Supporting Information

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SI Text

Supplemental Discussion. The ability to generate in vitro an AgrC mutant with dramatically altered ligand specificity as demonstrated in this work may have implications for the development of antimicrobial resistance in vivo by virulent staphylococci. Inhibitory, noncognate AIPs, or AIP–mimetic compounds have been proposed previously as a potential, novel class of antistaphylococcal therapeutics, because they would interfere with *agr* signaling, and thus density-dependent virulence gene regulation (1, 2). The genetic acquisition within AgrC, through as little as one residue change, of greatly broadened specificity combined with reduced sensitivity to inhibition would represent a mechanism of quickly evolved resistance to such inhibitory compounds, possibly limiting their therapeutic potential. For example, the I171K mutation in AgrC-I would diminish the inhibitory effect of an inverse agonist compound toward an *agr*-I strain, and would result in activation with a neutral antagonist compound, enabling the mutant organisms in either situation to evade interference of *agr* signaling. Importantly, this mutation would preserve autoinduction by the original cognate AIP, and thus density-dependent regulation of virulence gene expression, preserving pathogenicity.

In contrast to an altered specificity mutation, an AgrC mutation conferring constitutive activity would presumably compromise bacterial fitness in vivo for several possible reasons: the critical, density-dependent adaptive response to the host would be lost, and continued high-level expression of the *agr* system may pose an overwhelming burden on cellular metabolism. Constitutively active mutants, however, could conceivably be beneficial as a subpopulation within a mixed infection containing WT cells. Because noncognate AIPs inhibit *agr* expression but not growth, there is no selection for constitutive or altered specificity mutants during normal growth in vitro. Whether selection for such mutants could take place in vivo is unknown. To test these ideas, it will be of interest to study the effects of constitutive and altered specificity AgrC mutations on experimental *S. aureus* murine infections, and to analyze the influence of inhibitor peptides on such infections.

Supplementary Materials and Methods. ***DNA library and plasmid construction.*** The strains and plasmids used in this study are listed in Table S1, and primers are listed in Table S2. All constructs were sequenced by the Skirball DNA Sequencing Core Facility (New York, NY). The GeneMorph II Kit (Stratagene) was used to generate the randomly mutagenized *agrC-I* clone libraries. The first library PCR products covered AgrC-I residues 86 through 430 and were amplified by using primers SpeI-F and KpnI-R. The second library represented residues 1 through 193, and primers CI-F and ClaI-R were used. The amplification parameters were optimized per the manufacturer's instructions to obtain a mutation rate of 1 to 5 base changes per product, such that 1–2 residues were targeted. Amplified products were digested and inserted into pEG1 and pEG2 vectors, which contain a chloramphenicol (Cm) resistance cassette and the P*cad* promoter driving *agrC* expression. Ligation products were transformed into XL-10 Gold ultracompetent *Escherichia coli* cells (Stratagene). Before pooling, the mutation frequency and random mapping of mutations over the targeted *agrC* area were verified by sequence analysis of several nonselected clones. Miniprepped plasmids from pooled colonies were electroporated into the *S. aureus* P3-*tetK* selection strain RN11004 (see below), followed by growth on tryptic soy agar/tetracycline (4 or 6 μg/mL) plates. Selection for altered specificity mutants involved TSA-tetracycline plates containing 1:25 volume of filtered supernatants from a postexponential phase culture of an *agr*-III lab strain (providing AIP-III). Approximately 15,000 transformants were plated per library. Selected (tetracycline-resistant) clones were patched separately on tetracycline and Cm (10 μg/mL) plates, and plasmids from double-resistant clones were (*i*) outcrossed via transduction to the reporter strain RN10829, and (*ii*) miniprepped and transformed into *E. coli* strain DH5α for subsequent sequencing.

Additional cloning was performed by using *E. coli* DH5α. Site-directed *agrC-I* mutants were constructed by using primers covering engineered restriction sites as indicated in Table S1, and were inserted into pRN9160. *agrC-II-L202R* was constructed from an *agrC-II*-pUC18 subclone by inverse PCR closing on an engineered MluI site, followed by insertion into pRN9160. Clones were transformed into RN4220, our standard recipient for *E. coli* DNA, before transduction to RN10829. A chromosomal *agr* locus derivative containing a P3-*tetK* fusion and lacking *agrC*, *agrB*, and *agrD* was constructed by deleting *agrB* and *agrD* from pRN9253 (3) and inserting *tetK* (cloned from pT181) between P3 and RNAIII via the AvrII and BglII sites, creating plasmid pRN9255. The construct then was integrated into the *S. aureus* chromosome at the SaPI1 *att* site in an RN4220 derivative in which the endogenous *agr* locus was deleted, creating the P3-*tetK* selection strain RN11004.

Synthesis of AIPs. *S. aureus* AIPs were synthesized via solid-phase Boc-based chemistry (4) or by the recently developed Fmoc-based chemistry (2). All cyclized peptides were purified by RP-HPLC and characterized by mass spectrometry and amino acid analysis (Keck Facility, Yale University, New Haven, CT) to validate peptide composition and ensure >95% purity. AIPs were diluted in 25% propylene glycol/50 mM phosphate (pH 5.7) and were assayed in this buffer. AIP-containing supernatants from staphylococcal strains (Table S1) were prepared as described previously (5).

1. Mayville P, *et al.* (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. *Proc Natl Acad Sci USA* 96:1218–1223.
2. George EA, Novick RP, Muir TW (2008) Cyclic peptide inhibitors of staphylococcal virulence prepared by Fmoc-based thiolactone peptide synthesis. *J Am Chem Soc* 130:4914–4924.
3. Geisinger E, George EA, Muir TW, Novick RP (2008) Identification of ligand specificity determinants in AgrC, the Staphylococcus aureus quorum-sensing receptor. *J Biol Chem* 283:8930–8938.
4. Lyon GJ, Wright JS, Muir TW, Novick RP (2002) Key determinants of receptor activation in the agr autoinducing peptides of Staphylococcus aureus. *Biochemistry* 41:10095–11104.
5. Lyon GJ, Mayville P, Muir TW, Novick RP (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.

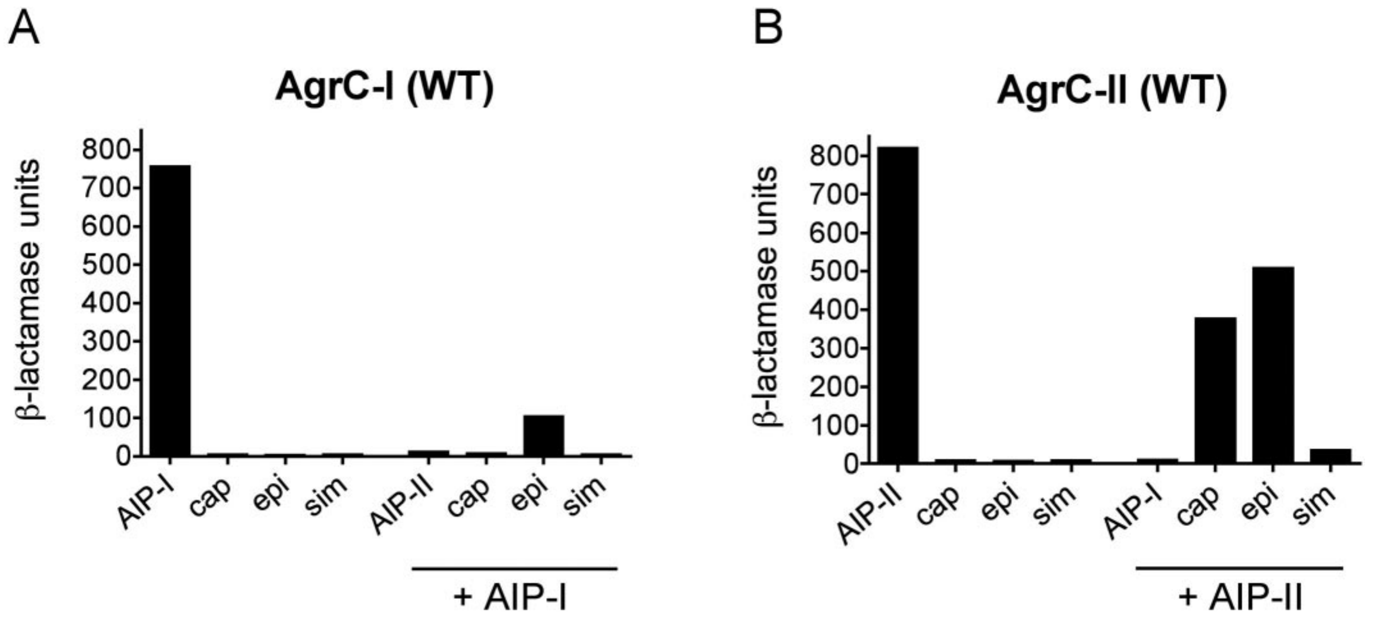

Fig. S1. Inhibition of WT AgrC by non-*aureus* staphylococcal AIPs. β-Lactamase reporter cells expressing WT AgrC-I (*A*) or AgrC-II (*B*) were incubated in the presence of the indicated AIP(s) and/or the indicated non-*aureus* AIP-containing culture supernatant (cap, *S. caprae*; epi, *S. epidermidis*; sim, *S. simulans*). Data are presented as β-lactamase units.

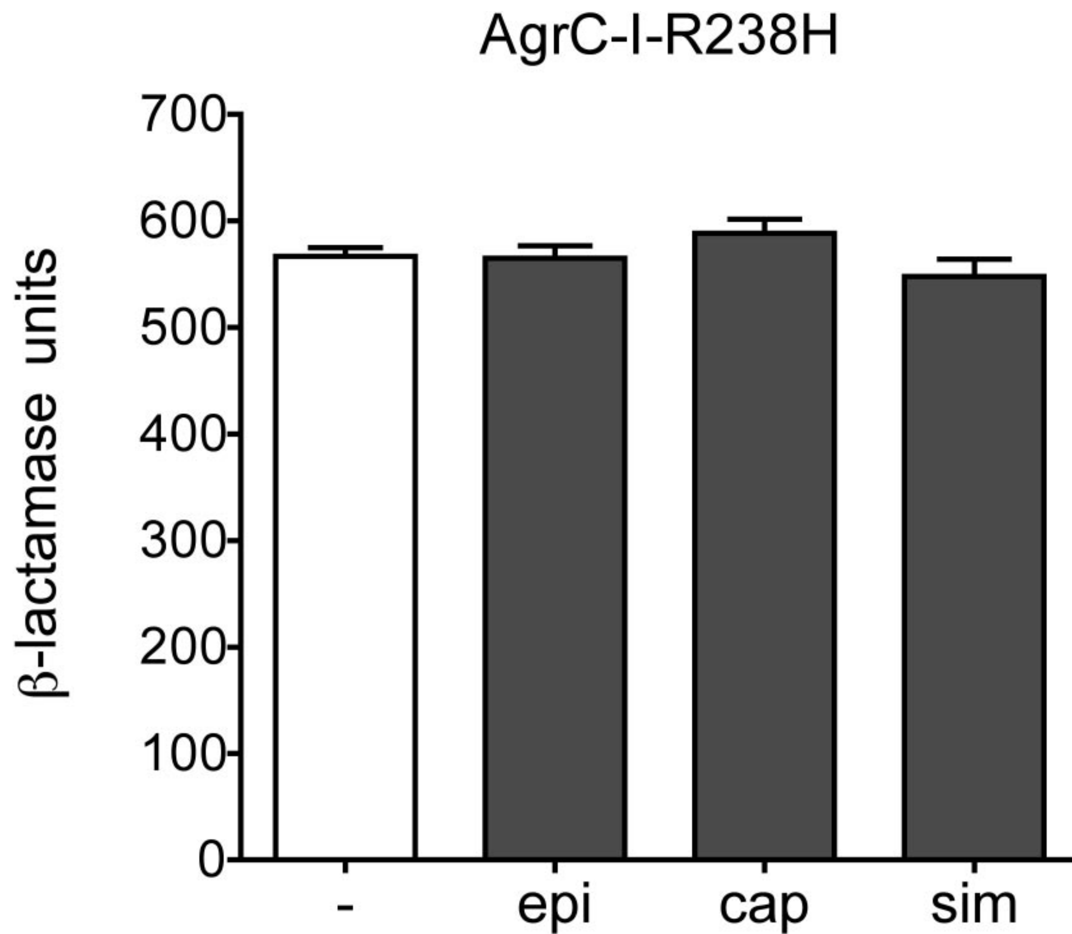

Fig. S2. Effect of non-*aureus* supernatants on AgrC-I-R238H. β-Lactamase reporter cells expressing AgrC-I-R238H were incubated in the absence or presence of the indicated non-*aureus* supernatant (cap, *S. caprae*; epi, *S. epidermidis*; sim, *S. simulans*). Data are presented as β-lactamase units $\pm$ SEM.

Table S1. Strains and plasmids

Strain or plasmid	Genotype or description	Ref. or source
S. aureus strains		
RN4220	Restriction-deficient mutant of strain 8325-4	1
RN7206	*agr*::*tetM* replacement in standard *agr*-I lab strain, RN6734	2
RN10829	RN7206 containing pRN9254 (P2-*agrA*; P3-*blaZ*) integrated into the SaPI1 *att* site (β-lactamase reporter strain)	3
RN11004	RN4220 with markerless *agr* deletion and containing pRN9255 integrated into the SaPI1 *att* site (selection strain)	This work
Other staphylococcal strains		
RN9503	*S. caprae agr-I*, predicted AIP sequence: YSTCSYYF	Unpublished
RN9509	*S. epidermidis agr-I*, AIP sequence: DSVCASYF	Unpublished
RN9516	*S. simulans agr-I*, predicted AIP sequence: YNPCLGFL	Unpublished
E. coli strains		
DH5α	Standard recipient for plasmid cloning	Promega
XL-10 Gold	Ultracompetent cells for library construction	Stratagene
Plasmids		
pT181	Contains *tetK* gene (dosage-dependent tetracycline resistance)	4
pRN9255	Shuttle/suicide vector pJC1111 (3) containing SaPI1 integration cassette and tetracycline selection construct (P2-*agrA*; P3-*tetK*-RNAIII)	This work
pRN9160	pCN51 (5) with Em marker replaced with Cm marker	6
pEG1	pRN9160 with *agrC-I*	This work
pEG2	pRN9160 with *agrC-I* containing engineered SpeI site	This work
pEG3	pRN9160 with *agrC-I* containing engineered ClaI site	This work
pEG4	pRN9160 with *agrC-I-I171K*	This work
pEG5	pRN9160 with *agrC-I-R180W*	This work
pEG6	pRN9160 with *agrC-I-S183F*	This work
pEG7	pRN9160 with *agrC-I-T197K*	This work
pEG8	pRN9160 with *agrC-I-L205R*	This work
pEG9	pRN9160 with *agrC-I-L205H*	This work
pEG10	pRN9160 with *agrC-I-M234L*	This work
pEG11	pRN9160 with *agrC-I-R238H*	This work
pEG12	pRN9160 with *agrC-I-R238C*	This work
pEG13	pRN9160 with *agrC-I-R238G*	This work
pEG14	pRN9160 with *agrC-I-R238E*	This work
pEG15	pRN9160 with *agrC-I-R238K*	This work
pEG16	pRN9160 with *agrC-I-Y241C*	This work
pEG17	pRN9160 with *agrC-I-D273N*	This work
pEG18	pRN9160 with *agrC-I-E286Q*	This work
pEG19	pRN9160 with *agrC-I-E292Q*	This work
pEG20	pRN9160 with *agrC-I-Q305H*	This work
pEG21	pRN9160 with *agrC-I-Q305R*	This work
pEG22	pRN9160 with *agrC-I-Q305E*	This work
pEG23	pRN9160 with *agrC-I-E306Q*	This work
pEG24	pRN9160 with *agrC-I-E306A*	This work
pEG25	pRN9160 with *agrC-I-E306R*	This work
pEG26	pRN9160 with *agrC-I-E306D*	This work
pEG27	pRN9160 with *agrC-I-E314Q*	This work
pEG28	pRN9160 with *agrC-I-R238E,E306R*	This work
pEG29	pRN9160 with *agrC-II*	This work
pEG30	pRN9160 with *agrC-II-L202R*	This work

1. de Azavedo JC, *et al.* (1985) Expression of the cloned toxic shock syndrome toxin 1 gene (tst) in vivo with a rabbit uterine model. *Infect Immun* 50:304–309.
2. Novick RP, *et al.* (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J* 12:3967–3975.
3. Geisinger E, George EA, Muir TW, Novick RP (2008) Identification of ligand specificity determinants in AgrC, the Staphylococcus aureus quorum-sensing receptor. *J Biol Chem* 283:8930–8938.
4. Projan SJ, Novick RP (1984) Reciprocal intrapool variation in plasmid copy numbers: A characteristic of segregational incompatibility. *Plasmid* 12:52–60.
5. Charpentier E, *et al.* (2004) Novel cassette-based shuttle vector system for gram-positive bacteria. *Appl Environ Microbiol* 70:6076–6085.
6. Adhikari RP, Novick RP (2008) Regulatory organization of the staphylococcal sae locus. *Microbiology* 154:949–959.

Table S2. Oligonucleotide primers

Primer	Sequence (5′–3′)*	RE site used
CI-F	CCAGCTGCAGGAAGTACCAAAAGAATTAACACAA	PstI
KpnI-R	GAGCTCGGTACCTTCATACATTCACATCCTTATGGCTAGTTG	KpnI
SpeI-F	GATTATGACTAGTCAGATTATTCTATACTGTGCTAAC	SpeI
ClaI-R	GAAGATCGATAAAAATATAGTGATACCAATAAAAATAAAAGAATACTGCCTTATTAC	ClaI
pSpeI-R	GAGCTGACTAGTCATAATCAACAAAATAGAATACC	XhoI
pSpeI-F	GAGAAGATCTCGACACCATATCTAATACTAAACAAAGG	XhoI
pClaI-F	GTTGATCGATATTAACATTTGTTATTTCTCAATTTCTCCTTAAAGAGATGAAATATAAACG	ClaI
R238E-F	GCAAGTTCGAACATGATTATGTCAATATCTTAACGACAC	BstBI
R238E-R	TCATGTTCGAACTTGCGCATTTCGTTGTTGATAGC	BstBI
R238K-R	GTAGCCCGGGCATGTCATCTTCTCGAATGTATTCTGAAAGTGTCGTTAAGATATTGACATAATCATGTTTGAACT-TGCGCATTTCGTTGTTG	XmaI
D273N-F	CATGCCCGGGCTACGTGATTATTTCAATAAAAATATTGTACCTATGAAAAACAATTTACAAATGAATGC	XmaI
E286Q-F	AATGGGATCCAGAATCTTAAAGTACGTGAAATTAAAGGC	BamHI
E286Q-R	AGATTCTGGATCCCATTTAATTTTATAGCATTCATTTG	BamHI
E292Q-R	ACGTAAGATCTTCGCAGTAATTAAGCCTTTAATTTGACGTACTTTAAGATTCTCGATACC	BglII
E306Q-F	GCGAAGATCTTACGTGCACAACAAATGAATATTCCGATTAGTATC	BglII
E306A-F	GCGAAGATCTTACGTGCACAAGCAATGAATATTCCGATTAGTATC	BglII
E306R-F	GCGAAGATCTTACGTGCACAACGAATGAATATTCCGATTAGTATC	BglII
E306D-F	GCGAAGATCTTACGTGCACAAGATATGAATATTCCGATTAGTATC	BglII
E306-R	GCACGTAAGATCTTCGCAGTAATTAAGCCTTTAATTTCAC	BglII
E314Q-F	GCGAAGATCTTACGTGCACAAGAAATGAATATTCCGATTAGTATCCAAATACCCGATGAAG	
BglII-F	TGCGAAGATCTTACGTGCACAAGAAATGAATATTCCG	BglII
CII-F	GTACCCGCTGCAGTAACGAATTTATACGAC	PstI
CII-R	GTTTTGGTACCTTCACATCCTTATGGCTAGTTG	KpnI
L202RCII-F	TTTCACGCGTAAAGAGATGAAATACAAACGAAATCAAG	MluI
L202RCII-R	CTTTACGCGTGAAAAGCGACATTATAAGTATTACAACAG	MluI
tetK-F	GGCTCCTAGGGTAAAGAGGTAAAATTGTTTAG	AvrII
tetK-R	GGCCAGATCTCTATTCAAACTGCTTTTCAGAACG	BglII
P3-R	GCGCCCTAGGCATCTCTGTGATCTAGTTATATTAAAA	AvrII
RIII-F	GCGCAGATCTTGATGGAAAATAGTTGATGAGTTG	BglII

*Restriction sites and half-sites are underlined