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Ligand-Receptor Recognition for Activation of Quorum Sensing in *Staphylococcus aureus*

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The accessory gene regulator (*agr*) locus controls many of the virulence toxins involved in *Staphylococcus aureus* pathogenesis, and can be divided into four specificity groups. AgrC is the only group-specific receptor to mediate both intra-group activation and inter-group inhibition. We studied the ligand-receptor recognition of the *agr* system in depth by using a luciferase reporter system to identify the key residues responsible for AgrC activation in two closely related *agr* groups, AgrC-I, and AgrC-IV. Fusion PCR and site-directed mutagenesis were used to screen for functional residues of AgrC. Our data suggest that for AgrC-IV activation, residue 101 is critical for activating the receptor. In contrast, the key residues for the activation of AgrC-I are located at residues 49~59, 107, and 116. However, three residue changes, T101A, V107S, I116S, are sufficient to convert the AIP recognizing specificity from AgrC-IV to AgrC-I.

***Keywords*: histidine protein kinases, ligands, protein structure, quorum sensing, transmembrane receptors, two-component system**

Two-component systems, consisting of a sensor histidine protein kinase and a response regulator, are perhaps the most widely used system by bacteria to sense environmental signals (Koretke *et al.*, 2000). Histidine protein kinases are potential targets for anti-infective therapy because they are not found in animals and they are less likely to induce resistance than antibiotics (Wolanin *et al.*, 2002). In Gram-positive bacteria, peptides are used as a signal ligands in some two-component signaling systems. These peptides are detected by transmembrane receptors (Lyon and Novick, 2004). However, the mechanism of ligand-receptor recognition is still unclear.

Most staphylococcal virulence toxins are controlled by the accessory gene regulator (*agr*) locus (Recsei *et al.*, 1986; Morfeldt *et al.*, 1988), which belongs to a quorum sensing system and is responsible for cell density via autoinducing peptide (AIP) signal molecules (Ji *et al.*, 1995). The *agr* locus consists of two divergent operons driven by the P2 and P3 promoters (Peng *et al.*, 1988; Janzon *et al.*, 1989). The P2 operon encodes AgrB and AgrD, the proteins essential for AIP production, and AgrC and AgrA, a two-component signaling system for sensing AIP signal molecules (Ji *et al.*, 1995; Novick *et al.*, 1995). AgrC, the histidine protein kinase, is autophosphorylated on a histidine residue only in response to AIP (Lina *et al.*, 1998). AgrC then transfers a phosphate group to an aspartate residue on the response regulator, AgrA. Phosphorylated AgrA binds to both the P2 and P3 promoter elements to activate transcription, resulting in an autoinduction feedback loop for the amplification of AIP production and the initiation of RNA III expression at critical AIP levels (Ji *et al.*, 1995; Otto, 2001; Koenig *et al.*, 2004). RNA III is a transcript of the P3 operon that up-regulates the expression of most extracellular toxins and down-regulates the surface protein genes in *Staphylococcus aureus* (Janzon and Arvidson, 1990; Novick *et al.*, 1993).

S. aureus strains are divided into four *agr* groups based their ability to cross-activate or inhibit *agr* expression (Ji *et al.*, 1997; Jarraud *et al.*, 2000; McDowell *et al.*, 2001). AIP activation of its cognate AgrC is highly sequence specific. AgrC is a transmembrane receptor composed of an extracellular sensing domain and a cytoplasmic signaling domain. The N-terminal region of AgrC contains the agonist AIP binding site and is the determinant of group specificity (Lina *et al.*, 1998; Lyon *et al.*, 2000). In addition, intra-domain chimeras of *agr* groups I and IV AgrC (AgrC-I and AgrC-IV) are functional, with the distal sensor domain of the receptor playing a primary role in responding to cognate AIP (Wright *et al.*, 2004). The *agr* groups I and IV are a good model to study ligand-receptor recognition in two-component systems because their AIPs (AIP-I and AIP-IV) differ only in residue 5 within the thiolactone ring, and their cognate AgrC receptors share 87% sequence identity with highly conserved transmembrane topology (Wright *et al.*, 2004).

In an earlier study, we showed that variations in the second extracellular loop of AgrC is sufficient to modify AIP recognition specificity (Chen *et al.*, 2004). This result was confirmed by two recently published papers (Geisinger *et al.*, 2008; Jensen *et al.*, 2008) and our present study. In addition, Geisinger *et al.* (2009) identified a single amino acid (I171) at third extracellular loop of AgrC-I. This study showed that I171 mutant plays a critical role in ligand-mediated inhibition with dramatically broadened activation specificity and reduced sensitivity to inhibition. However, the role of

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individual residues on AgrC activation is still unclear. In the present study, we used site-directed mutagenesis and fusion PCR to systematically exchange receptor amino acids between AgrC-I and AgrC-IV to construct a series of AgrC chimerical and mutant reporter strains. These reporter strains allowed us to determine the key residues involved in AgrC activation. Our data showed that three residue changes, T101A, V107S, I116S, are sufficient to convert the AIP recognizing specificity from AgrC-IV to AgrC-I.

Materials and Methods

Bacterial strains

The bacterial strains, plasmids, and part of the primers used are listed in Table 1. Unless stated otherwise, LB broth and plates were used for growth of *Escherichia coli* and *S. aureus* at 37°C. *E. coli* strain XL10-Gold (Stratagene, USA) was used for cloning. *S. aureus* cells were transformed by electroporation, as described previously (Schenk and Laddaga, 1992). Ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) were used for plasmid selection in *E. coli* and *S. aureus*, respectively.

Construction of luciferase reporter of AgrC

A modified *luxABCDE* operon, optimized for expression in Gram-positive bacteria, was excised from pSB2035 by *Sal*I-*Pvu*II and cloned into pSK5630, a stable low-copy-number shuttle vector, to generate a luciferase reporter system, pFJC0401 (Qazi *et al.*, 2001; Grkovic *et al.*, 2003). For convenience, the *Bam*HI site located between *luxDE* was removed from the modified *lux* operon by digesting with *Bam*HI, blunting with Klenow, and religating. Subsequent transcriptional fusions to the luciferase reporter gene were constructed by cloning the PCR fragment of *agr* P2P3 promoter region from chromosomal DNA of RN6390B into the *Bam*HI-*Sal*I sites of pFJC0401 using primers P2-F and P2-R to produce pP3*lux* reporter plasmid, pFJC0402. Because the *agrA* gene is conserved among various *agr* groups, an *agrA* fragment was cloned as the vector backbone sequence. For cloning of the *agrA* fragment, a *Kpn*I site located between *luxBC* of pFJC0402 was removed by digesting with *Kpn*I, blunting with T4 DNA polymerase, then religating to gene-

Table 1. Bacterial strains, plasmids, and primers

Strain/Plasmid/Primer	Description	Reference
Strains		
E. coli		
XL10-Gold	Ultra-competent cell for site-directed mutagenesis	Stratagene
S. aureus		
RN4220	Restriction-deficient derivative of 8325-4	Novick (1991)
RN6390B	Group I prototype	Novick (1991)
RN6911	*agr*-null derivative of RN6390B	Novick *et al.* (1993)
TC21	Group IV prototype	This study
Plasmids		
pUC18	Cloning vector for site-directed mutagenesis, Ap^r	Norrander *et al.* (1983)
pSB2035	*luxABCDE* with modification for high expression in gram-positive, Ap^r, Cm^r	Qazi *et al.* (2001)
pSK5630	Stable low-copy-number shuttle vector, Ap^r, Cm^r	Grkovic *et al.* (2003)
pFJC0401	*luxABCDE* operon excised from pSB2035 and inserted into *Sal*I-*Pvu*II sites of pSK5630	This study
pFJC0402	*agr* P2P3 promoter fragment cloned into the *Bam*HI-*Sal*I sites of pFJC0401	This study
pFJC0501	Removal *Kpn*I site of pFJC0402	This study
pFJC0601	*agrA* gene fragment cloned into the *Bam*HI-*Sma*I sites of pFJC0501 and introduced a *Kpn*I site	This study
Primers[a]		
P2-F (*Sal*I)	5'-CGCGTCGACTCACATCTCTGTGATCTAGT	
P2-R (*Bam*HI)	5'-CGGGATCCACCACTCTCCTCACTGTCAT	
agrA-F (*Bam*HI, *Kpn*I)	5'-CGGGATCCTAGGTACCATAAGGATGTGAATGT**ATG**	
CA-R (*Sma*I)	5'-TCCCCCGGGACAACGAAGTATGGTGCTAT	
g1C-F (*Bam*HI)	5'-CGGGATCC**GTG**GAATTATTAAATAGTTA	
g4C-F (*Bam*HI)	5'-CGGGATCC**GTG**GAATCATTAAATAGTTA	
agrC-R (*Kpn*I)	5'-GGGGTACCTAGTTGTTAATAATTTCAAC	
luxA-QF	5'-GTGGCGTGACTTTGTAT	
luxA-QR	5'-AGAAACGGCATGACATC	
gyrB-QF	5'-CTGATGCCGATGTGGA	
gyrB-QR	5'-GGTGCTCAGGGTTCATT	

[a] The underlined nucleotides were restriction enzyme sites, and the initiation codon of *agrA* or *agrC* were in bold

rate pFJC0501. The *agrA* fragment was amplified by PCR from chromosomal DNA of RN6390B using primers agrA-F and CA-R, and ligated into the *Bam*HI-*Sma*I sites of pFJC 0501, which was introduced into a *Kpn*I site neighboring the *Bam*HI site. This plasmid, pFJC0601, was used as the vector for the expression of the AgrC and AgrC mutant receptors in an *agr*-null strain, RN6911. Two *agrC* genes were amplified using PCR from chromosomal DNA of prototypical *agr* groups I and IV strains, RN6390B and TC21, respectively, using the forward primers g1C-F and g4C-F, and the conserved reverse primer agrC-R. They were then cloned into the *Bam*HI-*Kpn*I sites of pFJC0601 to produce AgrC-I and AgrC-IV reporters. In the resultant constructs, the *luxABCDE* operon was driven by *agr* P3 promoter and the *agrCA* genes were driven by *agr* P2 promoter. Construction of plasmids using PCR and removal of restriction enzyme sites were verified by restriction analysis and DNA sequencing. Sequencing was performed at the DNA Sequencing Core Lab of our institutes. The pFJC0601 derivatives were electroporated into *S. aureus* strain RN4220, and then electroporated into RN6911.

Construction of AgrC mutants

The N-terminal sensor domain was hybridized between groups I and IV *agrC* by fusing the AgrC N-terminus of one group to the AgrC C-terminus of another group. Fusion sites in AgrC were chosen within inside loop regions between the computationally predicted transmembrane helices. The two fragments were PCR amplified individually from corresponding chromosomal DNA templates, the homologous sequence at the fusion site was used as an internal primer for the subsequent fusion PCR step, and the resulting PCR fusion product was amplified with a pair of external *agrC* primers. Blunt end ligations were performed to fuse non-homologous regions between two groups of *agrC*. Two fragments were amplified by PCR using phosphorylated primer as the internal primer and equal amounts of N-terminal and C-terminal encoding PCR fragments were blunt end ligated with T4 DNA ligase. Complete *agrC* was amplified from the ligation mixture by PCR with external primers. The resulting full *agrC* chimera PCR fragments were then cloned into the *Bam*HI-*Kpn*I sites of pFJC0601.

Site-directed mutagenesis was performed to exchange the amino acid residues of AgrC between AgrC-I and AgrC-IV. The *agrC* genes of *agr* group I and IV were excised from AgrC-I and AgrC-IV reporters, respectively, by *Bam*HI-*Kpn*I and then subcloned into pUC18 to generate the templates for site-directed mutagenesis. The mutations were carried out using the QuickChange Site-directed Mutagenesis kit (Stratagene). The resulting mutations were then cloned back into pFJC0601 using *Bam*HI-*Kpn*I sites.

Preparation of culture supernatants

S. aureus strains were grown overnight at 37°C with shaking at 200 rpm in 3 ml of CYGP broth (Novick, 1991). Cells were centrifuged at 2,880×g at 4°C for 10 min. The culture supernatants were filtered (0.22 μm filter, Corning) then boiled for 10 min, and the filtrate was stored at -80°C. Synthetic peptides were provided by Prof. Paul Williams (University of Nottingham, UK).

Activation assays

Assays for exogenous AIP (crude culture supernatants and synthetic peptides) activation were performed by bioluminescence on the TopCount NXT system (Perkin-Elmer Biosystems, USA). Strain RN6911 harboring AgrC reporters were grown overnight with shaking at 37°C in CYGP broth and diluted with fresh medium to 1/100. They were grown for an additional 2 h, then 190 μl of reporter cells were added to 96-well plates in duplicate, followed by 10 μl AIPs, and incubated at 37°C. Both the cell density at 595 nm and the bioluminescence were measured after 90 min. The half maximal effective concentration (EC_{50}) values were calculated by fitting the data to a sigmoidal dose-response (variable slope) curve using the Prism 4 (GraphPad Software, USA).

RNA isolation and real-time quantitative PCR analysis

AgrC-I reporters were grown overnight with shaking at 37°C in CYGP broth and diluted with 5 ml fresh medium to 1/100. They were grown for an additional 2 h, then a 5% volume of various concentrations of AIP-I were added and shaken at 200 rpm at 37°C for 90 min. Both the cell density and the bioluminescence were measured in 96-well plates and approximately 2×10^9 cells were harvested at the same time for RNA isolation. Total RNA was isolated using the RNeasy Protect Bacteria Mini kit (QIAGEN Inc., Germany) and processed according to the enzymatic lysis and proteinase K digestion protocol with on-column DNase digestion. A total of 250 μg/ml lysostaphin (Sigma, USA) and 1.5 mg/ml proteinase K (Roche, Germany) were used for preparing cell lysates. RNA concentration and purity were determined by spectrophotometry at 260 nm. DNase I and RNasin treatment (Promega Inc., USA) were used to remove any residual DNA contaminants. Approximately 1 μg of purified RNA was reverse transcribed to cDNA using ImProm-II Reverse Transcription kit (Promega). Real-time quantitative PCR analysis was performed in triplicate on a Rotor-Gene 3000 system (Corbett Research, Australia) according to the manufacturer's instructions using a TITANIUM *Taq* PCR kit (Clontech, USA) and SYBR Green I (Cambrex, USA) to determine the level of mRNA. The expression of *gyrB* in each treatment was used as the endogenous control to normalize the input RNA amount and the efficiencies of reverse transcription. The following primers were used: luxA-QF and luxA-QR, gyrB-QF and gyrB-QR. The relative quantifications were analyzed by using the "comparative quantitation" function of the Rotor-Gene 3000 software (version 6.0.38) (Corbett Research). The level of *luxA* mRNA from the AgrC-I reporter cells in the absence of AIP was defined as one.

Computational method

A transmembrane topology of AgrC-I and -IV (accession number: NC_007795, AF288215, respectively) was predicted by using the TMHMM2.0 algorithm (Krogh *et al.*, 2001).

Statistical analyses

All values from activation assays of supernatants represent the analysis of four independent experiments performed in duplicate. Results are presented as Means±SEM percentage

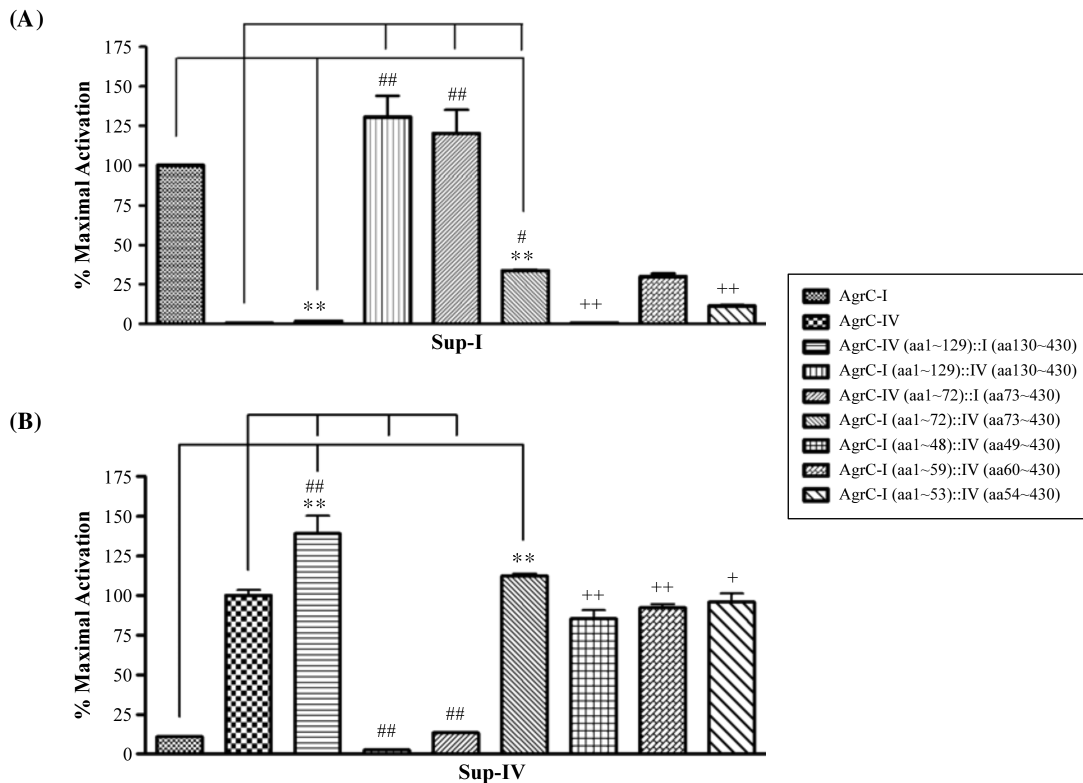

Fig. 1. *agr* Activation of AgrC-I and -IV wild-types and chimerical receptors by *agr* group I supernatant (Sup-I) (A) and *agr* group IV supernatant (Sup-IV) (B). Data represent percent maximal bioluminescence activity (relative light units/cell density, RLU/OD_{595}). Results of different mutant receptors were compared and symbols denote statistical significance when compared with AgrC-I ($^{*}P<0.05$; $^{**}P<0.01$), AgrC-IV ($^{\#}P<0.05$; $^{\#\#}P<0.01$) (shown with connecting lines), or AgrC-I(aa1~72)::IV(aa73~430) ($^{+}P<0.05$; $^{++}P<0.01$).

of wild type AgrCs to their cognate AIP. All tests were performed using Prism 4 (GraphPad Software). Mean percent values of AgrC mutants were compared to those of wild type AgrC using one-way ANOVA followed by *post hoc* comparisons using Dunnett's test. $P<0.05$ was considered significant.

Results

Bioluminescence reporter of AgrC

We determined *agr* group specificity between AIP and AgrC receptors based on the bioluminescence activity of the P3 promoter. The AgrC-I and AgrC-IV reporters could be activated by their corresponding supernatants as expected, but AgrC-I was also slightly activated by the group IV supernatant (Fig. 1). To assess if the bioluminescence reporter of AgrC represents the response of P3 promoter to AIP, we performed a real-time quantitative PCR to check the relationship between the bioluminescence activity and the mRNA levels of *luxA* of AgrC-I reporter triggered by P3 promoter. Dose-response analysis showed that the receptor activation kinetics (determined by measuring the mRNA levels of *luxA*) is linear between 1.2 and 39.1 nM synthetic AIP-I (data not shown). Bioluminescence activities also showed a good correlation with the mRNA levels of *luxA* of AgrC-I reporter in this concentration range (correlation coefficients, r^2=0.98) (data not shown). For ease of screening a large number of constructs, crude supernatants were used for the activation assay. To confirm the role of critical residue(s) and to determine the receptor kinetics precisely, synthetic AIPs were used in determining the half maximal effective concentration (EC_{50}) values.

Functional sensor domain of AgrC

To test whether the divergent N-terminal sensor domain of AgrC contains the determinant of AIP specificity, we constructed AgrC chimeras by fusing the N-terminus of AgrC from one group to the C-terminus of AgrC from another group based on the results of TMHMM2.0 algorithm (Fig. 2). Two AgrC chimeras, AgrC-I(aa1~129)::IV(aa130~430) and AgrC-IV(aa1~129)::I(aa130~430), could be activated by group I and IV supernatants, respectively (Fig. 1), indicating that the key determinants affecting the AgrC specificity by AIP are located in the N-terminal 129 residues. Two other AgrC chimeras, AgrC-I(aa1~72)::IV(aa73~430) and AgrC-IV(aa1~72)::I(aa73~430), could be activated by group IV and I supernatants, respectively. However, both were also slightly activated by supernatants of the opposite group (Fig. 1). We concluded that amino acid residues 1~129 of AgrC-I and -IV can respond to its group specific AIP, with residues 73~129 playing a more important role.

In contrast to AgrC-IV which could not respond to group

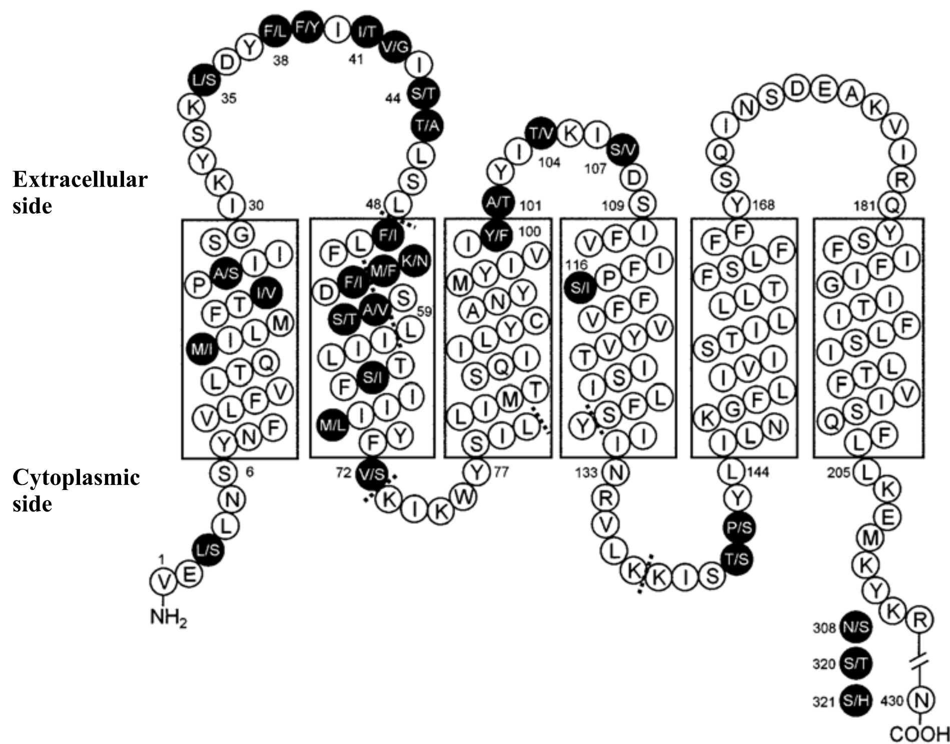

Fig. 2. Transmembrane topology of AgrC-I and -IV predicted by TMHMM2.0. Six transmembrane segments (TM, residues in the boxes) are connected by three extra- and two intracellular loops. Black circles indicate residues which differ between AgrC-I and -IV (residues in I/IV, respectively). Dotted lines containing positions 73~80 or 130~137 indicate the homologous region for fusion PCR of the two fragments, and dotted lines between positions 48~49, 53~54, and 59~60 indicate the ligation sites of the two fragments.

I supernatant, AgrC-I(aa1~72)::IV(aa73~430) retained 33.6% activity for the activation of AgrC-I by group I supernatant (Fig. 1), indicating that the N-terminus of AgrC-I fragment contains the specific activation of AgrC-I. To assess the source of residual activity, we fused segments of this region between *agrC*-I and -IV by performing blunt end ligations after first moving the ligation site to the end of the first extracellular loop. The resulting constructs, AgrC-I(aa1~48)::IV(aa49~430), only responded to group IV supernatant (Fig. 1). However, an extended AgrC-I fragment, AgrC-I(aa1~59)::IV(aa60~430), responded not only to group IV supernatant but its response to group I supernatant was also similar to that obtained with AgrC-I(aa1~72)::IV(aa73~430), indicating that the residual response to group I supernatant was located within residues 49~59 (Fig. 1). We further constructed AgrC-I(aa1~53)::IV(aa54~430), whose ligation site was moved to the middle of residues 49~59, and found a 3-fold reduction in response to group I supernatant compared to AgrC-I(aa1~72)::IV(aa73~430), with a weak activity of about 11.3% (Fig. 1). This result indicated that, although the residual activity was due to residues 49~53 and 54~59, the latter residues play a major role.

The key residue required for receptor activation

Within the residues 73~129 of AgrC, there are only five differences between AgrC-I and AgrC-IV. According to the topological prediction algorithms, four of these five residues may be located within the second extracellular loop (Fig. 2). Site-directed mutagenesis was used to change these four residues between AgrC-I and -IV singly and in different combinations. A four residue replacement mutant, AgrC-I (Y100F, A101T, T104V, S107V), could respond to both supernatants but its response was less than the wild type AgrC to their cognate supernatant. The activity of AgrC-I (Y100F, A101T, T104V, S107V) was similar to that of AgrC-I (A101T, T104V, S107V) (67% vs. 74% for group I supernatant, 64% vs. 59% for group IV supernatant, respectively, $p>0.05$), indicating that residue 100 does not play a major role in response to group IV specificity (Table 2).

We therefore focused on residues 101, 104, and 107 to assess which residue plays the most critical role. Two residue combination mutants of these three residues showed that AgrC-I(A101T, T104V) and AgrC-I(A101T, S107V), but not AgrC-I(T104V, S107V), resulted in a significantly increased response to group IV supernatant, implying that residue 101 mutation can broaden the specificity response to include both group supernatants. This observation was further supported by results obtained with single mutants of these four residues, among which only AgrC-I(A101T) resulted in a significant response to group IV supernatant. Of note is that AgrC-I(S107V) showed reduced activity in response to group I supernatant by retaining only one-third of the wild type activity. Besides these four residues within the second extracellular loop, residue 116 also differs between

Table 2. *agr* Activation of AgrC-I and -IV and their mutants in response to *agr* group I and IV supernatants or to synthetic AIP-I and -IV

Wild-type and mutant AgrCs	% Maximal Activation[a] (Means±SEM)		EC_{50}, nM (95% confidence intervals)	
	Sup-I	Sup-IV	AIP-I	AIP-IV
AgrC-I	100.0±0.4	10.9±0.3	23 (19-27)	291 (266-319)
AgrC-I(Y100F, A101T, T104V, S107V)	66.7±3.7**	64.2±3.3**		
AgrC-I(A101T, T104V, S107V)	73.9±4.9	58.6±3.9**		
AgrC-I(A101T, T104V)	156.3±12.6**	110.4±7.7**		
AgrC-I(A101T, S107V)	90.5±3.3	78.1±2.6**		
AgrC-I(A101T, S116I)	84.1±10.2	93.7±8.2**		
AgrC-I(T104V, S107V)	85.8±9.4	2.4±0.3		
AgrC-I(S107V, S116I)	2.9±0.1**	0.5±0.0		
AgrC-I(Y100F)	114.3±2.0	29.7±1.6	15 (12-20)	160 (148-174)
AgrC-I(A101T)	127.2±7.2*	105.9±0.7**	27 (20-36)	67 (62-73)
AgrC-I(T104V)	102.3±3.2	25.6±0.3	19 (13-26)	219 (198-242)
AgrC-I(S107V)	36.0±0.5**	0.4±0.0	180 (110-292)	653 (590-723)
AgrC-I(S116I)	11.5±0.6**	1.0±0.1	387 (334-447)	628 (574-687)
AgrC-I(A101T, S107V, S116I)	85.0±3.1	122.8±5.8**	53 (41-67)	54 (43-68)
AgrC-IV	0.7±0.0	100.0±3.3	521 (488-557)	58 (49-68)
AgrC-IV(F100Y)	6.9±0.5	95.2±3.7	230 (203-261)	52 (45-60)
AgrC-IV(T101A)	0.5±0.0	0.5±0.0##	32988 (5-2.120e+008)	3102 (383-25124)
AgrC-IV(V104T)	2.2±0.2	74.4±3.6##	363 (334-394)	43 (40-47)
AgrC-IV(V107S)	0.6±0.0	71.9±4.8##	463 (436-493)	89 (73-108)
AgrC-IV(I116S)	11.6±0.8##	71.4±5.4##	255 (211-308)	136 (124-148)
AgrC-IV(F100Y, T101A)	0.7±0.1	0.5±0.0##	3815 (3295-4418)	1324 (1148-1530)
AgrC-IV(T101A, V104T)	0.6±0.0	0.7±0.1##		
AgrC-IV(T101A, V107S)	0.6±0.1	0.6±0.1##	1029 (901-1174)	654 (568-753)
AgrC-IV(T101A, I116S)	3.2±0.3	0.6±0.1##	244 (184-325)	1083 (978-1199)
AgrC-IV(V104T, V107S)	2.3±0.2	73.0±2.5##		
AgrC-IV(T101A, V104T, V107S)	0.7±0.1	0.5±0.0##		
AgrC-IV(V107S, I116S)	102.5±5.9##	75.6±2.8##	62 (50-79)	144 (129-160)
AgrC-IV(T101A, V107S, I116S)	80.2±4.7##	2.9±0.1##	54 (43-68)	506 (473-541)

[a] Sup-I and -IV, group I and IV supernatants, respectively. All values from activation assays of supernatants represent the analysis of four independent experiments performed in duplicate. Results are presented as Means±SEM percentage of wild type AgrCs to their cognate AIP. Symbols denote statistical significance when compared with AgrC-I (*$P<0.05$; **$P<0.01$) or AgrC-IV (##$P<0.01$). The half maximal effective concentration (EC_{50}) values were calculated by fitting the data to a sigmoidal dose-response (variable slope) curve using the Prism 4 (GraphPad Software, USA).

AgrC-I and -IV among residues 73~129. To our surprise, AgrC-I(S116I) resulted in a 10-fold reduced activity in response to group I supernatant. Taken together, these results suggested that residues 107 and 116 of AgrC-I play a role in receptor activation by group I supernatant. A double replacement mutant at residues 107 and 116 of AgrC-I almost eliminated all activity of the receptor. This showed that these two residues have synergy effect in combination. Concordant results were obtained by dose-dependent activation assays using synthetic AIPs (Table 2).

Because results of the AgrC-I mutants showed that residue 101 could exhibit broadened specificity, a series of constructs were made to include 1 to 3 residue mutants of residues 100, 101, 104, 107, and 116 of AgrC-IV in different combinations. In contrast to other 4 single amino acid mutants, which retained between 70~95% response to Agr-IV supernatant, AgrC-IV(T101A) had almost no response to group IV supernatant. Dose-dependent activation assays also showed significant (53 to 63-fold) loss in response of AgrC-IV(T101A) to synthetic AIP-IV and -I (EC_{50} of AIP-IV and -I, 3102 vs. 58 in wild type, and 32988 vs. 521 nM, respectively) (Table 2). We, therefore, created additional mutants to assess which could regain activity to AIP. AgrC-IV (F100Y, T101A) could recover about 2 to 9-fold activity in response to synthetic AIP-IV and -I, respectively. AgrC-IV (T101A, I116S) could gain the response to synthetic AIP-I better than AgrC-IV(T101A, V107S), at 135 vs. 32-fold, respectively [EC_{50} of AIP-I, 244 and 1029 vs. 32988 nM in AgrC-IV(T101A)](Table 2).

An extra mutant construct, AgrC-IV(T101A, V107S, I116S), could gain the response to synthetic AIP-I by 611-fold compared to AgrC-IV(T101A) (EC_{50} of AIP-I, 54 vs. 32988 nM, respectively), in contrast to its response to synthetic AIP-IV which only increased by 6-fold (EC_{50} of AIP-IV, 506 vs. 3102 nM, respectively). These results indicated that the double mutant of residues 107 and 116 by Ser dramatically affected AgrC-IV(T101A) activation by AIP-I. In fact, AgrC-IV(V107S, I116S) readily gained the activity in response to AIP-I compared to wild type AgrC-IV (EC_{50} from 521 to 62 nM), while its response to AIP-IV was slightly reduced (EC_{50} from 58 to 144 nM). Replacement of its Thr by Ala at residue 101 reduced the response to

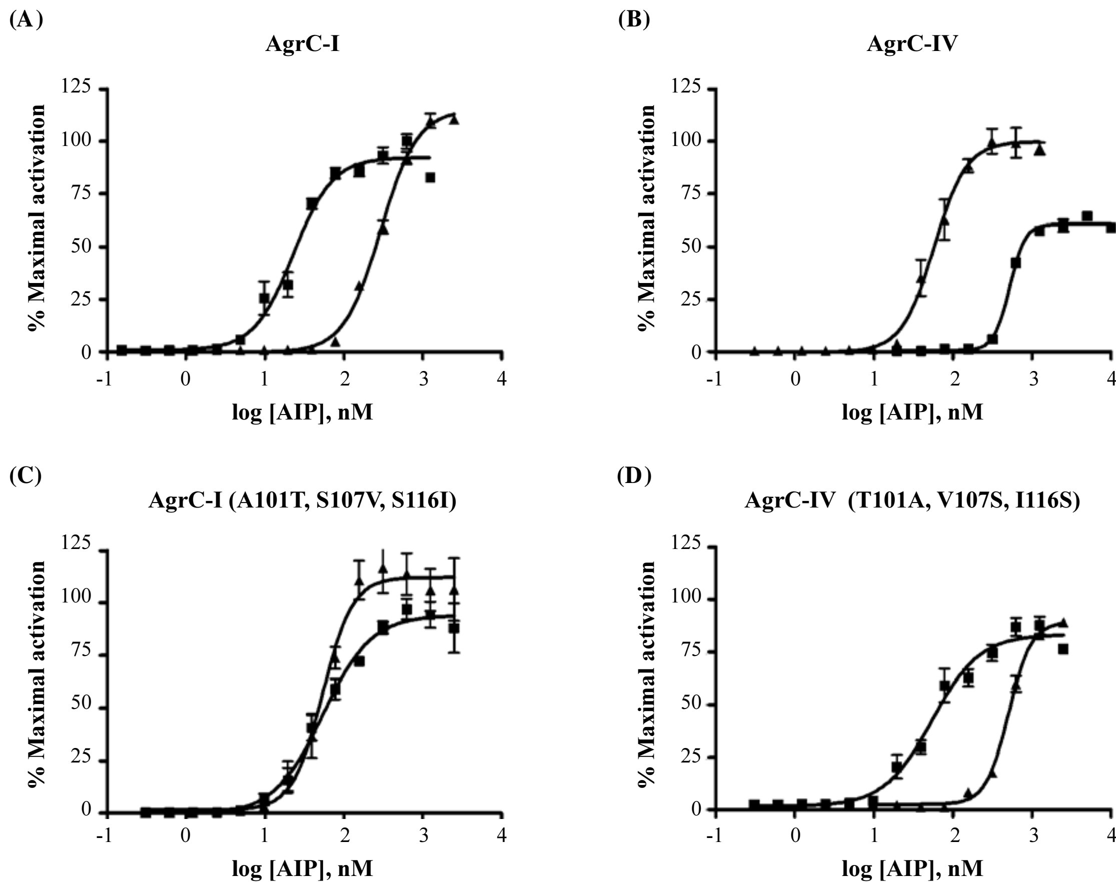

Fig. 3. Bioluminescence dose-response curves for the activation of the reporter constructs, AgrC-I (A), AgrC-IV (B), AgrC-I(A101T, S107V, S116I) (C), and AgrC-IV(T101A, V107S, I116S) (D) by synthetic AIP-I (■) and -IV (▲). Data are shown as percent maximal bioluminescence activity.

AIP-IV (EC_{50} from 144 to 506 nM) but not to AIP-I (EC_{50} from 62 to 54 nM) (Table 2), indicating that AIP-I could retain its activity with small Ala at residue 101 of AgrC but not AIP-IV to activate AgrC. The dose-response profile of AgrC-IV(T101A, V107S, I116S) was more similar to that of AgrC-I than AgrC-I(A101T, S107V, S116I) when compared to wild type AgrC-IV, probably due to the contribution of activity by AIP-I at residues 49~59 of AgrC-I as described above (Fig. 3).

The result of AgrC-I(aa1~72)::IV(aa73~430, T101A) also confirmed that residue 101 is the key residue for activation in AgrC-IV because this mutation completely eliminated the activity in response to group I and IV supernatants compared to its parental construct, AgrC-I(aa1~72)::IV(aa73~430), even though the 72 N-terminal sequence of these two chimera receptors belonged to AgrC-I (Fig. 4).

The role of second extracellular loop

To assess the role of the second extracellular loop of AgrC on group specificity, we constructed another two AgrC-I mutants, AgrC-I(aa100~109 II, FLTLRKYTTD) and AgrC-I (aa100~109 III, FSYIITISHS), whose second extracellular loop were replaced by *agr* group II and III sequences, respectively. These two constructs were activated by group I and IV supernatants (Fig. 4) instead of group II or III supernatants (no activity, data not shown), suggesting that the second extracellular loop is not the sole determinant of specificity and also illustrated that the conformation of AgrC-I is more flexible for activation.

Discussion

AIP activation of its cognate AgrC is highly sequence specific. The AIPs-I and -IV differ only in residue 5 within the thiolactone ring (Asp and Tyr, respectively) and this position is not only required for the whole activity of AIP-I/IV but also is the specificity-determining residue that may interact with the residue on the extracellular loop of AgrC to activate the receptor (Gether and Kobilka, 1998; Wright *et al.*, 2004). Our results showed that residue T101 of AgrC-IV is important for the whole activity of the receptor. When residue T101 of AgrC-IV was replaced by Ala, an amino acid with a small, non-reactive side chain (Betts and Russell, 2003), it caused a dramatic decrease in response to both AIP-I and AIP-IV. In contrast, residue T101 of AgrC-I is the specificity-determining residue for AIP-IV but also

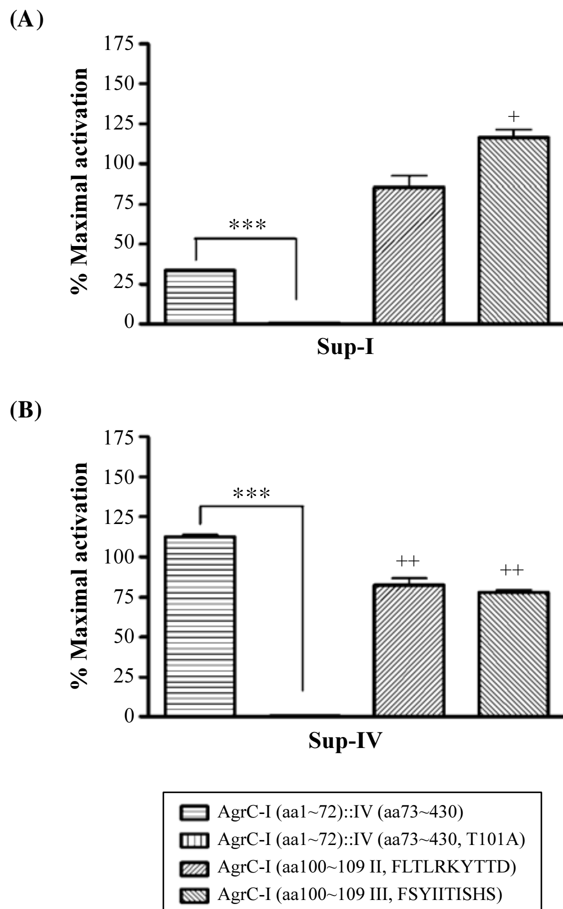

Fig. 4. Confirmation of the role of residue 101 and the second extracellular loop of AgrC on the response to Sup-I (A) and Sup-IV (B). *agr* activation assays of AgrC-I(aa1~72)::IV(aa73~430) with residue T101 changed to Ala, and AgrC-I derivatives, whose second extracellular loop (aa100~109) was replaced by *agr* group II and III amino acid sequences. Statistical significance was calculated by AgrC-I(aa1~72)::IV(aa73~430, T101A) with AgrC-I (aa1~72)::IV(aa73~430) ($^{***}p<0.001$) and by comparing AgrC-I (aa100~109 II, FLTLRKYTTD) and AgrC-I(aa100~109 III, FSYIITISHS) with AgrC-I ($^{+}p<0.05$; $^{++}p<0.01$).

maintains the interaction with AIP-I, since AgrC-I(A101T) can broaden the response to both group AIPs. Because a single amino acid change at the same residue 101 on AgrC-I and -IV have opposite responses, we speculate that the specificity-determining residue of AIP-I/IV may interact with the residue 101 of AgrC-I and -IV to activate the receptor.

Only the results of residue 101 still cannot explain the specific activation between the cognate AIP and AgrC. Unlike AgrC receptors, transmembrane G protein-coupled receptors (GPCRs) are well-characterized. They contain seven transmembrane helices (TM) and mediate signal transduction in response to a wide range of stimuli (Gether and Kobilka, 1998). A two-state model for GPCR activation has been proposed, with the receptor existing in inactive and active conformation states. Agonist-dependent receptor activity is accomplished by agonists binding with the inactive conformation state, which shifts the receptor to the active conformation state (Gether, 2000). The inactive conformation state of several receptors is maintained by the conformational constraints upon the stabilizing intrahelices interactions (Gether, 2000). Individual residues within the extracellular loop or transmembrane helices of GPCRs have been identified as important for relaxing the conformational constraint of the receptor (Lawson and Wheatley, 2004).

In AgrC-I, a single replacement of S107 with Val or S116 with Ile decreased the activity of the wild-type receptor. A S107V, S116I double mutant almost eliminated the activity in response to group I and IV supernatants but did not change the specificity. Based on the two-state model of GPCR we hypothesize that these two hydrophobic substitutions increased the conformational constraints and maintained the inactive conformation state in response to AIP. In addition, single replacement of V107 or I116 with Ser in AgrC-IV is insufficient to activate by AIP-I. Only a V107S, I116S double mutant in AgrC-IV broadened its response to AIP-I, and even to group III supernatant to some extent (data not shown). This may indicate that the Ser 107 and 116 of AgrC-IV allowed for easier relaxing of the conformational constraint of the receptor to gain the ligand-dependent receptor activity and is the specificity-determining residue for AIP-I. Comparison of the results of AgrC-I (aa1~48)::IV(aa49~430)and AgrC-I(aa1~59)::IV(aa60~430) showed that residues 49~59 of AgrC-I sequences can broaden AgrC-IV responseto group I supernatant, indicating that residues 49~59 of AgrC-I sequences may also play a similar function as Ser 107 and 116, all of them are the specificity-determining residues for AIP-I. Therefore, we propose that residues 49~59 in TM2, residue 107 in second extracellular loop, and residue 116 in TM4 of AgrC are involved in stabilizing intrahelices interactions. Thus, even when the AgrC-I second extracellular loop was replaced by group II and III sequences, its conformation still maintained AgrC-I-like characteristics, which allow for easier activation by group I and IV supernatants. This conformational flexibility for activation in AgrC-I may have evolutionary advantages by allowing it to maintain AIP-AgrC recognition during evolutionary mutations and may explain why *agr* group I strains predominated worldwide (van Leeuwen *et al.*, 2000). However, the reason that activation of AgrC-IV by AIP-I is more difficult than the reverse may be caused by the conformational constraints of AgrC-IV and by the weaker interaction of charged Asp of AIP-I with T101 of AgrC-IV than the aromatic Tyr of AIP-IV.

Inhibition of AgrC from all 4 *agr* groups by AIP-I/IV(5A) has been reported by different research groups (McDowell *et al.*, 2001; Lyon *et al.*, 2002). Geisinger *et al.* (2009) recently identified additional aspects of cross-inhibition based on the constitutively active AgrC-I variants, and defined AIP-I/IV 5A) as neutral antagonist. Its inhibition on the native receptor is only by competition with an activating ligand. Based on these findings, we speculate that the mechanism of *agr* intergroup inhibition in AgrC-I/IV may be caused by competitive binding of the AIP to the same hydrophobic site on the receptor, but the specific contact with residue 101 does not activate the receptor. Since, when the specificity-determining residue of AIP-I/IV at position 5 was replaced by small Ala, it lost the ability to interact with residue

101 of AgrC-I and -IV, but it retained the binding patch to bind to the receptor and then convert it to a potent inhibitor. Therefore, we predicted that either AgrC-I(A101T) or AgrC-IV(V107S, I116S) mutant constructs could be reactivated by AIP-I/IV(5A). The former has been shown by Jensen *et al*. (2008). However, our results of AgrC-V (T101A) differ from those of Jensen et al., whose data showed that their AgrC-IV(T101A) mutant did not lose response to AIP-IV dramatically, but their AgrC-IV(V104T) did (57-fold) (Jensen *et al.*, 2008). We cannot explain the conflicting results at the present time.

Part of our results also differ from those of a recent study by Geisinger *et al*. (2008), who reported that double replacement of Y100 and A101 in AgrC-I with Phe and Thr, respectively, can broaden the specificity response to include both group AIPs, and that the activation of AgrC-I appears to depend on the steric interactions of the specificity-determining side chains in both receptor and ligand residues. However, residue 100 of AgrC-I and -IV, Tyr and Phe, both contains a bulky aromatic side chains. Therefore, steric interactions with ligands could not account for this position. A potential explanation for the difference is that Geisinger *et al*. (2008) did not perform single mutation at residue 100 or 101 of AgrC-IV and based their report on double residue mutants, and therefore thought that these two residues have similar roles in AgrC activation. In addition, ours is the first report of the critical residues within residues 49~59 on TM2 which could also contribute to the group I activation and, although the second extracellular loop is important for specificity, it is not the sole determinant.

A number of AIP-I/IV variants have been tested by other researchers (Lyon *et al.*, 2002; Geisinger *et al.*, 2008), whose results, together with ours, suggest that the conformational constraints of AgrC-IV only allow AIPs containing a bulky aromatic side chain, Tyr or Phe, at position 5 to interact with T101 of AgrC-IV, but not AIPs containing either a hydrophobic side chain (Leu) or a small side chain (Ala) for activation. In contrast with the conformational constraints of AgrC-IV, AgrC-I is easier to activate by both AIPs even when its residue 101 is Ala. Therefore, most of the tested substitutions at position 5 of AIP-I/IV could activate AgrC-I, except small Ala, and the contribution of substituted residues with charged or hydrophobic side chains, Asp, Leu, and Phe, is better than the bulky Tyr (Lyon *et al.*, 2002; Geisinger *et al.*, 2008). However, all of the specificity-determining residues between receptors and ligands may interact directly or indirectly *via* a noncovalent interaction, including contributions to the exterior of the contact pocket, influencing the position and/or orientation of other critical residues on the receptors (Greenspan and Di Cera, 1999; Ballesteros *et al.*, 2001). Further analysis is in progress to prove our model.

In conclusion, our detailed exploration of the mechanism of AIP-AgrC recognition contributes to the understanding of AIP-AgrC recognition in particular, and to ligand-receptor recognition in the two-component signaling system in general. The finding of key residues required for receptor activation also provides information for designing potential structure-based inhibitors.

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References

Ballesteros, J.A., L. Shi, and J.A. Javitch. 2001. Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. *Mol. Pharmacol.* 60, 1-19.

Betts, M.J. and R.B. Russell. 2003. Amino acid properties and consequences of substitutions, p. 289-316. *In* I.C. Gray and M.R. Barnes (eds.), Bioinformatics for geneticists.

Chen, F.J., A. Cockayne, K. Winzer, W.C. Chan, H.J. Lo, and P. Williams. 2004. Probing AgrC-AIP interactions in *Staphylococcus aureus*. Cell-Cell Communication in Bacteria (2nd). Abstr. 76A. American Society for Microbiology, Banff, Alberta, Canada.

Geisinger, E., E.A. George, T.W. Muir, and R.P. Novick. 2008. Identification of ligand specificity determinants in AgrC, the *Staphylococcus aureus* quorum-sensing receptor. *J. Biol. Chem.* 283, 8930-8938.

Geisinger, E., T.W. Muir, and R.P. Novick. 2009. *agr* Receptor mutants reveal distinct modes of inhibition by staphylococcal autoinducing peptides. *Proc. Natl. Acad. Sci. USA* 106, 1216-1221.

Gether, U. 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr. Rev.* 21, 90-113.

Gether, U. and B.K. Kobilka. 1998. G protein-coupled receptors. II. mechanism of agonist activation. *J. Biol. Chem.* 273, 17979-17982.

Greenspan, N.S. and E. Di Cera. 1999. Defining epitopes: It's not as easy as it seems. *Nat. Biotechnol.* 17, 936-937.

Grkovic, S., M.H. Brown, K.M. Hardie, N. Firth, and R.A. Skurray. 2003. Stable low-copy-number *Staphylococcus aureus* shuttle vectors. *Microbiology* 149, 785-794.

Janzon, L. and S. Arvidson. 1990. The role of the delta-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *EMBO J.* 9, 1391-1399.

Janzon, L., S. Lofdahl, and S. Arvidson. 1989. Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. *Mol. Gen. Genet.* 219, 480-485.

Jarraud, S., G.J. Lyon, A.M. Figueiredo, L. Gerard, F. Vandenesch, J. Etienne, T.W. Muir, and R.P. Novick. 2000. Exfoliatin-producing strains define a fourth *agr* specificity group in *Staphylococcus aureus*. *J. Bacteriol.* 182, 6517-6522.

Jensen, R.O., K. Winzer, S.R. Clarke, W.C. Chan, and P. Williams. 2008. Differential recognition of *Staphylococcus aureus* quorum-sensing signals depends on both extracellular loops 1 and 2 of the transmembrane sensor AgrC. *J. Mol. Biol.* 381, 300-309.

Ji, G., R.C. Beavis, and R.P. Novick. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl. Acad. Sci. USA* 92, 12055-12059.

Ji, G., R. Beavis, and R.P. Novick. 1997. Bacterial interference caused by autoinducing peptide variants. *Science* 276, 2027-2030.

Koenig, R.L., J.L. Ray, S.J. Maleki, M.S. Smeltzer, and B.K. Hurlburt. 2004. *Staphylococcus aureus* AgrA binding to the

RNAIII-*agr* regulatory region. *J. Bacteriol.* 186, 7549-7555.

Koretke, K.K., A.N. Lupas, P.V. Warren, M. Rosenberg, and J.R. Brown. 2000. Evolution of two-component signal transduction. *Mol. Biol. Evol.* 17, 1956-1970.

Krogh, A., B. Larsson, G. von Heijne, and E.L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567-580.

Lawson, Z. and M. Wheatley. 2004. The third extracellular loop of G protein-coupled receptors: more than just a linker between two important transmembrane helices. *Biochem. Soc. Trans.* 32, 1048-1050.

Lina, G., S. Jarraud, G. Ji, T. Greenland, A. Pedraza, J. Etienne, R.P. Novick, and F. Vandenesch. 1998. Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus*. *Mol. Microbiol.* 28, 655-662.

Lyon, G.J., P. Mayville, T.W. Muir, and R.P. Novick. 2000. Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. *Proc. Natl. Acad. Sci. USA* 97, 13330-13335.

Lyon, G.J. and R.P. Novick. 2004. Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* 25, 1389-1403.

Lyon, G.J., J.S. Wright, T.W. Muir, and R.P. Novick. 2002. Key determinants of receptor activation in the *agr* autoinducing peptides of *Staphylococcus aureus*. *Biochemistry* 41, 10095-10104.

McDowell, P., Z. Affas, C. Reynolds, M.T. Holden, S.J. Wood, S. Saint, A. Cockayne, P.J. Hill, C.E. Dodd, B.W. Bycroft, W.C. Chan, and P. Williams. 2001. Structure, activity and evolution of the group I thiolactone peptide quorum-sensing system of *Staphylococcus aureus*. *Mol. Microbiol.* 41, 503-512.

Morfeldt, E., L. Janzon, S. Arvidson, and S. Lofdahl. 1988. Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. *Mol. Gen. Genet.* 211, 435-440.

Norrander, J., T. Kempe, and J. Messing. 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* 26, 101-106.

Novick, R.P. 1991. Genetic systems in staphylococci. *Methods Enzymol.* 204, 587-636.

Novick, R.P., S.J. Projan, J. Kornblum, H.F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh. 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. *Mol. Gen. Genet.* 248, 446-458.

Novick, R.P., H.F. Ross, S.J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh. 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.* 12, 3967-3975.

Otto, M. 2001. *Staphylococcus aureus* and *Staphylococcus epidermidis* peptide pheromones produced by the accessory gene regulator *agr* system. *Peptides* 22, 1603-1608.

Peng, H.L., R.P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert. 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J. Bacteriol.* 170, 4365-4372.

Qazi, S.N., E. Counil, J. Morrissey, C.E. Rees, A. Cockayne, K. Winzer, W.C. Chan, P. Williams, and P.J. Hill. 2001. *agr* Expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. *Infect. Immun.* 69, 7074-7082.

Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R.P. Novick. 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. *Mol. Gen. Genet.* 202, 58-61.

Schenk, S. and R.A. Laddaga. 1992. Improved method for electroporation of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 73, 133-138.

van Leeuwen, W., W. van Nieuwenhuizen, C. Gijzen, H. Verbrugh, and A. van Belkum. 2000. Population studies of methicillin-resistant and -sensitive *Staphylococcus aureus* strains reveal a lack of variability in the *agrD* gene, encoding a staphylococcal autoinducer peptide. *J. Bacteriol.* 182, 5721-5729.

Wolanin, P.M., P.A. Thomason, and J.B. Stock. 2002. Histidine protein kinases: key signal transducers outside the animal kingdom. *Genome Biol.* 3, reviews3013.

Wright, J.S., 3rd, G.J. Lyon, E.A. George, T.W. Muir, and R.P. Novick. 2004. Hydrophobic interactions drive ligand-receptor recognition for activation and inhibition of staphylococcal quorum sensing. *Proc. Natl. Acad. Sci. USA* 101, 16168-16173.