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Identification of Ligand Specificity Determinants in AgrC, the *Staphylococcus aureus* Quorum-sensing Receptor*[S]

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Activation of the *agr* system, a major regulator of staphylococcal virulence, is initiated by the binding of a specific autoinducing peptide (AIP) to the extracellular domain of AgrC, a classical receptor histidine protein kinase. There are four known *agr* specificity groups in *Staphylococcus aureus*, and we have previously localized the determinant of AIP receptor specificity to the C-terminal half of the AgrC sensor domain. We have now identified the specific amino acid residues that determine ligand activation specificity for *agr* groups I and IV, the two most closely related. Comparison of the AgrC-I and AgrC-IV sequences revealed a set of five divergent residues in the region of the second extracellular loop of the receptor that could be responsible. Accordingly, we exchanged these residues between AgrC-I and AgrC-IV and tested the resulting constructs for activation by the respective AIPs, measuring activation kinetics with a transcriptional fusion of *blaZ* to the principal *agr* promoter, P3. Exchange of all five residues caused a complete switch in receptor specificity. Replacement of two of the AgrC-IV residues by the corresponding residues in AgrC-I caused the receptor to be activated by AIP-I nearly as well as the wild type AgrC-I receptor. Replacement of two different AgrC-I residues by the corresponding AgrC-IV residues broadened receptor recognition specificity to include both AIPs. Various types of intermediate activity were observed with other replacement mutations. Preliminary characterization of the AgrC-I-AIP-I interaction suggests that ligand specificity may be sterically determined.

Signal transduction is a universal modality for the communication of environmental information to the interior of a cell. It typically involves a transmembrane receptor whose extracellular domain senses an external signal and transmits it to a cytoplasmic transmitter domain, setting in motion an intracellular response that usually involves phosphotransfer. In bacteria, the primary signaling pathway is relatively simple, usually involving a receptor histidine protein kinase and a single cytoplasmic protein, the response regulator, a transcription factor whose activity depends on its phosphorylation state. Although hundreds of such two-component signaling modules have been identified in bacteria, specific ligands are known for only a very few (1), and evidence relating to determinants of ligand binding specificity has been obtained for but a handful of cases. One example is the histidine protein kinase-associated bacterial aspartate receptor, Tar. This receptor uses three arginine residues to sense aspartate (2), suggesting that sensing involves electrostatic interactions between ligand and receptor, and one of these arginines was found to be mutated in a recently isolated Tar variant with altered specificity for novel attractants (3). Another intensively studied receptor is the O_2-sensing heme protein, FixL, of *Rhizobia*, for which O_2 binding to the permanently bound heme allosterically modifies the activity of the histidine kinase domain (4); in this case, discrimination of dioxygen from other small molecules has been proposed to depend on the ability of a critical arginine residue to hydrogen bond with its target ligand (5). Determinants of specificity in histidine protein kinases that sense macromolecular ligands such as peptides are unknown.

Staphylococci use a key two-component system, encoded in the quorum-sensing *agr* locus, to coordinate with population density the expression of a large set of accessory protein genes, many of which are involved in pathogenesis (6–9). The *agr* signal receptor histidine protein kinase, AgrC, is activated by a unique ligand, the AIP,[2] a short thiolactone peptide. The peptide binds to the receptor by means of a conserved C-terminal hydrophobic patch and activates it by one or more specific secondary contacts involving other residues (10, 11). Activation of AgrC leads to phosphorylation of the response regulator AgrA, followed by up-regulation of the *agr* effector molecule, RNAIII, which initiates a cascade of regulatory changes in virulence gene expression. The *agr* system is widely, perhaps universally, conserved among the staphylococci (12), and homologs have been identified in several other bacterial species (13, 14).[3] Sequence variants have evolved for which the specificity of the ligand-receptor interaction has been changed. This variation has led to the identification of four specificity groups in *Staphylococcus aureus* (15, 16) and two or more in each of several other staphylococcal species (12). As a general rule, only the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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[2] The abbreviations used are: AIP, autoinducing peptide; SBA, sheep's blood agar; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

[3] K. Winzer, personal communication.

TABLE 1
Strains and plasmids

Strain or plasmid	Genotype or description	Reference
***S. aureus* strains**		
RN4220	Restriction-deficient mutant of strain 8325-4	Ref. 30
RN6734	8325-4 agr^+ ϕ 13 lysogen of RN6390B	Ref. 31
RN7206	RN6734 with *tetM* replacing *agr*	Ref. 20
RN10828	RN7206 containing pRN9253 integrated into the SaPI1 *att* site (hemolysis assay strain)	This work
RN10829	RN7206 containing pRN9254 integrated into the SaPI1 *att* site (β-lactamase reporter strain)	This work
***E. coli* strain**		
DH5α	Standard recipient for plasmid cloning	Promega
Plasmids		
pRN9231	pCN47 (32) carrying promoter P2, insertion site for *agrC*, and P2 terminator	This work
pRN9232	pRN9231 with *agrC-I*	This work
pRN9233	pRN9231 with *agrC-IV*	This work
pRN9234	pRN9231 with *agrC-IV 100–116grI*	This work
pRN9235	pRN9231 with *agrC-IV V104T,V107S,I116S*	This work
pRN9236	pRN9231 with *agrC-IV F100Y,T101A,V104T,V107S*	This work
pRN9237	pRN9231 with *agrC-IV F100Y,T101A*	This work
pRN9238	pRN9231 with *agrC-IV V104T,V107S*	This work
pRN9239	pRN9231 with *agrC-IV V104T,I116S*	This work
pRN9240	pRN9231 with *agrC-IV V107S,I116S*	This work
pRN9241	pRN9231 with *agrC-IV V104T*	This work
pRN9242	pRN9231 with *agrC-IV V107S*	This work
pRN9243	pRN9231 with *agrC-IV I116S*	This work
pRN9244	pRN9231 with *agrC-I 100–116grIV*	This work
pRN9245	pRN9231 with *agrC-I Y100F,A101T*	This work
pRN9246	pRN9231 with *agrC-I A101T*	This work
pRN9247	pRN9231 with *agrC-I Y100F*	This work
pRN9248	pRN9231 with *agrC-I T104V, S107V,S116I*	This work
pRN9249	pRN9231 with *agrC-I S107C*	This work
pRN9250	pRN9231 with *agrC-I S116C*	This work
pRN9251	pRN9231 with *agrC-I S107A,S116A*	This work
pRN9252	pRN9231 with *agrC-I S107V,S116I*	This work
pJC1000	Group I *agr* locus cloned in pUC18	This work
pJC1111	Shuttle/suicide vector containing SaPI1 integration cassette	This work
pRN9253	pJC1111 carrying hemolytic reporter construct (P2-*agrB,D,A*; P3-RNAIII)	This work
pRN9254	pJC1111 carrying β-lactamase reporter construct (P2-*agrA*; P3-*blaZ*)	This work

single cognate receptor-ligand interaction results in activation; most heterologous interactions inhibit activation of the receptor, although a few are inert.[4] It is particularly remarkable that a wide variety of thiolactone peptides can inhibit any given receptor competitively, but only a single one can activate it (17).

In previous studies, we have constructed AgrC chimeras in which we have exchanged the N- and C-terminal sensor subdomains among the four *S. aureus* specificity groups and observed that ligand recognition specificity was largely determined by the C-terminal subdomain (18). In the present study we wished to localize the specificity determinant further and have chosen to examine the two most closely related groups, I and IV, which are weakly cross-reactive. Both AIPs are octapeptides, which differ by a single aminoacyl residue (Asp *versus* Tyr), and their cognate AgrC sensor domains differ by 27 aminoacyl residues (see Fig. 1). Four of these 27 are located in a central extracellular loop, and a fifth is adjacent to it (19). Using site-directed replacements, we have found that exchange of these five residues completely switches specificity. To analyze further which of these five were critical, we constructed several combinations in each of the two parental AgrCs. Replacement of Val^{107} and Ile^{116} (see Fig. 1) in AgrC-IV by the corresponding AgrC-I residues (both serines) caused AgrC-IV to be activated by AIP-I nearly as well as the wild type AgrC-I. Other combinations appeared to broaden the specificity so that the hybrid AgrC was activated equally well by both peptides; *e.g.* replacement of Phe^{100} and Thr^{101} in AgrC-I by the corresponding AgrC-IV residues, Tyr and Ala, respectively, had this effect. Other replacements appeared to reduce the response of AgrC to either peptide. In general, we observed that AgrC-I activation appears to depend on the bulkiness of the specificity-determining side chains in both receptor and ligand residues, suggesting that sterically determined interactions may play an important role in receptor activation.

[4] J. S. Wright and R. P. Novick, unpublished data.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

The *S. aureus* strains used in this study, listed in Table 1, are derivatives of NCTC8325. RN6734 is our standard laboratory strain and is *agr* group I (15); RN7206 is a derivative of RN6734 in which the *agr* locus has been replaced by *tetM* (20). *Escherichia coli* strain DH5α was used for cloning. All of the clones were first transformed into RN4220, our standard recipient for *E. coli* DNA, before transduction to other strains. *S. aureus* cells from overnight GL plates (21) containing the appropriate selective antibiotics (erythromycin 10 μg/ml or cadmium 0.1 mM) were used as inocula for all experiments. Subsequent growth in CYGP broth (21) without antibiotics was performed at 37 °C with shaking.

Plasmid and Reporter Construction

The plasmids used in this study (listed in Table 1) were prepared by cloning PCR products obtained from oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) as listed in supplemental Table S1. All of the clones were sequenced by the Skirball DNA Sequencing Core Facility. Plasmid pRN9231,

which was used as the backbone vector for pRN9232-9252, was constructed from pCN47 and contains the *agr*P2P3 intergenic region, the transcriptional terminator region from the *agr*P2 transcript, and restriction sites for insertion of AgrC mutants. The *agr*P2P3 intergenic region was cloned from the RN6734 genome and oriented such that promoter P2 drives expression of the cloned *agrC*. The *agr*P2 transcriptional terminator was also cloned from the RN6734 genome and inserted downstream of the *agrC* cloning site. Mutations in *agrC*-IV were introduced via primers that covered an endogenous AflIII site in the *agrC* gene; the amplified products were subcloned into pUC18, followed by insertion of the entire *agrC* mutant gene into pRN9231 via PstI and KpnI sites. Certain derivative mutants were subsequently constructed by using an internal SspI site to swap parts of the mutant or wild type sequence. Mutations in *agrC*-I were constructed in a similar fashion to that described for *agrC*-IV but employed either an engineered BsrGI site or an endogenous NsiI site instead of the AflIII site.

Two chromosomal *agr* locus derivatives were constructed from pJC1000, which contains the group I *agr* locus cloned from RN6734. The construct used in the SBA hemolysis assay was created by deleting *agrC* from pJC1000 via inverse PCR. The β-lactamase reporter construct was created by deleting *agrB* and *agrD* from the SBA hemolysis construct and replacing RNAIII with the *blaZ* gene, amplified from pCN41 with AvrII and SphI sites, thus producing an *agr*P3-*blaZ* fusion with *agrA* alone expressed from *agr*P2. The resulting constructs were moved into a suicide vector, pJC1111, which contains a cadmium resistance cassette and the SaPI1 integration cassette, creating plasmids pRN9253 and pRN9254.

Synthesis of AIPs

AIPs were synthesized via solid phase Boc-based chemistry as described (11) or by Fmoc-based chemistry, utilizing a new linker for Fmoc thioester synthesis that generates an ester at the C terminus of the linear peptide.[5] In this Fmoc-based method, cyclization was performed by a one-pot disulfide reduction, O to S acyl shift, and transthioesterification. All of the cyclized peptides were purified by reverse phase high pressure liquid chromatography and characterized by mass spectrometry and amino acid analysis (Keck Facility, Yale University) to validate peptide composition and ensure >95% purity.

Activation and Inhibition Assays

SBA Hemolysis Assay—Derivatives of strain RN10828 containing the plasmid-borne *agrC* mutants were subcultured into CYGP, normalized for cell density, and spotted on SBA plates, followed by overnight growth at 37 °C.

β-Lactamase Assay—Derivatives of strain RN10829 containing the plasmid-borne *agrC* mutants were grown to mid-exponential phase and transferred to microtiter plates. Synthetic peptides in 25% propylene glycol, 50 mM phosphate (pH 5.7) were added to cells at various concentrations followed by incubation with shaking at 37 °C for 60 min in a ThermoMax microplate reader (Molecular Devices) with monitoring of cell density at 650 nm. Assay of β-lactamase expression from the chromosomal P3-*blaZ* reporter fusion was performed by the nitrocefin method as described (22). Assay data were normalized to a percentage of maximal activation and plotted as initial β-lactamase reaction velocity *versus* log peptide concentration. Prism 4.0 (GraphPad, San Diego) was used to fit individual agonist or antagonist dose-response curves via nonlinear regression to the following four-parameter logistic equation,

$$E = \text{basal} + \frac{E_{max} - \text{basal}}{1 + 10^{\log EC_{50} - \log[A] n_H}} \quad \text{(Eq. 1)}$$

where E denotes effect, [A] denotes the agonist concentration, n_H denotes the midpoint slope, EC_{50} denotes the midpoint location parameter, and E_{max} and basal denote the upper and lower asymptotes, respectively. For inhibition curves, the midpoint location parameter from the above equation reflects the IC_{50}.

RESULTS

Localization of the Activation Specificity Determinant of AgrC-I Versus AgrC-IV—Building on information obtained from the analysis of intergroup chimeras, in which activation specificity for AgrC-I and -IV was localized to the C-terminal half of the sensor domain of the receptor, we analyzed the sequence variation between the two groups in this subdomain. Of the seven total amino acid residue differences, five are located in or adjacent to the second extracellular loop of the receptor (residue positions 100, 101, 104, 107, and 116; Fig. 1) and thus could logically contribute to ligand interaction. To determine whether group specificity localizes to this region, we replaced these five residues in AgrC-IV for those of AgrC-I via site-directed mutagenesis and tested the resulting mutant for its response to AIP-I, using two different cell-based assays of *agr* activity. The first involves a qualitative readout of the *agr*-dependent hemolytic activity of *S. aureus* tester strains on SBA plates. These strains express a mutant receptor cloned to a plasmid and contain the rest of the *agr* locus in the chromosome at the SaPI1 attachment site (23). Activation of AgrC by the endogenous AIP-I induces RNAIII leading to activation of hemolysin genes (*e.g. hla*; Fig. 2*A*). The second utilizes a tester strain containing chromosomal *agrA* and a β-lactamase reporter fused to the *agr*P3 promoter. Here, activation of AgrC by exogenously administered AIP induces β-lactamase production (Fig. 2*B*). *agr* groups I and IV are the most closely related, and the respective receptors exhibit weak cross-activation by the noncognate AIPs; because this cross-activation is much weaker when AgrC-IV is challenged with AIP-I than in the converse direction, we chose to focus our initial mutational analysis on AgrC-IV. As seen on SBA, expression of the constructed mutant receptor (AgrC-IV 100–116grI)[6] results in the appearance of full hemolytic activity in response to endogenously produced AIP-I, reflecting a striking increase in activity compared with wild type AgrC-IV (Fig. 2*C*, *spots 3* and *2*). When quantitated by β-lactamase reporter activity in response to synthetic AIP-I, this dramatic shift in specificity was represented as a

[5] George, E. A., Novick, R., and Muir, T. W. (2008) *J. Am. Chem. Soc.*, in press.

[6] 100–116grI refers to residue replacements in AgrC-IV by the corresponding residues of AgrC-I at positions 100, 101, 104, 107, and 116. 100–116grIV refers to the reciprocal substitutions in AgrC-I.

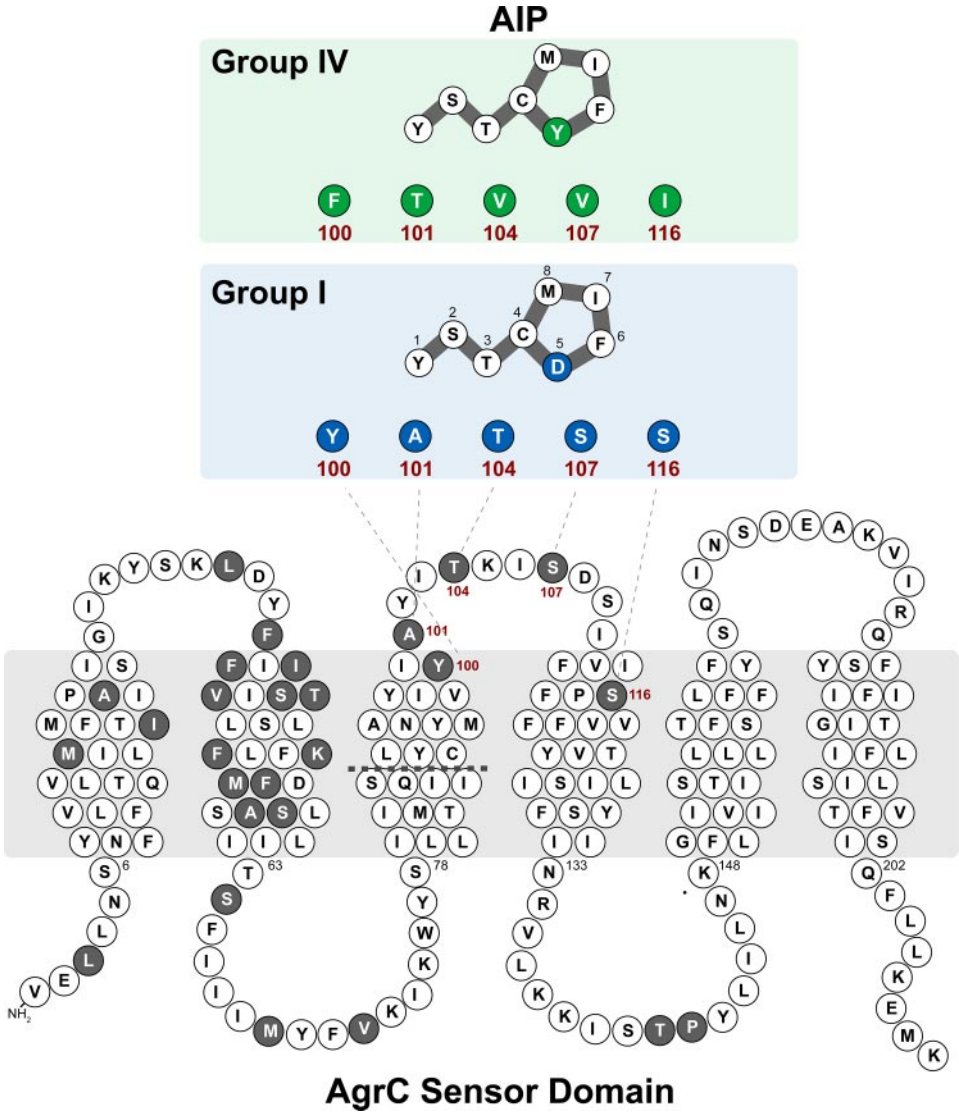

FIGURE 1. **AgrC transmembrane topology and sequence divergence between groups I and IV.** AgrC is composed of a group-specific, transmembrane sensor domain and a conserved, cytoplasmic transmitter domain. The predicted topology of the isolated AgrC-I sensor domain (19, 29) is shown, with residues divergent from AgrC-IV highlighted in *gray*. Analysis of intergroup chimeras constructed approximately at the *thick dotted line* demonstrated that the C-terminal half of the sensor domain mediates cognate specificity (18). The five divergent residues in this subdomain in or adjacent to the second extracellular loop were analyzed in this study; their numerical positions and identities in AgrC-I (*blue*) and AgrC-IV (*green*) are noted. Juxtaposed with the respective receptor residues are cartoon depictions of AIPs-I and -IV. The unique, fifth endocyclic residue on the AIPs that defines group specificity is colored.

sharp decrease in EC_{50} value compared with wild type AgrC-IV (1500 nM *versus* 26 nM, respectively (Fig. 2*D* and Table 2). This represents a level akin to that of AgrC-I (EC_{50} of 11 nM). When the corresponding five residues of AgrC-IV were substituted for those in AgrC-I, the resulting mutant (AgrC-I 100–116grIV) similarly demonstrated a full shift in specificity (Fig. 2*E* and Table 2). Activation specificity for AIP-I *versus* -IV thus localizes to the region of the second extracellular loop of AgrC.

Determinants of Specificity for AIP-I—To identify the minimal requirements for ligand specificity in this region, we divided the five critical residues into two groups, analyzing residues at position 100 and 101 as one set and those at positions 104, 107, and 116 as another set. This grouping was based on the overall characteristics of these residues in AgrC-I; the proximal set consists of hydrophobic residues (Tyr and Ala), whereas the distal set consists of polar residues (Thr, Ser, and Ser). We again employed site-directed mutagenesis to replace these residue subsets in AgrC-IV with the corresponding residues of AgrC-I. When amino acids of the distal subset (positions 104, 107, and 116) in AgrC-IV were substituted with those of AgrC-I, we found a dramatic increase in the activation of the mutated AgrC-IV (AgrC-IV V107S,I116S) by AIP-I (Fig. 2, *C* and *D*), as represented by a decrease in EC_{50} value from 1500 to 37 nM (Table 2). This change was accompanied by a decrease in activation by AIP-IV (EC_{50} from 16 to 45 nM), further suggesting a change in specificity. A similar result was obtained by replacing just two of these three (Val^{107} and Ile^{116}) with serines, with activation by AIP-I and AIP-IV represented by EC_{50} values of 50 and 56 nM, respectively. Note that the resulting EC_{50} values in both cases are very nearly equal for both AIPs (37 nM *versus* 45 nM and 50 nM *versus* 56 nM, respectively; see Table 2). We next constructed single amino acid replacements involving the three distal residues, at positions 104, 107, and 116, respectively. Activation tests of these, as listed in Table 2, showed that the replacement of isoleucine 116 with serine sharply decreased sensitivity to AIP-IV and increased sensitivity to AIP I correspondingly. The other two single replacements had little if any effect, suggesting that residue 116 could be an important determinant of receptor specificity. This was supported by a comparison of AgrC-IV V104T,V107S, I116S (five-residue configuration: $F_{IV}T_{IV}T_IS_IS_I$) with AgrC-IV V104T,V107S ($F_{IV}T_{IV}T_IS_II_{IV}$); the former mutant showed a dramatic increase in activation by AIP-I and a decrease in activation by AIP-IV compared with the latter. Finally, we constructed an AgrC-IV mutant with AgrC-I replacement of the proximal residue subset (at positions 100 and 101). This mutation did not significantly change activation by AIP-I but greatly decreased activation by AIP-IV (Table 2), suggesting that Tyr^{100} and Ala^{101} are not important for AIP-I recognition but may play a role in the response to AIP-IV, as discussed below.

Determinants of Specificity for AIP-IV—We next constructed and analyzed reciprocal replacements in AgrC-I. Surprisingly, when the distal three aminoacyl residues that conferred activa-

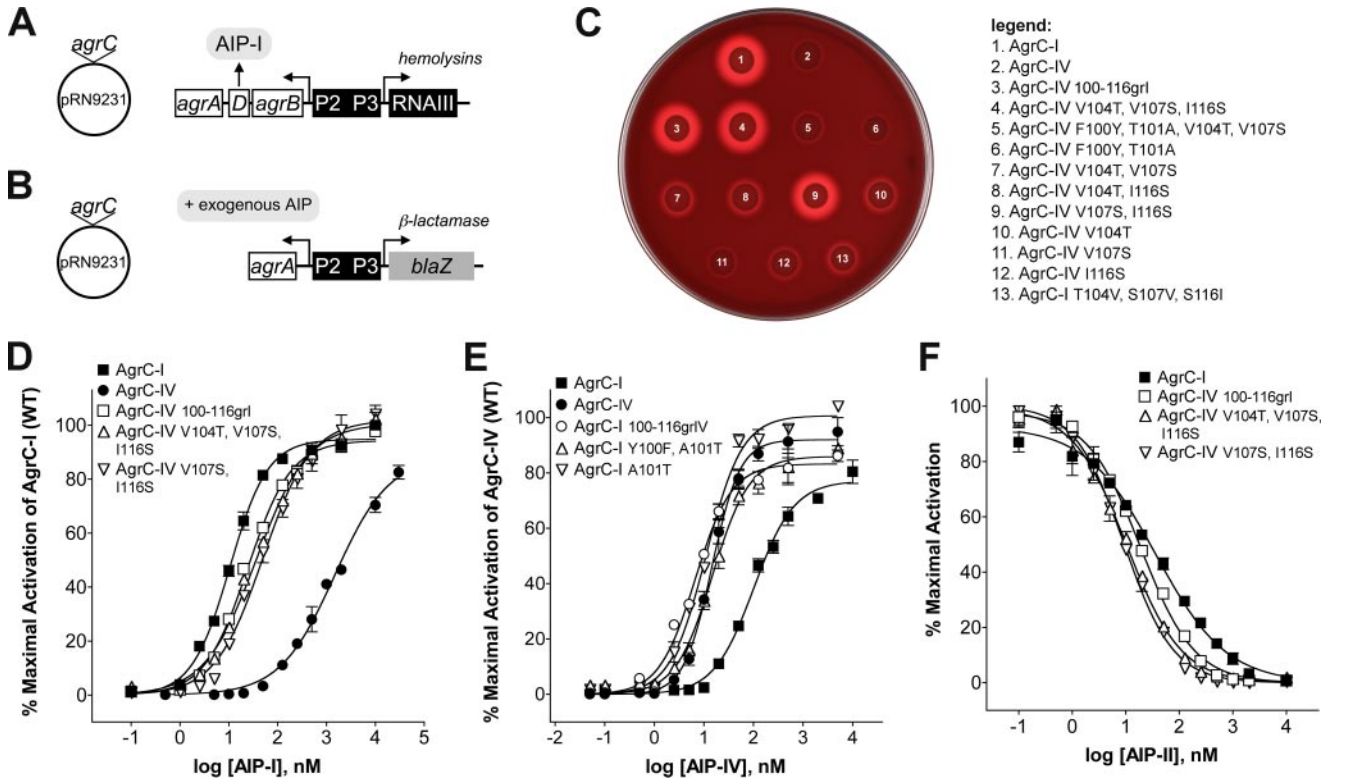

FIGURE 2. **Activation and inhibition analysis of AgrC site mutants.** *A* and *B*, constructs used for the SBA hemolysis and β-lactamase reporter assays. In both assays, AgrC is expressed from derivatives of plasmid pRN9231. In the SBA hemolysis assay, *S. aureus* cells contain a chromosomal group-I *agr* construct deleted for *agrC*, represented in *A*, and produce AIP-I endogenously; AgrC activation leads to downstream activation of RNAIII-dependent hemolysin genes. In the β-lactamase reporter assay, synthetic AIP is added to cells harboring a chromosomal construct encoding *agrA* and a P3-*blaZ* fusion, diagrammed in *B*. *C*, hemolytic activity on SBA plate. Zones of clearance surrounding the spots represent hemolysin activity as a consequence of receptor activation by AIP-I. *D–F*, β-lactamase dose-response curves. *D* and *E* represent dose-dependent activation of wild type and mutant AgrC receptors by AIP-I and AIP-IV, respectively. *F* represents dose-dependent inhibition of wild type AgrC-I and AgrC-IV mutants by AIP-II in the presence of a constant concentration (125 nM) of AIP-I. The data are shown as a percentage of maximal activation at each concentration ± S.E.

tion specificity to AIP-I were substituted by the corresponding residues of AgrC-IV, the resulting mutant (AgrC-I T104V,S107V, S116I) did not exhibit an increase in specificity for AIP-IV (Table 2). In fact, this residue configuration ($Y_IA_IV_{IV}V_{IV}I_{IV}$), in both receptor backgrounds (AgrC-I T104V,S107V,S116I and AgrC-IV F100Y,T101A), generally led to greatly reduced receptor sensitivity to either AIP. On the other hand, when either or both of the proximal residues at positions 100 and 101 in AgrC-I were replaced with the corresponding AgrC-IV residues, an increase in activation by AIP-IV was observed in the resulting mutants (EC_{50} values from 100 to 17, 11, or 15 nM; see Table 2 and Fig. 2*E*), suggesting that these two residues were important for recognition of AIP-IV. It is noted, however, that the resulting EC_{50} values for AIP-I were unaffected. An alternative possibility, therefore, is that rather than switching specificities, the amino acid replacements at positions 100 and 101 broadened receptor specificity, causing equal sensitivity to the two AIPs.

Effect of Activation Specificity Mutations upon Response to AIP-II—Although the exchange of residues between AgrC-I and AgrC-IV generally caused an increase in activation by the corresponding noncognate AIP with a concomitant decrease in activation by the original cognate ligand, some replacement mutants retained the ability to be activated by the original cognate AIP. Where a shift in activation specificity was observed, the change in specificity was expected to be limited to these two groups. It was possible, however, that increased sensitivity to a closely related AIP in either case may extend to more distantly related peptides, allowing activation by a normally inhibitory peptide. To address this issue, we tested the entire set of mutants for activation by AIP-II, a strong antagonist of both AgrC-I and IV (10). None of the mutants could be activated by AIP-II at any concentration tested (up to at least 10 μM; Table 2). Importantly, this result demonstrates that the broadened specificity, proposed above, of the AgrC-I mutants containing proximal subset AgrC-IV replacements does not extend to the distantly related peptide, AIP-II.

Our general hypothesis regarding the AgrC-AIP interaction contends that activation requires a specific contact between ligand and receptor and that the absence of such a contact results in competitive inhibition. Accordingly, modification of receptor residues that participate in the putative activating contact should not affect the ability of the receptor to be inhibited by antagonist peptides, so long as the substitutions do not affect general peptide binding. To test this idea, we treated those mutant receptors that demonstrated the most dramatic shift in activation specificity (AgrC-IV 100–116grI; AgrC-IV V104T, V107S,I116S; and AgrC-IV V107S,I116S) with a constant dose of AIP-I while challenging them with increasing concentrations of AIP-II. The resulting inhibition curves and IC_{50} values (Fig. 2*F* and Table 2) demonstrate that mutations in the specificity region of AgrC-IV do not affect the inhibition of this receptor

TABLE 2
Response of AgrC-I and -IV receptor mutants to various AIPs

	AIP-I	AIP-IV	AIP-II	AIP-I/IV 5N	AIP-I/IV 5L
Activation (EC_{50}, nM)					
AgrC-I	11 (9–12)[a]	100 (86–130)	—[b]	85 (61–120)	20 (15–28)
AgrC-IV	1500 (1000–2100)	16 (13–20)	—	—	—
AgrC-IV 100–116grI	25 (22–28)	140 (130–160)	—	180 (160–200)	290 (170–490)
AgrC-IV V104T,V107S,I116S	37 (31–43)	45 (38–55)	—	130 (110–160)	19 (17–22)
AgrC-IV V107S,I116S	50 (40–63)	56 (45–71)	—	150 (130–170)	29 (23–36)
AgrC-IV V104T,I116S	240 (180–330)	68 (56–83)	—	620 (520–730)	120 (100–150)
AgrC-IV I116S	320 (270–370)	94 (76–120)	—	390 (330–470)	110 (89–130)
AgrC-IV V104T,V107S	600 (480–750)	19 (15–24)	—	460 (350–600)	480 (280–850)
AgrC-IV F100Y,T101A,V104T,V107S	700 (590–840)	390 (330–460)	—	1600 (1300–2000)	—
AgrC-IV V104T	790 (660–940)	19 (16–22)	—	2300 (1400–3900)	1300 (920–1700)
AgrC-IV F100Y,T101A	1100 (1000–1300)	620 (560–670)	—	—	
AgrC-IV V107S	1300 (1000–1600)	22 (19–26)	—	440 (320–600)	
AgrC-I 100–116grIV	44 (34–58)	7 (6–8)	—		
AgrC-I Y100F,A101T	8 (7–9)	15 (12–18)	—		
AgrC-I A101T	11 (10–12)	11 (10–13)	—		
AgrC-I Y100F	8 (7–10)	17 (14–19)	—		
AgrC-I T104V,S107V,S116I	520 (440–620)	290 (250–330)	—		
AgrC-I S107C	41 (36–47)	500 (470–530)	—		
AgrC-I S116C	62 (51–75)	470 (410–530)	—		
AgrC-I S107AS116A	53 (44–64)	45 (32–63)	—		
AgrC-I S107V,S116I	450 (330–610)	100 (85–120)	—		
Inhibition (IC_{50}, nM)					
AgrC-I	—	—	37 (27–50)	—	—
AgrC-IV	—	—	52 (43–63)	83 (72–96)	24 (16–36)
AgrC-IV 100–116grI	—	—	19 (17–21)	—	—
AgrC-IV V104T,V107S,I116S	—	—	10 (7–13)	—	—
AgrC-IV V107S,I116S	—	—	9 (8–10)	—	—

[a] The parentheses indicate 95% confidence intervals.
[b] —, no activation observed at any concentration up to at least 10 μM/no inhibition observed because peptide is activator.

by the antagonist AIP-II and therefore imply that general binding of peptides to the receptor is unaffected.

Probing the Specificity-determining Residues of AgrC-I and AIP-I/IV—As mentioned above, the AgrC residues that were shown above to determine specificity of activation by AIP-I display interesting differences in overall polarity between groups I and IV: in AgrC-I, these residues (Thr^{104}, Ser^{107}, and Ser^{116}) are hydrophilic, whereas those in AgrC-IV (Val^{104}, Val^{107}, and Ile^{116}) are hydrophobic. This polarity pattern in the receptor appears to match the polarity of the unique, endocyclic residue that defines group specificity in the respective cognate AIPs: an aspartate in AIP-I and a tyrosine in AIP-IV (Fig. 1). Previous work done in our laboratory revealed that conservative replacement of the unique AIP residue, asparagine for the group I aspartic acid (AIP-I/IV 5N) and phenylalanine for the group IV tyrosine (AIP-I/IV 5F), did not affect the activity of these peptides toward their cognate receptors (11). These findings suggest that group I-specific activation may occur through a hydrophilic interaction, whereas group IV-specific activation may involve a hydrophobic interaction. To test this idea, we analyzed variants of AIP-I and -IV that contained polar or hydrophobic modifications at the critical, specificity-determining fifth residue position for activation of the wild type and mutant receptors.

The first peptide, containing an asparagine (AIP-I/IV 5N), moderately activated AgrC-I but did not activate AgrC-IV (Fig. 3*A* and Table 2), as observed previously (11). However, the AgrC-IV mutants, most notably those in which residues at positions 104, 107, and/or 116 were switched to those of group I, were able to respond to this peptide, reaffirming the importance of these residues in determining activation specificity. The second peptide we tested was an AIP-I/IV variant containing a leucine at the fifth position. As shown in Fig. 3*B* and Table 2, this peptide, AIP-I/IV 5L, unexpectedly had strong activity toward AgrC-I (EC_{50} 20 nM) but did not detectably activate AgrC-IV; instead, it was a potent AgrC-IV inhibitor (Table 2). This peptide activated most AgrC-IV-I replacement mutants, as did AIP-I/IV 5N. These results thus illustrate that the activating interaction between AIP-I and AgrC-I cannot be explained on the basis of polarity.

An alternative hypothesis is that the structural organization of the ligand specificity region may govern the ability of the receptor to accommodate various AIPs and that this could be responsible for ligand selectivity. To test this idea in the context of *agr* group I, we introduced changes to AgrC-I that focused on the side chain bulkiness of the two critical serine residues in the ligand specificity region of this receptor and analyzed the resulting effect on activation by AIP-I. According to the above hypothesis, mutations that decrease polarity but do not affect the size of the substituted residue should not affect specificity. First, individual mutations in AgrC-I Ser^{107} and Ser^{116} that convert these residues to cysteine, effectively changing one atom (the side chain hydroxyl to a sulfhydryl), each caused a moderate (4–5-fold) decrease in response to AIP-I (Fig. 3*C* and Table 2). Next, an AgrC-I derivative was constructed in which both serines were simultaneously replaced with alanines (AgrC-I S107A,S116A). When tested for activation by AIP-I, a similar, modest shift in activity was observed (Fig. 3*D* and Table 2). Thus the sterically similar aliphatic side chain of alanine appears to substitute adequately for the polar hydroxyl groups of the critical serines, in agreement with the conclusion that elimination of polarity does not greatly interfere with activation. When serines 107 and 116 were replaced with the bulkier group IV residues (valine and isoleucine), however, the resulting mutant demonstrated a substantially larger, 40-fold decrease in activation by AIP-I (Fig. 3*D* and Table 2), consistent with the hypothesis that the bulkiness of the critical receptor

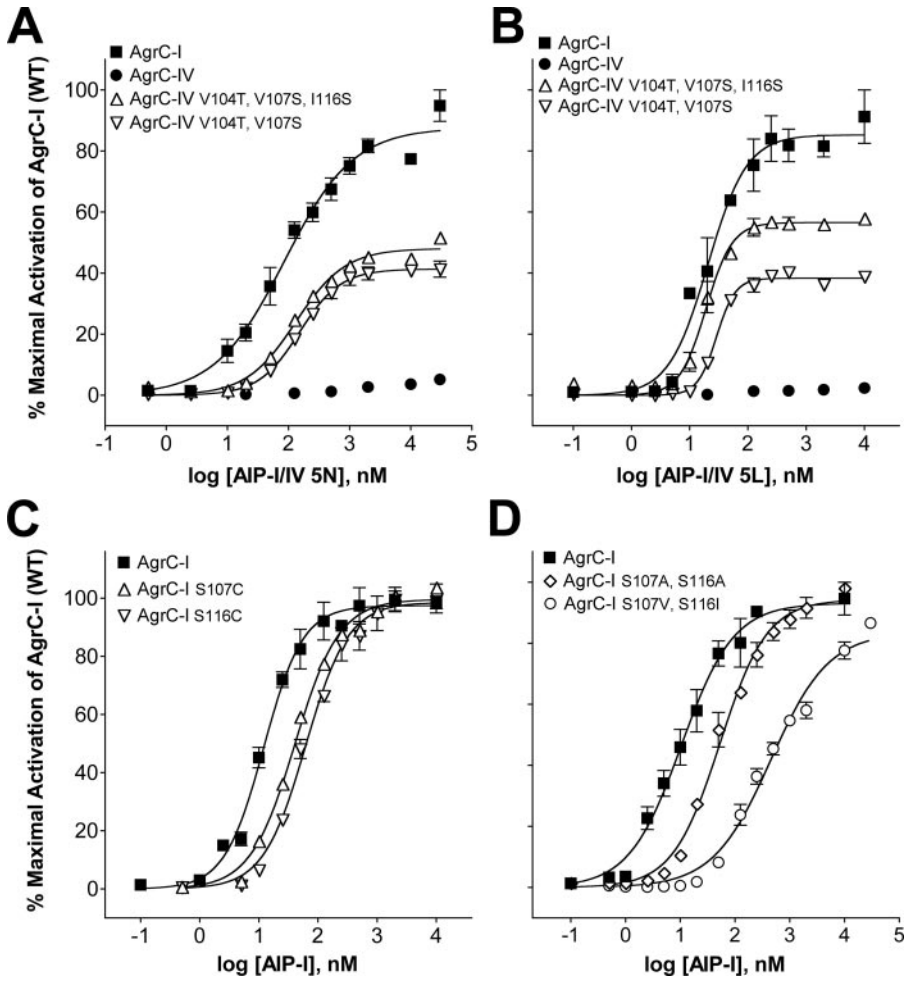

FIGURE 3. **Probing the group I/IV AgrC and AIP specificity regions.** *A* and *B*, β-lactamase reporter cells expressing wild type AgrC or AgrC-IV mutants were incubated with increasing concentrations of the indicated AIP. *C* and *D*, β-lactamase reporter cells expressing wild type or mutant AgrC-I were incubated with varying concentrations of AIP-I. The data are shown as percentage of maximal activation at each concentration ± S.E.

residue side chains dictates ligand selectivity through steric interactions.

DISCUSSION

In this study, we have localized the determinant of activation specificity for *agr* groups I and IV to the four divergent amino acid residues in a central extracellular loop of the polytopic transmembrane *agr* receptor, AgrC, plus a fifth divergent residue adjacent to this loop. Exchange of these five residues between the two receptors caused a total switch in the specificity of activation by the corresponding AIPs. Initial experiments suggested that the three amino acids at positions 104, 107, and 116 were responsible for recognition by AgrC-I of AIP-I, because switching these three AgrC residues between the two receptors also switched activation specificities. These three residues, a threonine and two serines, are polar, whereas the corresponding residues in AgrC-IV, two valines and an isoleucine, are hydrophobic, and the only difference between the AIPs is an aspartate (polar) in AIP-I as opposed to a tyrosine (hydrophobic) in AIP-IV. Thus we hypothesized that activation specificity involved a hydrophilic (for group I) or hydrophobic (for group IV) interaction. This hypothesis, however, was not supported by additional experimental data; an AIP-I/IV variant, 5L, whose specificity determinant was replaced with the unrelated hydrophobic residue leucine, strongly activated AgrC-I but not AgrC-IV. An alternative possibility is that sterically determined interactions dictate ligand specificity in this system, and this idea may be supported, at least for group I, by our finding that the AgrC-I S107A,S116A double mutant demonstrated only moderately reduced activation by AIP-I when compared with the wild type receptor. A similar result can be found in a study of the mammalian μ-opioid receptor, a polytopic G protein-coupled receptor that also senses a peptide ligand (24). When an amino acid near its third extracellular loop, tryptophan, was replaced with the corresponding residue of the δ-opioid receptor (leucine) or a charged residue (lysine), a switch in specificity toward δ-opioids was observed, suggesting that the interaction between specificity determinants on ligand and receptor did not depend on polarity and instead involved steric hindrance. It seems likely, therefore, that the chemical and steric properties of the various residue side chains on peptide ligands and receptors make different relative contributions to the overall conformation of the activated peptide-receptor complex.

We also observed that replacement of AgrC-I residues Tyr^{100} and/or Ala^{101} by the corresponding AgrC-IV residues Phe^{100} and/or Thr^{101} considerably increased the activation of AgrC-I by AIP-IV, suggesting that these two residues were important for AIP-IV recognition and therefore that the two AIPs might bind to different sites on the receptor. However, it was also observed that these two replacements did not affect activation of either receptor by AIP-I; indeed, both receptors with Phe^{100} or Tyr^{101} were equally sensitive to either of the AIPs, suggesting that these two sites had more to do with the overall breadth of AIP recognition. We have proposed elsewhere that the *agr* groups evolved from a common ancestor and that this evolution must have been concerted in that matching interactions between AgrC and AIP and that AIP processing specificity had to be maintained (25). This implies that the first step toward evolutionary divergence would have to involve broadening of specificity. Because *agr* groups I and IV are the most closely related and because *agr*-I is vastly more common than *agr*-IV (26, 27), it seems likely that *agr*-IV is an evolutionary offshoot of *agr*-I

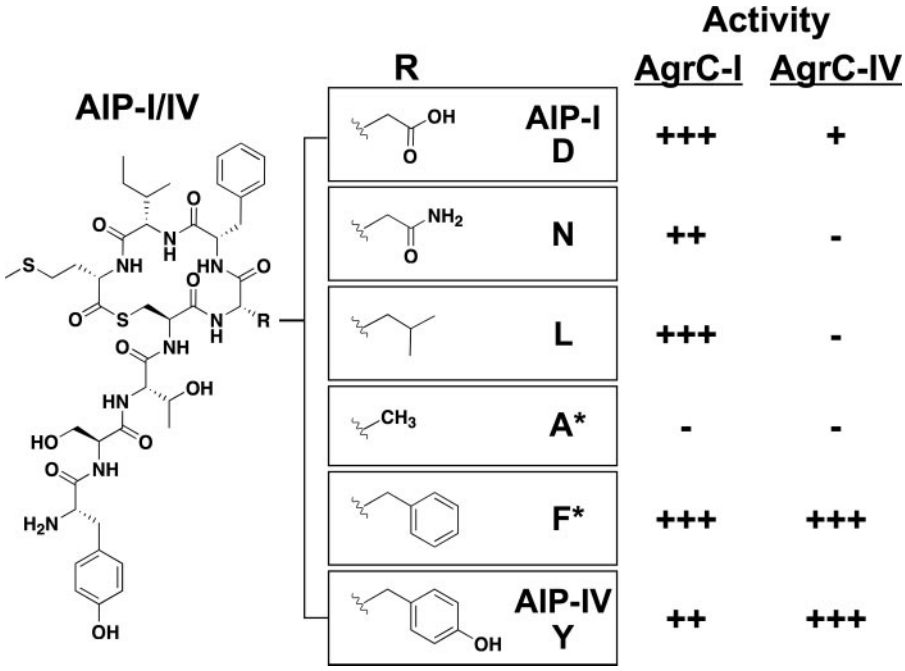

FIGURE 4. **Summary of effects of AIP-I/IV specificity determinant modifications on AgrC-I and -IV activation.** The chemical structure of AIP-I/IV is shown, with an *R* representing the site of replacement at the unique, fifth position. The side chain substitutions are listed with the corresponding activities with the wild type AgrC-I and -IV receptors. *Asterisks* denote substitutions not reported in this work but tested previously (11).

and that their divergence could well have involved mutations affecting AgrC residues 100 and 101.

A considerable number of AIP-I/IV variants were tested in this (Fig. 4) and an earlier study (11). These results together suggest that in general AgrC-I exhibits a lower selectivity for ligands than does AgrC-IV. The specificity-determining receptor residues of AgrC-I (Thr, Ser, and Ser) are generally less bulky than the corresponding AgrC-IV residues (Val, Val, and Ile), and if receptor activation were driven by a steric interaction, the former combination would probably accommodate a larger range of side chain sizes in the peptide ligand than the latter. It is noted, however, that for AIPs I and IV, an alanine substitution at the specificity-determining position is not sufficient for activity on either receptor, because the resulting peptide is a general AgrC inhibitor (11); thus a larger side chain appears to be required. Another possibility that could account for the higher selectivity of AgrC-IV is that its interaction with the cognate AIP involves specific benzene stacking. Maximal AgrC-IV activation is seen with an AIP containing an aromatic side chain at the fifth position; the native residue is tyrosine, and a derivative with phenylalanine at this position has precisely the same activity (11), and an important specificity-determinant on AgrC-IV is a phenylalanine. We of course do not rule out the possibility that other mechanisms are involved in this and other AIP-receptor interactions. Structural analysis of the AIP-AgrC complex would ultimately elucidate the particular mechanism used for receptor activation by the cognate AIP in each case.

Although it is implicit in the foregoing discussion that the five critical AgrC residues represent actual AIP interaction sites, there is no direct evidence for this, and it is therefore possible that they act indirectly, *e.g.* by influencing the conformation or presentation of direct contact residues elsewhere on the receptor. Indeed, previous results have suggested that other sites in the receptor have a role in ligand recognition/binding, and this is supported by the results presented here with the AIP I/IV 5L derivative, which strongly activates AgrC-I but activates the AgrC-IV derivative containing the five critical AgrC-I residues quite weakly (Table 2). The cysteine-substituted AgrC-I mutants may help to distinguish between a direct or indirect role of the amino acid residues at those critical sites and to probe the ligand-receptor interaction further. Experiments to label the thiols introduced by these mutations are in progress.

In perspective, the relatedness of the receptors we chose to analyze facilitated the precise identification of determinants of activation specificity. It is likely that many geographically scattered motifs on a polytopic sensor protein such as AgrC or G protein-coupled receptors contribute to the differential responsiveness to peptide ligands (28) and for relatively unrelated receptors, substitution of one or two single residues would be unlikely to cause a full switch in receptor specificity. This is likely to be the case for other *agr* specificity pairings involving groups II and III, in which both AgrC and AIP display considerably more sequence divergence than do the closely related groups I and IV. Identifying specificity determinants in these divergent receptors would require the use of more powerful approaches, such as structural analysis or random mutagenesis and genetic selection, and experiments in these directions are currently in progress.

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