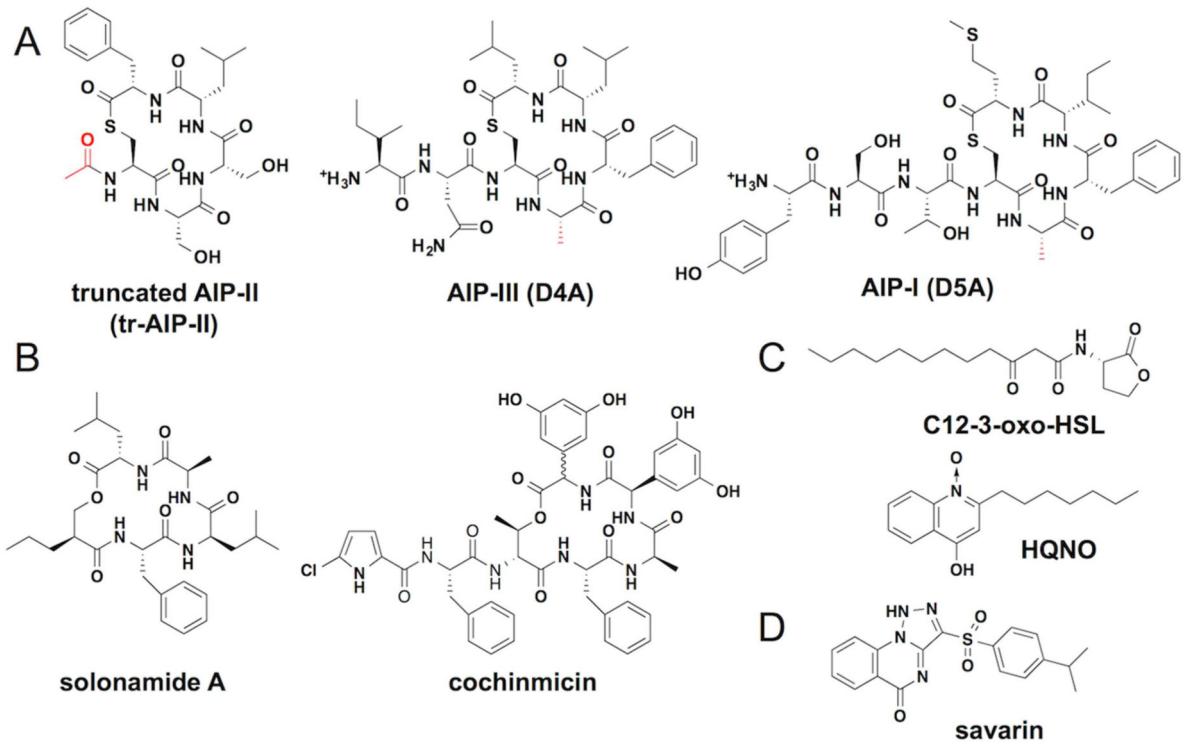

Figure 7. Synthetic molecules and natural products that target agr

(A) Global AgrC inhibitors derived from native AIPs. Groups that differ from the wild-type AIP are highlighted in red. (B) AIP-mimicking natural products. (C) The *Pseudomonas* autoinducers 3-oxo-C12-HSL and HQNO. (D) The AgrA-targeting lead compound, savarin.