

Hydrophobic interactions drive ligand-receptor recognition for activation and inhibition of staphylococcal quorum sensing

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Two-component systems represent the most widely used signaling paradigm in living organisms. Encoding the prototypical two-component system in Gram-positive bacteria, the staphylococcal *agr* (accessory gene regulator) operon uses a polytopic receptor, AgrC, activated by an autoinducing peptide (AIP), to coordinate quorum sensing with the global synthesis of virulence factors. The *agr* locus has undergone evolutionary divergence, resulting in the formation of several distinct inter- and intraspecies specificity groups, such that most cross-group AIP-receptor interactions are mutually inhibitory. We have exploited this natural diversity by constructing and analyzing AgrC chimeras generated by exchange of intradomain segments between receptors of different *agr* groups. Functional chimeras fell into three general classes: receptors with broadened specificity, receptors with tightened specificity, and receptors that lack activation specificity. Testing of these chimeric receptors against a battery of AIP analogs localized the primary ligand recognition site to the receptor distal subdomain and revealed that the AIPs bind primarily to a putative hydrophobic pocket in the receptor. This binding is mediated by a highly conserved hydrophobic patch on the AIPs and is an absolute requirement for interactions in self-activation and cross-inhibition of the receptors. It is suggested that this recognition scheme provides the fundamental basis for *agr* activation and interference.

staphylococci | *agr* | intradomain chimera | autoinducing peptide

Two-component signaling systems function in the sensing of the cell's external environment and are probably the most widely used signaling paradigm in living organisms (1). Many two-component signaling systems in Gram-positive bacteria use polytopic transmembrane receptors that are activated by autoinducing peptides (AIPs) (2, 3). Although these systems regulate virulence in staphylococci (*agr*) (4) and enterococci (*fsr*) (5), competence in bacilli (6) and pneumococci (*com*) (7), and bacteriocin production in lactic acid bacteria (*pin* and *ssp*) (8, 9), constituting a major component of the regulatory biology of these organisms, the mechanism(s) by which peptides bind to and activate the respective receptors are unknown.

We have found the staphylococcal *agr* (accessory gene regulator) system to be particularly amenable to mechanistic investigation in this context for two reasons: first, because it is conserved throughout the staphylococci but has undergone a highly significant evolutionary divergence, resulting in four (or more) different specificity variants in *Staphylococcus aureus* and at least 20 others in the non-*aureus* species (10–12); and second, because the 7- to 10-aa AIPs of different staphylococcal species form a close family with a conserved structure consisting of a 5-aa thiolactone ring (a lactone ring in one case) and a linear 2- to 5-aa “tail” (13–16). Because the AIP from any one specificity group generally inhibits *agr* activation in the other groups (10, 11, 15, 17, 18), one is provided with a unique and powerful set of preexisting tools with which to analyze receptor–ligand interactions, particularly significant biologically because

the predicted mutual exclusion may be the evolutionary driving force for divergence and speciation in the staphylococci.

In earlier studies, we developed a convenient method for synthesizing the AIPs (13) and constructed a series of group-specific reporter strains with which to test the activity of these molecules (19). Using this combination of chemistry and genetics, we have characterized the four known *S. aureus* AIPs (20), performed a detailed structure function analysis of AIP-II (13) (peptides and receptors are identified by a roman numeral suffix designating their specificity group), and shown that the interactions between activating and inhibiting peptides are competitive (14). A fundamental question raised by these studies is how a wide variety of divergent peptides can each competitively inhibit the same receptor, whereas only the single cognate AIP can activate it.

We exploited the natural diversity of the *agr* system to generate a series of molecular chimeras in the AgrC receptor domain, whose behavior might be informative with respect to this question. Segments of the proximal and distal halves of the polytopic N-terminal sensor domain were switched among different receptor specificity types, corresponding to the four known *S. aureus agr* groups. These intradomain chimeras were then tested for activation and inhibition specificity against chemically synthesized native AIPs and a battery of AIP analogs. This approach localized the region of receptor–ligand specificity and identified the orientation of the receptor–ligand interface. Remarkably, however, two of the chimeric receptors could not be inhibited by any AIP tested and had entirely lost activation specificity (being activated by all but three of the peptides tested, even those that strongly inhibit all four of the native receptors). These chimeras allowed us to uncover a key component of AgrC–AIP recognition, a seemingly promiscuous, hydrophobic interaction, which may explain the mechanism of staphylococcal cross-group interference.

Materials and Methods

Bacterial Strains and Growth Conditions. Staphylococcal strains are derivatives of NCTC8325. *Escherichia coli* strain JM109 was used for cloning. RN6734 is our standard laboratory strain and is *agr* group I (11). RN7206 is a derivative of RN6734 in which the *agr* locus has been replaced by *tetM* (21). RN4220 is a heavily mutagenized derivative of *S. aureus* that readily accepts foreign DNA (22). Overnight cultures of *S. aureus* on GL media [3 g/liter casamino acids/3 g/liter yeast extract/5.9 g/liter NaCl/3.3 ml/liter 60% sodium lactate/40 ml/liter 25% (vol/vol) glycerol/15 g/liter agar] (23) containing antibiotics when necessary (chloramphenicol, tetracycline, and erythromycin at 5 or 10 μ g/ml) were routinely used as inocula. *S. aureus* were grown in CYGP broth (10 g/liter

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Abbreviations: AIP, autoinducing peptide; HK, histidine kinase.

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casamino acids/10 g/liter yeast extract/5 g/liter glucose/5.9 g/liter NaCl/60 mM glycerophosphate) (23) in the absence of antibiotics with shaking at 37°C, and cell growth was monitored by a Klett–Summerson (Klett, Long Island City, NY) colorimeter with a green (540 nm) filter. Media used for *E. coli* were Luria broth and LB agar with ampicillin used for selection at 100 μg/ml.

Construction of Receptor Chimeras. Plasmid pRN7128 served as the vector for expression of the receptor chimeras in RN7206. pRN7128 is characterized in detail in refs. 14 and 20 but, in brief, carries the P2::*agrCA* genes, a P3::*blaZ* reporter, an *ori* from pT181 (for replication in *S. aureus*), the *ermC* gene from pE194 (for selection in *S. aureus*), and an ampicillin/ColE1 *ori* fragment from pUC19 (for replication and selection in *E. coli*). Plasmid pRN7128 has a unique *Afl*III restriction site at the boundary between the sensor and signaling domain motifs in *agrC*. The three other *agrC* receptors have been individually cloned to pRN7128 and are characterized in detail in refs. 14 and 20. Two-step PCR (24) with appropriate primers and receptor templates was used to construct the chimeric fusions. The PCR fragments were then ligated back into pRN7128 by using an upstream *Pst*I site and the *Afl*III site. Sequences of the chimeras were confirmed by dye-terminator DNA sequencing chemistry (Skirball DNA Sequencing Core Facility). pRN7128 derivatives were electroporated into *S. aureus* RN4220 (25) and moved by phage transduction into an *agr*-null strain, RN7206, to eliminate the effects of endogenous AIPs on function. The AgrC receptor histidine kinases (HKs) are referred to in the text as AgrC-N (N corresponding to the *agr* specificity group) and the chimeras are referred to as AgrC-N::C (designating AgrC group N for the N terminus of the receptor domain and AgrC group C for the C terminus).

Synthesis of AIPs. All AIPs were chemically synthesized on a *t*-butoxycarbonyl-amino acyl-3-mercapto-propionamide-4-methylbenzhydramine-copoly(styrene-1% DVB) [Boc-AA-(COS)-MBHA] support and characterized as described in ref. 20. The 3-thiopropionic acid linker on MBHA resin is labile to hydrogen fluoride (HF)-cleavage conditions, thereby releasing linear thioester peptides upon global deprotection. After removal of HF, the crude peptide product was precipitated by using cold diethyl ether (Et₂O), washed thoroughly with Et₂O, and dissolved in 45% acetonitrile (CH₃CN)/50% H₂O/0.1% trifluoroacetic acid (TFA). After lyophilization, linear thioester peptides were dissolved again in CH₃CN and H₂O with 0.1% TFA and cyclized in solution by the addition of at least 1× vol of 0.2 M phosphate buffer at pH 7. After cyclization (>90%), the peptides were purified by RP-HPLC and characterized by analytical RP-HPLC, mass spectrometry, amino acid analysis, and thioester base-lability studies, which collectively demonstrated that all peptides were of the correct composition and >95% pure. In addition, two-dimensional ¹H NMR analysis yielded complete assignments for the synthetic peptides. The concentrations of stock solutions were calculated based on amino acid analysis, with the peptides dissolved in 45% CH₃CN/55% H₂O/0.1% TFA. Peptide stocks were made in 25% propylene glycol/50 mM phosphate (pH 5.7) at a known concentration and serially diluted into the same buffer for assays with cells.

Activation and Inhibition Assays. Strain RN7206 containing the *agrC* receptor chimeric fusions were grown with shaking at 37°C in CYGP to a cell density of 140 Klett (mid-exponential phase) for assay of β-lactamase expression from the P3::*blaZ* reporter fusion. Synthetic peptides in 25% propylene glycol/0.05 M phosphate (pH 5.7) were added alone (activation) or together (inhibition) at various concentrations to 96-well plates and incubated with shaking at 37°C for 60 or 90 min in a ThermoMax microplate reader (Molecular Devices) with monitoring of cell density at 650 nm. Assay of β-lactamase expression was performed by the nitrocefin method as described in ref. 4. The data from the β-lactamase assay

were normalized to percent maximal activation and plotted as initial β-lactamase reaction velocity versus log peptide concentration. By using PRISM 3.0 (GraphPad, San Diego), individual agonist or antagonist concentration–response curves were fitted via non-linear regression to the following four-parameter Hill equation:

$$E = \text{Basal} + \frac{E_{\text{max}} - \text{Basal}}{1 + 10^{(\text{LogEC}_{50} - \text{Log}[A])^{n_H}}}$$

where *E* denotes effect, [*A*] denotes the agonist concentration, *n_H* denotes the midpoint slope, EC₅₀ denotes the midpoint location parameter, and *E_{max}* and Basal denote the upper and lower

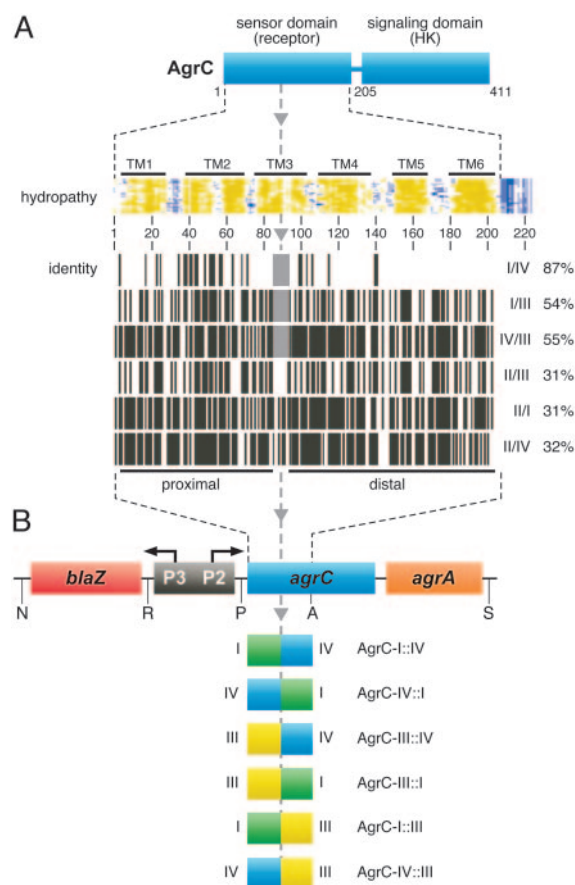


Fig. 1. Comparison of the AgrC receptor domains and strategy for subdomain chimera construction. (A) The domain organization of AgrC. A hydropathy plot of all known AgrC receptor sequences (residues 1–227) was performed by using the SEQVU 2.1 program (Garvan Institute of Medical Research, Darlinghurst, Australia). Yellow shading represents predicted hydrophobic regions, and blue shading represents hydrophilic regions. Putative transmembrane-spanning domains (TM1–6) were identified by several topological prediction algorithms (31–35) and are consistent with previous topological (12) and *phoA* fusion (26) analyses. A corresponding plot illustrates amino acid sequence identity between combinations of the four *S. aureus* AgrC receptors. For each residue, a black bar represents sequence variation, and a white bar represents sequence identity. The percentage of sequence identity for each pair in the receptor region (amino acids 1–205) is also denoted. The location of the junction in AgrC used for the construction of the subdomain chimeras is indicated by a dashed line as well as gray shading in the identity plot and corresponds to the amino acid sequence Q₈₆L₈₇L₈₈L₈₉Y₉₀C₉₁A₉₂N₉₃, which is identical in AgrCs I, III, and IV. (B) At the top is a diagram of the *agrCA* reporter construct with *blaZ* transcriptionally fused to the P3 promoter (restriction sites: N, *Nar*I; R, *Eco*R I; P, *Pst*I; A, *Afl*III; S, *Sph*I). The six subdomain chimeras for which results are reported are illustrated below. In each case, the recombinant *agrC* gene was cloned to the reporter construct replacing the receptor domain-encoding region of the native gene. Chimeras involving AgrC-II were also constructed but were essentially inactive and are not presented.

Table 1. Response of AgrC chimeras to various AIPs

	AgrC-I::IV	AgrC-III::IV	AgrC-IV::I	AgrC-III::I	AgrC-I::III	AgrC-IV::III
	Activation (EC ₅₀)*					
AIP-I	+ (1,220 nM)	–	++ (26 nM)	+ (1,700 nM)	+	++
AIP-II	–	–	–	–	+ (3,100 nM)	+ (640 nM)
AIP-III	–	–	–	–	++ (48 nM)	++ (4 nM)
AIP-IV	++ (6 nM)	+ (340 nM)	++ (120 nM)	–	++	++
AIP-I D5N	–	–	+ (440 nM)	–	++	++
AIP-I D5A	–	–	–	–	++	++
TrAIP-II [†]	–	–	–	–	++ (160 nM)	++ (50 nM)
AIP-II F9A	–	–	–	–	–	–
AIP-II L8A	–	–	–	–	–	–
AIP-II N3A	–	–	–	–	++	++
LiAIP-I [‡]	–	–	–	–	+ (3,100 nM)	+ (6,700 nM)
LiAIP-II	–	–	–	–	–	+
	Inhibition [§]					
AIP-I	/	+	/	/	/	/
AIP-II	+	+	+	+	/	/
AIP-III	–	+	+	+	/	/
AIP-IV	/	/	/	+	/	/
AIP-I D5N	+	+	/	+	/	/
AIP-I D5A	–	–	–	–	/	/
TrAIP-II	+	+	+	+	/	/
AIP-II F9A	–	–	–	–	–	–
AIP-II L8A	–	–	–	–	–	–
AIP-II N3A	–	–	–	–	/	/
LiAIP-I	–	–	–	–	/	/
LiAIP-II	–	–	–	–	–	/

++, Activation observed at <200 nM; +, activation observed at >200 nM/inhibition observed; –, no activation at <10,000 nM/no inhibition observed; /, AIP is an agonist and was not tested for inhibition.

*Where indicated, EC₅₀ values were obtained as described in *Materials and Methods*.

[†]Truncated AIP; contains only the macrocycle without the tail residues.

[‡]Linear AIP; peptide without the thioester bond.

[§]In all cases, the most potent agonist for a given chimeric receptor was used for inhibition analysis. IC₅₀ values are omitted because AIP agonists were used at different concentrations and are thus incomparable. Therefore, inhibition is only qualitatively scored in this section of the table.

asymptotes, respectively. For inhibition curves, the midpoint location parameter from the above equation reflects the IC₅₀.

Results

Design and Construction of Functional AgrC Chimeras. Because the receptor has a highly conserved C-terminal cytoplasmic HK domain (residues 206–411) and a highly divergent N-terminal polytopic transmembrane domain (residues 1–205) (11), it was predicted and confirmed that the N-terminal domain would be the sensor and would determine receptor specificity (14, 20). Although the sensor domains are divergent, their transmembrane topology appears to be highly conserved, as judged by the spacing of the putative hydrophobic α -helices (Fig. 1A). Thus, it seemed likely that the exchange of segments within this domain would generate functional chimeric receptors, whose behavior might be informative with respect to the localization of peptide recognition sites, specific contacts, etc. Accordingly, we have constructed 10 of 12 possible chimeric derivatives by exchanging the proximal and distal halves of the sensor domains, using a single conserved motif for all of the exchanges.

The chimeric AgrC constructs were transduced into an *agr*-null *S. aureus* strain and analyzed for functionality by testing them for their ability to activate a β -lactamase reporter fused to the *agr*-RNAIII promoter in response to various AIPs, as diagrammed in Fig. 1B. Some of the constructs had detectable receptor activity, whereas others did not; indeed, there was an inverse relationship between the functionality of the chimera and the degree of sequence divergence of the two parental molecules. Thus, the chimeras between the two most closely related AgrCs, I and IV (87% sequence identity), were both functional, those involving AgrC-II, which is most distant from the others (32% sequence identity) were

not, and those involving AgrC-III and either I or IV (54% sequence identity) were functional but had unpredictable activities.

Orientation of the AgrC–AIP Interface. We first examined the activity of the chimeras between the closely related AgrC-I and AgrC-IV receptors. The AgrC-I::IV and -IV::I subdomain chimeras were activated by both AIPs I and IV and inhibited by AIP-II and by a truncated (“tailless”) AIP-II variant, with kinetics similar to those observed with the two native receptors (Table 1 and Fig. 2A and B). However, AIP-I was 50- to 100-fold more potent on the AgrC-IV::I receptor than on the AgrC-I::IV receptor, whereas the opposite was true for AIP-IV (Table 1), indicating that the major determinant of AIP recognition is in the distal subdomain of the sensor, as previously suggested by Lina *et al.* (26) (see Fig. 1A). AIPs I and IV differ at only a single residue, D vs. Y at position 5, within the thiolactone ring (see Fig. 3), indicating that it is the ring moiety of the AIP that makes the defining group-specific contact with the distal subdomain. This conclusion was supported by results with an AIP-I/IV variant having asparagine at this position (D5N). AIP-I D5N activates AgrC-I but, unlike native AIP-I, inhibits AgrC-IV (20). Revealingly, it has precisely the same effects with the AgrC-IV::I and -I::IV chimeras, activating the former and inhibiting the latter (Fig. 2C and D). However, AgrC-I::IV was activated by AIP-I much more strongly (EC₅₀ of 1,220 nM) than was the native AgrC-IV [EC₅₀ of 26,000 nM (20)], suggesting that the proximal subdomain also contributes a significant role in receptor function, including specificity. These results suggest that the AIP is oriented with its ring moiety facing the distal region of the receptor.

AgrC Chimeras That Show Increased Specificity but Decreased Responsiveness. The properties of the chimeras involving AgrCs I and IV and AgrC-III were very much dependent on which subdomain

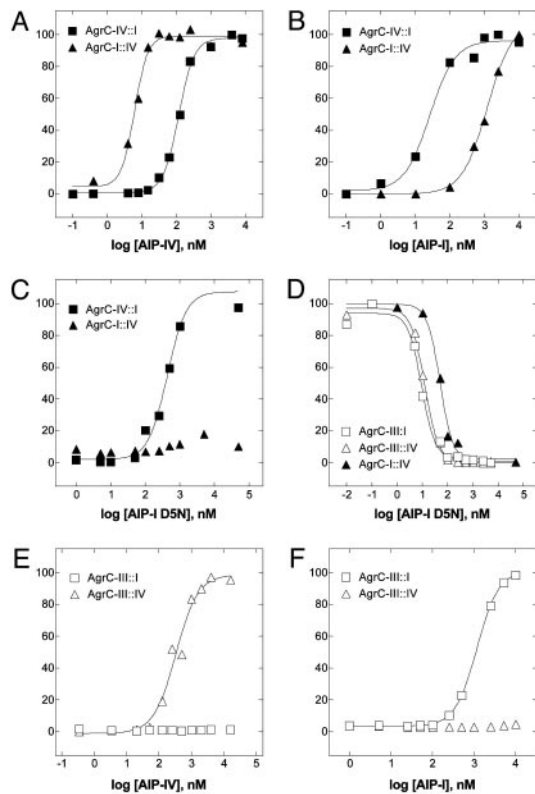


Fig. 2. Dose–response curves for various constructs and AIPs. In each case, the indicated AIP was added in increasing concentrations to an exponential phase culture containing the indicated reporter. Samples were removed after 1–1.5 h of incubation at 37°C and assayed for β -lactamase activity by the nitrocefin method as adapted to the microtiter format (11). The y axis for all graphs indicates the percent maximal activation, and the x axis represents the concentration of the AIP. A–C, E, and F represent dose-dependent activation of the AgrC chimeras with various AIPs. In D are the results of an inhibition test with AIP-I D5N. In this case, AIP-I or -IV was added at 1,000 nM along with the inhibitor.

of AgrC-III was present. In the case of the III::I and III::IV chimeras activation specificity again followed the distal subdomain: III::I was activated by AIP-I and III::IV was activated by AIP-IV, but the AIP concentrations required were 100- to 1,000-fold higher than with the native receptors (Fig. 2 E and F). Moreover, there was no detectable cross-activation by AIPs IV and I on III::I and III::IV, respectively; on the contrary, AIP-IV cross-inhibited III::I and AIP-I cross-inhibited III::IV. Both of these chimeras were also inhibited by AIPs II and III, similarly to the inhibition seen with native AgrCs I and IV (20). Again, the results support the localization of the AgrC-I and -IV specificity determinants to the distal subdomain and imply that the proximal subdomain has an important role (in this case, greatly diminishing the responsiveness but increasing the selectivity of the receptor).

AgrC Chimeras That Lack Activation Specificity. The results with the I::III and IV::III chimeras were, to say the least, surprising. Akin to the behavior of the chimeras described above, the I::III and IV::III chimeras were preferentially responsive to AIP-III. However, both of these chimeras were also strongly activated by other AIP analogs. These included AIPs I, II, and IV, which are strong inhibitors of AgrC-III (13), and AIP-I D5A and truncated AIP-II (trAIP-II), which are strong inhibitors of all four AgrCs (13, 20). Even linear AIPs I and II, which are generally inert in this system (11, 16), were found to activate one or the other of these chimeras, albeit weakly. Moreover, no AIP or AIP derivative tested could inhibit activation of either receptor. Consistent with the previous results, it appears

<i>S. aureus</i> -I*	Y	S	T	C	D	F	I	M	8
<i>S. aureus</i> -IV*	Y	S	T	C	Y	F	I	M	8
<i>S. aureus</i> -III*		I	N	C	D	F	L	L	7
<i>S. aureus</i> -II*	G	V	N	A	C	S	S	L	9
<i>S. capitis</i> -I	G	A	N	P	C	Q	L	Y	9
<i>S. capitis</i> -II	G	A	N	P	C	A	L	Y	9
<i>S. epidermidis</i> -I*	D	S	V	C	A	S	Y	F	8
<i>S. epidermidis</i> -II	K	Y	N	P	C	S	N	Y	9
<i>S. epidermidis</i> -III	K	Y	N	P	C	A	S	Y	9
<i>S. warnerii</i> -I*	Y	S	P	C	T	N	F	F	8
<i>S. caprae</i> -I	G	Y	S	T	C	S	Y	F	9
<i>S. caprae</i> -II	G	Y	R	T	C	N	T	Y	9
<i>S. lugdunensis</i> -I*		D	I	C	N	A	Y	F	7
<i>S. lugdunensis</i> -II		D	M	C	N	G	Y	F	7
<i>S. carnosus</i> -I	K	Y	N	P	C	V	G	Y	9
<i>S. simulans</i> -I	K	Y	N	P	C	L	G	F	9
<i>S. simulans</i> -II	K	Y	Y	P	C	F	G	Y	9
<i>S. intermedius</i> -I*	R	I	P	T	S	T	G	F	9
<i>S. auricularis</i> -I	K	A	K	T	C	T	V	L	9
<i>S. auricularis</i> -II	K	T	K	T	C	T	V	L	9
<i>S. arlettae</i> -I	G	V	N	P	C	G	V	W	9
<i>S. gallinarum</i> -I*	V	G	A	R	P	C	G	G	10
<i>S. xylosum</i> -I	G	A	K	P	C	G	G	F	9
<i>S. cohnii cohnii</i> -I	G	G	K	V	C	S	A	Y	9
<i>S. cohnii urealyticum</i> -I	S	V	K	P	C	T	G	F	9

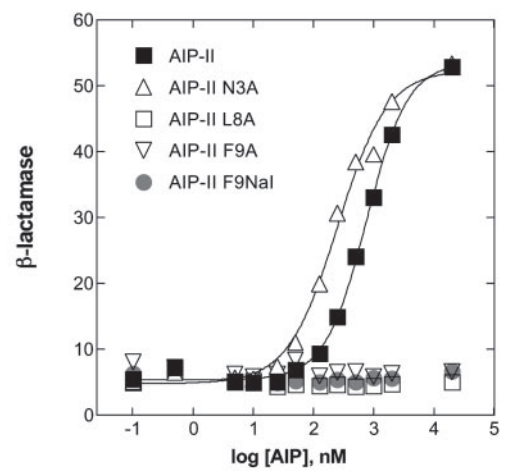
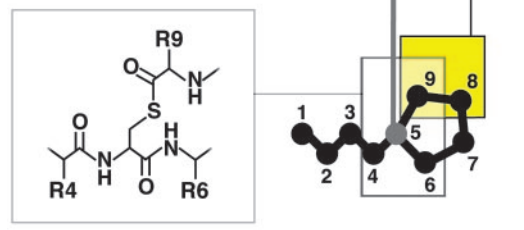


Fig. 3. Sequence, hydrophobicity, and structure of various known and predicted AIPs. (A) At the top is displayed the sequences and hydrophobicity profiles of the 24 known or predicted AIPs. Most or all of the staphylococcal AIPs are 7–10 aa in length. In the alignment, hydrophilic (polar) residues are colored blue, and hydrophobic residues (nonpolar) are colored yellow. A shaded box marks the conserved cysteine, which is always 5 aa from the C terminus, and a black outline traces the conserved hydrophobic macrocycle residues. N3 in AIP-II and D5 and Y5 in AIP-I and -IV are highlighted because they are critical for activation (13, 20). Note that AIPs I and IV differ by only this amino acid and that *Staphylococcus intermedius* contains a serine in place of the conserved cysteine. Sequences marked with an asterisk are those that have been confirmed by *in vitro* synthesis and mass spectroscopy (11, 13–15, 36) (M. Kalkum, personal communication). The rest are predicted from the corresponding *agrD* sequences (12). Because of variation in the N-terminal tail region, most of the unconfirmed AIPs have been given an arbitrary length of 9 aa. Below this is a schematic model of a generic 9-aa AIP and a magnified view of the thiolactone bond. As a point of reference, highlighted residues are identified on the AIP model. (B) A representative dose–response plot with different AIP-II analogs against the AgrC-IV::III chimera demonstrates the importance of the conserved hydrophobic macrocycle residues in receptor recognition. The x axis represents the concentration of AIP, and the y axis indicates raw β -lactamase activity.

native cognate receptors, may also not be sufficient for cross-inhibition either. For example, AIP-III is a poor antagonist of AgrC-I:IV but a strong inhibitor of the native AgrC-I and -IV receptors, and the lactam analog of AIP-II neither inhibits nor significantly activates AgrC-II but acts as a potent inhibitor of AgrC-I, -III, and -IV (13), suggesting that specific contacts or structural features of the peptide may be involved for inhibition, in at least some cases, as well as for activation. This finding implies that inhibition may not be simply a matter of blocking access of the activating peptide to its site on the receptor but may also require specific contacts in addition to binding. We also note that one of the AIPs, that of *S. cohnii urealyticum*, is predicted to have a C-terminal alanine, yet supernatant from this strain presumed to contain this AIP does not inhibit activation of the *S. aureus agr* groups (unpublished data). It has not yet been determined whether this peptide is a self-activator; its further study promises to be enlightening with respect to our model.

A Model for AgrC–AIP Recognition. We propose a simple model for AgrC–AIP recognition based on the remarkable activities of these chimeras (Fig. 4). Driven by the bulky hydrophobic amino acids in the macrocycle, any AIP can be accommodated into a promiscuous binding pocket within the receptor. We note that the EC₅₀ and IC₅₀ values for activation and inhibition are comparable (14, 20), suggesting that the binding energy for either is approximately the same. It is therefore suggested that both cognate and heterologous AIPs bind competitively and with equal strength to the receptor. The conserved hydrophobic patch in the macrocycle interacts with an as-yet-unidentified hydrophobic pocket that might be comprised of both the proximal and distal moieties of the AgrC receptor domain. Further analysis on this feature may be complicated by the fact that the receptor is also likely to exist as a preformed dimer as demonstrated for other two-component receptor HKs and methyl-accepting chemotaxis proteins (MCPs) (29, 30). A dimer with two (or more) AIP binding sites could also account for the previously observed cooperativity of the *agr* response both for activation and inhibition (13, 14, 20). Ligand binding is likely accompanied by conformational changes in the AIP and/or the receptor for activation and, perhaps, for inhibition, but it is not known what these changes may comprise or where they may occur. Clearly, these features will need to be addressed in future studies by more rigorous analysis.

A Potential Role for AgrC Chimeras in the Discovery of Quorum-Sensing Inhibitors. Although our understanding of the significance of quorum-sensing competition on the biology of staphylococci has remained elusive, this characteristic inhibitory effect has shown therapeutic potential: A single dose of an AIP antagonist blocks the

formation of an experimental murine abscess (13), and we expect that fuller understanding of the AIP–AgrC interaction will enable the design of more potent inhibitors. The properties of the chimeric receptors analyzed in this study may significantly aid in this strategic approach by serving in a gain-of-function (activation) rather than a loss-of-function (inhibition) screen of the *agr* response. For example, compounds that activate the highly promiscuous AgrC-IV::III chimera, which would be readily identifiable in a high-throughput screen, may represent potent inhibitors of the native receptors.

Accommodation of Diverse Ligands Provides the Fundamental Basis for Quorum-Sensing Interference and May Drive the Generation of *agr* Diversity. A fundamental conclusion from this work is that AgrC has the intrinsic ability to recognize any structurally appropriate ligand, and that cognate AIP–AgrC interactions may be a consequence of conformational specificity rather than rigid allele-specificity. If receptor activation were dependent on highly allele-specific AIP–AgrC interactions, we probably would have been unsuccessful at generating functional chimeric receptors by wholesale exchange of receptor subdomains, because residues required for activation in both the receptor and the AIP would have likely been mismatched. Instead, we created chimeras from receptors sharing only 54% sequence identity that were activated in response to various AIPs, indicating that the hallmark of AgrC–AIP recognition for activation may not be allele-specific but dependent on subtle conformational variations.

We propose that accommodation of diverse ligands by the receptor is a prerequisite for the evolution of cognate recognition and may provide the mechanism for interference by noncognate AIPs. Coevolution of *agrC* and *agrD* may be triggered by mutations in one component that reduce or eliminate AgrC–AIP activity but do not abolish binding, so that activity could then be restored by a compensatory mutation(s) in the other component. Hence, maintenance of the AgrC–AIP interaction would be vital to evolutionary divergence, and mutations that eliminate AgrC–AIP binding altogether would presumably represent evolutionary dead-ends. We suggest that an accommodating AIP-binding pocket may serve as a molecular scaffold for the preservation of this interaction. This defining characteristic would permit the evolution of *agr* diversity and provide a mechanism for AgrC–AIP competitive inhibition and, perhaps, biological isolation.

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