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

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Two-component signaling systems involving receptor-histidine kinases are ubiquitous in bacteria and have been found in yeast and plants. These systems provide the major means by which bacteria communicate with each other and the outside world. Remarkably, very little is known concerning the extracellular ligands that presumably bind to receptor-histidine kinases to initiate signaling. The two-component *agr* signaling circuit in *Staphylococcus aureus* is one system where the ligands are known in chemical detail, thus opening the door for detailed structure–activity relationship studies. These ligands are short (8- to 9-aa) peptides containing a thiolactone structure, in which the α-carboxyl group of the C-terminal amino acid is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid from the C terminus of the peptide. One unique aspect of the *agr* system is that peptides that activate virulence expression in one group of *S. aureus* strains also inhibit virulence expression in other groups of *S. aureus* strains. Herein, it is demonstrated by switching the receptor-histidine kinase, AgrC, between strains of different *agr* specificity types, that intragroup activation and intergroup inhibition are both mediated by the same group-specific receptors. These results have facilitated the development of a global inhibitor of virulence in *S. aureus*, which consists of a truncated version of one of the naturally occurring thiolactone peptides.

Staphylococcus aureus infections are a major cause of morbidity and mortality in community and hospital settings. Consequently, the emergence of methicillin-resistant and, more recently, vancomycin-resistant strains of *S. aureus* represents an enormous threat to public health. *S. aureus* pathogenesis primarily involves the secretion of toxins that damage or lyse host cells or interfere with the immune system, enzymes that degrade tissue components, and cell wall-associated proteins that may be involved in adhesion and protection against host defenses. Synthesis of many of these virulence factors is controlled by a global regulatory locus, *agr* (1, 2). This locus contains a two-component module that is activated by a secreted autoinducing peptide (AIP) in a cell density-dependent manner. This type of regulation falls under the rubric of the ever-broadening field of quorum sensing (3), whereby a population of bacteria responds in concert when a critical cell density is reached. In the case of *S. aureus,* as cells enter postexponential phase, the AIP reaches a threshold concentration that turns on the *agr* (virulence) response. This process is mediated by the five genes in the *agr* locus, *agrB, D, C, A,* and *rnaiii* (Fig. 1). The P2 promoter drives the transcription of the *agrB, D, C,* and *A* genes, which provide the cytosolic, transmembrane, and extracellular components of a quorum-sensing/autoinduction circuit. The *agrD* gene product is a propeptide that is probably processed and secreted by AgrB, an integral membrane protein (4). The resultant mature AIP binds to the transmembrane receptor-histidine kinase coded by *agrC*. Binding of the AIP triggers phosphorylation of AgrC on a histidine residue. The response regulator, AgrA, accepts the phosphate group from AgrC and, in conjunction with a second transcription factor, SarA (5, 6), activates transcription from the *agr* P2/P3 promoters. The P3 transcript, RNAIII, mediates up-regulation of secreted virulence factors as well as down-regulation of surface proteins (7–9).

The AIP–AgrC receptor pair shows considerable interstrain sequence variation, which must have resulted from evolutionary covariation of this region of the chromosome to retain the specificity of the receptor–ligand interaction. *S. aureus* strains can be divided into at least four *agr* specificity groups (10, 11). These strains appear to compete with each other at the level of *agr* expression, as each AIP inhibits the *agr* response in strains belonging to other groups. This type of bacterial interference is unusual, because it affects the expression of a subset of genes rather than inhibiting growth (10). Because of the intergroup inhibitory effects of the AIPs, we are now redefining "AIP," an acronym for "autoinducing peptide," to mean the mature AgrD peptides and their synthetic or modified variants. This usage is irrespective of whether the AIP acts as an autoinducer or as an inhibitor of *agr* expression.

Structure–activity analysis of the AIPs from *S. aureus* has begun to elucidate their mechanism of action (10, 12). These peptides contain a thiolactone ring structure (10) in which the α-carboxyl group of the C-terminal amino acid is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid from the C terminus of the peptide (see example in Fig. 1) (12). There are usually three or four amino acids N-terminal to the cysteine, which are collectively referred to as the "tail" of the AIP. The high-energy thiolactone linkage appears to be necessary for activation; the corresponding lactone and lactam analogues of the Group II AIP are inactive, as is the linear version of this and other AIPs (4, 12). However, the lactam and lactone analogues (but not the linear version) are potent intergroup inhibitors, arguing that the high-energy thiolactone linkage is not required for intergroup inhibition, but that the ring structure is important for both types of activity. It has also been shown in a murine s.c. abscess model that the Group II AIP attenuates the virulence of a Group I strain, which suggests possible therapeutic value for the AIPs (12). *Agr* is conserved throughout the staphylococci, and studies of the *Staphylococcus epidermidis*

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Abbreviations: *agr*, accessory gene regulator; AIP, *agr*-autoinducing or -inhibiting peptide.

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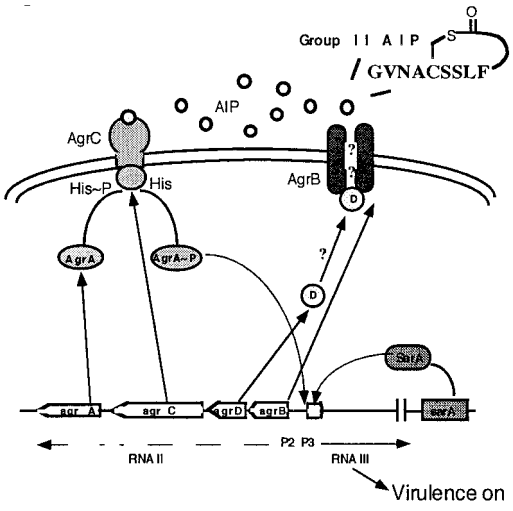

Fig. 1. The *agr* locus is depicted, showing the various components and their putative actions in the signaling network.

AIPs have generally confirmed what is known for *S. aureus* (13, 14).

There are two remarkable features of this system. First, the AIPs show major sequence diversity yet explicitly cross-inhibit *agr* activation with IC_{50}s that are in the same range as the EC_{50}s of the cognate AIPs. Second, analogs with oxygen or nitrogen in place of sulfur in the ring structure (i.e., the lactone or lactam vs. the thiolactone) are potent intergroup inhibitors, but neither activate nor inhibit their cognate receptors, at least when tested at concentrations a hundred-fold higher than the activating and inhibiting concentrations of the natural ligands. In earlier studies (4, 10, 12), *agr* activation and inhibition were studied in strains containing the chromosomal *agr* locus, sometimes by using a plasmid-carried *agr*P3::β-lactamase (*blaZ*) reporter. In these studies, the endogenous peptide was produced and its concentration varied over time, which made precise studies of activation and inhibition difficult. Furthermore, the experimental design precluded the separation of the *agr* locus from any other possibly group-specific factors that might have a role in inhibition or activation. In the present study, we demonstrate that the group specificity of the response to the AIP (inhibition or activation) is, in fact, determined solely by the AIP–receptor interaction. In other words, AIP-specific inhibition as well as activation occurs at the level of the receptor, AgrC. These results have led to the development of a variant AIP that inhibits autologous as well as heterologous *agr* expression and is therefore a global inhibitor of the virulence response in *S. aureus.* This variant, a truncated Group II peptide lacking the tail, is proposed to act by interfering with AIP binding to the receptor.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains used are listed in Table 1. *S. aureus* cells were grown in CYGP broth (15) with shaking at 37°C. Overnight cultures on GL plates (15) were routinely used as inocula. Cell growth was monitored with a Klett–Summerson colorimeter with a green (540-nm) filter (Klett, Long Island City, NY) or a THERMOmax microplate reader (Molecular Devices) at OD_{650}. Antibiotics used were ampicillin (100 μg/ml) for *Escherichia coli* and erythromycin (10

Table 1. Strains and plasmids

Strains or plasmids	References	Comments
S. aureus		
RN4220	15	Mutant of 8325-4 that accepts foreign DNA
RN6390b	15	Group I prototype
RN6734	15	Group I strain, ϕ-13 lysogen of 6390b
RN6911	8	Group I RN6390b, with *tetM* replacing *agr* (*agr*-null)
RN7206	8	Group I prototype, RN6734, with *tetM* replacing *agr*
RN6607	10	Group II prototype
RN9120	Unpublished	Group II with *tetM* replacing *agr* (*agr*-null)
RN8465	10	Group III prototype
RN4850	Unpublished	Group IV prototype from University of Minnesota strain KG*
RN9121	Unpublished	Group IV with *tetM* replacing *agr* (*agr*-null)
RN9033	This study	RN6911 with pRN7035
CA1-I (RN9222)	This study	RN6911 with pRN7062
CA1-I (RN9365)	This study	RN7206 with pRN7062
CA1-II (RN9366)	This study	RN9120 with pRN7062
CA2-II (RN9372)	This study	RN9120 with pRN7105
CA2-I (RN9367)	This study	RN7206 with pRN7105
CA4-IV (RN9371)	This study	RN9121 with pRN7107
CA4-I (RN9380)	This study	RN7206 with pRN7107
S. epidermidis		
RN2375	This study	Clinical isolate
S. warnerii		
RN3178	This study	Clinical isolate
E. coli		
DH5α		Cloning strain
Plasmids		
pRN7035	This study	Vector containing only *agr*-P3::*blaZ* fusion
pRN7062	This study	Vector containing group I *agr*CA and agr-P3::*blaZ*
pRN7105	This study	Vector containing group II *agr*CA and agr-P3::*blaZ*
pRN7107	This study	Vector containing group IV *agr*CA and agr-P3::*blaZ*

*Kindly provided by Dr. Patrick Schlievert.

μg/ml) and tetracycline (5 μg/ml) for *S. aureus*. Biotyping of *staphylococci* was performed at the Tisch Clinical Microbiology Laboratory, New York University Medical School, which confirmed the species classification of the non-*aureus* strains.

Construction of Strains and Plasmids. A shuttle vector, pRN7035, was used that contains plasmid replicons for *E. coli* and *S. aureus* along with antibiotic resistance cassettes: ampicillin for *E. coli* and erythromycin for *S. aureus*. This plasmid was constructed by cloning a PCR product containing the 180-nt *agr* P2P3 region into the pUC polylinker site of plasmid, pRN7034, which contains a β-lactamase reporter gene. In the new construct, the P3 promoter is fused to the β-lactamase reporter gene. Plasmids pRN7062, pRN7105, and pRN7107 were constructed by cloning PCR fragments containing *agrC*, *agrA*, and downstream termination signals from the various groups, into the *Pst*I or *Pst*I/*Sph*I sites of the pUC polylinker of pRN7035. Genomic DNA from a prototypical strain of each group was used as template in these reactions. The following PAGE-purified primers (Integrated DNA Technologies, Coralville, IA) were used in the PCR reactions: for pRN7062, forward primer, 5′-CCAACTGCAG-GAAGTACCAAAAGAATTAACACAA (*Pst*I site underlined), reverse primer, 5′-TTATACTGCAGACGTTTGC-CAACATTACAAGAGG (*Pst*I site underlined), for pRN7105, forward primer, 5′-TTTGAACTGCAGAAAGTACCCGCT-GAATTAACG (*Pst*I site underlined), reverse primer, 5′-GGTGAAGCATGCAGTTTGCCAACATTACAAGAGG-TTGAACAAGCATTTTAA, (*Sph*I site underlined), for pRN7101, forward primer, 5′ TCTTAACTGCAGAAGTT-GAAATACCTAAAGAATTAA CTCAATTACACG (*Pst*I site underlined), reverse primer, 5′-GGTGAAGCATGCAGT-TTGCCAACATTACAAGAGGTTGAACAAGCATTTTAA, (*Sph*I site underlined). Plasmid DNA from the corresponding *E. coli* DH5α derivative was introduced into the restriction-defective *S. aureus* strain, RN4220, by the protoplast method (15). Plasmids were then moved onto various genetic backgrounds by phage transduction (15). Note that RN9365 (CA1-I) (Table 1) is an additional strain with Group I *agrC* and *agrA* in a different Group I strain background. This strain was tested, and the results for RN9222 (CA1–1) were reproduced in this strain.

Preparation of AIP-Containing Supernatants. *S. aureus* strains were grown in CYGP broth with shaking at 37°C for 9 h starting with an inoculum of ≈3×10^7 cells/ml in 10 ml of broth. Cells were removed by centrifugation at 4°C, and the supernatant was filtered (0.22-μm filter, Gelman). The filtrate was stored at −80°C and used as a source of AIP. The group-specific activity of each supernatant correlated with that of the corresponding synthetic peptide.

Synthetic Peptides. All peptides used in this study have been previously described (12), except for the Group II truncated thiolactone peptide, Ac-Cys-Ser-Ser-Leu-Phe-CO, which was synthesized by using the transthioesterification method (12). After reverse-phase HPLC purification of the peptide, the product was characterized as the expected *N*-acetylated thiolactone-peptide by electrospray MS and two-dimensional ^{1}H NMR spectroscopy: observed mass = 580.0 ± 1.0 Da, predicted (average) mass = 579.7 Da; ^{1}H NMR (400 mHz, DMSO-d_6) ∂ 0.71 (Leu-4, γ−CH), 1.07 (Leu-4, δ−CH), 1.30 (Leu-4, β-CH), 2.79 and 3.17 (Cys-1, β-CH), 2.95 and 3.30 (Phe-5, β-CH), 3.61 (Ser-3, β-CH), 3.65 (Ser-2, β-CH), 4.08 (Leu-4, α−CH), 4.24 (Ser-2, α−CH), 4.27 (Cys-1, α−CH), 4.29 (Ser-3, α−CH), 4.67 (Phe-5, α−CH), 7.14–7.31 (Phe-5, Ar), 8.04 (Ser-3, NH), 8.18 (Leu-4, NH), 8.31 (Ser-2, NH), 8.34 (Cys-1, NH), 8.98 (Phe-5, NH) ppm.

***Agr* Autoinduction and Inhibition Assay.** *Agr*-null derivatives were used with a plasmid containing a constant *agr* P2P3::β-lactamase (*blaZ*) fusion plus a group-specific *agrAC* module. Assays were performed with CYGP cultures in early exponential phase (≈2×10^8 cells/ml). Culture supernatants (1/10 volume) or synthetic peptides in 10 mM phosphate buffer at pH 5.8 (concentration of stock solution determined by UV absorbance) were added, and cultures were incubated with shaking at 37°C for an additional 60 or 90 min, followed by determination of *agr* activation by β-lactamase assay (4). For inhibition tests, the activator and inhibitor were added simultaneously. EC_{50} and IC_{50} values were calculated from the sigmoidal dose-response curves by using nonlinear regression analysis with the program PRISM (GraphPad, San Diego). All assays were performed at least in duplicate.

Reverse-Phase HPLC Analysis of δ−Toxin Levels. A modification of the assay originally described by Otto and coworkers (13) was used to quantify δ−toxin in supernatants from various strains. Early exponential-phase cultures were treated with increasing concentrations of synthetic peptides and then allowed to grow for 9 h. Supernatants, obtained by centrifugation, were analyzed by reverse-phase HPLC on a Hewlett–Packard 1100 series instrument with diode array detection. Typically, 400-μl aliquots of supernatant were injected onto a 1-ml Pharmacia Resource PHE column, which was eluted at 1 ml/min as follows: isocratic at 35% buffer B in buffer A for 3 min, which allowed the majority of the UV-absorbing material to wash through the column, followed by a linear gradient of 35–50% B in A over 20 min, where A = 0.1% trifluoroacetic acid (TFA) in water and B = 0.1% TFA in 90% acetonitrile/10% water. This optimized gradient achieved excellent separation of δ−toxin from other components (>95% pure by HPLC and MS), which permitted direct quantitation by peak integration. δ−Toxin is usually *N*-formylated, and the additional 29 Da of the *N*-formyl group was included in the calculated mass. We observed the following masses on purified fractions of δ−toxin: *S. aureus* Group III, observed mass = 3,007.1 ± 2.2 Da, predicted (average) mass = 3,006.5 Da; *S. epidermidis* strains, observed mass = 2,848.7 ± 1.5 Da, predicted (average) mass = 2,848.4 Da, and *Staphylococcus warnerii* strains, observed mass = 2,900.1 ± 0.8 Da, predicted (average) mass = 2,899.4 Da.

Results

Reconstitution of *agr* Signaling in *agr*-Null Host Backgrounds. Shuttle vectors containing *agrC* and *agrA* under control of the *agr*-P2 promoter, along with a β-lactamase reporter gene driven by the *agr*-P3 promoter, were introduced into *agr*-null strains (see strains, Table 1). We reasoned, on the basis of prior studies (4, 10), that these constructs would be sufficient for signaling to occur in the *agr*-null backgrounds in response to exogenously added AIP. This was confirmed as shown in Table 2 and by example in Fig. 2. Two strains were used in these experiments, RN9222 (CA1-I) and RN9372 (CA2-II), containing the cloned *agrCA*/β-lactamase in the corresponding *agr*-null *S. aureus* strain (all reconstituted strains are provided with a descriptor in the format CAx-y, which indicates Group x *agrC* and *agrA* on the Group y *agr*-null background). In both cases, dose-dependent activation of the *agr* response was observed on addition of the cognate synthetic AIP (for example, see Fig. 2*A*). Moreover, the calculated EC_{50} values for *agr* activation in the two reconstituted strains were similar (Table 2). No dose-dependent *agr* activation was observed in the control strains, RN9033 and RN9416, which contain the vector alone (for example, see Fig. 2*A*).

Reconstitution of Cross-Strain Inhibition in the Absence of AgrB. As previously demonstrated (10), *S. aureus* culture supernatants inhibit *agr* activation in heterologous strains. Our prior study by

Table 2. Activation and inhibition by AIPs and an AIP analog

	RN9222 (CA1-I)	RN9366 (CA1-II)	RN9372 (CA2-II)	RN9367 (CA2-I)
Activation EC_{50}, nM				
Group I AIP	40 ± 9	23 ± 7	—	—
Group II AIP	—	—	34 ± 6	28 ± 14
Inhibition IC_{50}, nM*				
Group I AIP	—	—	26 ± 7	135 ± 52
Group II AIP	90 ± 30	78 ± 12	—	—
	RN9222(CA1-I)*	RN9372(CA2-II)*	RN8465(GroupIII)†	RN9371(CA4-IV)‡
Inhibition IC_{50}, nM for Group II AgrD truncated thiolactone				
Group II AIP, truncated	272 ± 67	209 ± 39	10 ± 1	188 ± 50

All values include an error of ±SEM determined from at least two assays.
*These values were obtained with a constant concentration, 100 nM, of the autologous (activating) AIP.
†All results determined for the Group III strain RN8465 were from HPLC analysis of δ toxin. All other results were from β-lactamase assays.
‡Group IV supernatant was used as a source of activating AIP in these analyses.

using a reporter gene assay in strains containing the endogenous *agr* locus (12) demonstrated that synthetic AIPs could reproduce these effects. However, the site of action for the inhibition seen with these peptides was not determined. Although the most likely site seemed to be AgrC, the diversity of sequences among inhibitory peptides and their analogs suggested that some mechanism other than competitive blocking of activator binding could be responsible. One possibility would be binding to the putative processing–secretion factor, AgrB, causing interference with production or secretion of the activator. Alternatively, the peptides could bind to any range of targets on or within the cells and could interfere with the *agr* signaling pathway up- or downstream of AgrC activation.

As shown in Fig. 2*B*, RN9222 (CA1-I) was grown with Group I AIP at 100 nM along with various concentrations of the Group II AIP. A dose-dependent inhibition response was seen with an IC_{50} value of 90 ± 30 nM (Table 2), thus demonstrating that inhibition occurs in the absence of AgrB. Similar experiments were performed with RN9372 (CA2-II), yielding an IC_{50} for inhibition by the group I AIP of 26 ± 7 nM (Table 2). These results, in two different *agr* specificity groups, confirm that AgrB cannot be the target of inhibition, as the new strains lack AgrB.

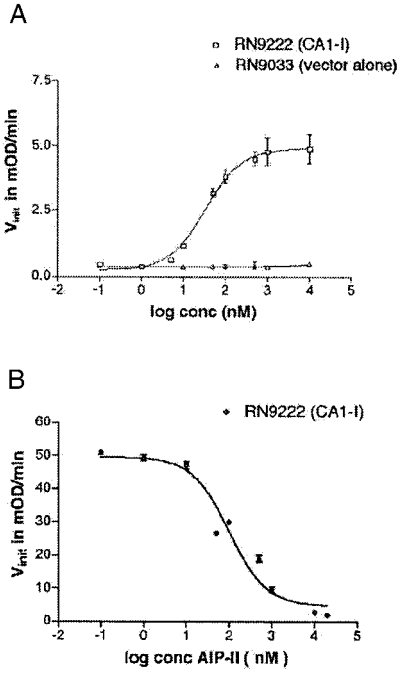

Fig. 2. Synthetic thiolactone peptides are biologically active in reconstituted strains. Shown are representative data for activation (*A*) and inhibition (*B*) of the *agr* response by synthetic AIPs. Degree of activity based on β-lactamase activity is shown as a plot of V_{init} (initial velocity) vs. peptide concentration. (*A*) Activation of the *agr* response in RN9222 (CA1-I) by Group I AIP. The RN9033 (vector alone) control is shown. (*B*) Inhibition of the *agr* response in RN9222 (CA1-I) by Group II AIP in the presence of activating Group I AIP at 100 nM.

The *agrAC* Two-Component Module Is Necessary and Sufficient for Group-Specific Activation and Inhibition. We next asked whether AgrC determines the group specificity of the AIP response. This question was addressed by testing the two-component module in *agr*-null host backgrounds derived from different *agr* groups. Transfer of the group-specific signaling phenotype with *agrCA* would argue against any other group-specific determinant. Accordingly, two strains, RN9366 (CA1-II) and RN9367 (CA2-I), were tested for their ability to respond to supernatants from $agr^{\pm}$ strains, as well as to Group I and II AIPs.

The two strains were compared with the appropriate controls, RN9222 (CA1-I) and RN9372 (CA2-II), for activation by postexponential phase supernatants from a series of $agr^{\pm}$ strains and by AIPs. As illustrated in Fig. 3, only supernatant from the Group II *agr* wild-type strain, RN6607, was able to activate β-lactamase expression in RN9372 (CA2-II) and RN9367 (CA2-I). In addition, similar EC_{50} values for activation were obtained for both strains by using Group II AIP (Table 2). Analogous results were obtained by using RN9222 (CA1-I) and RN9366 (CA1-II): these were activated by wild-type Group I supernatants (RN6734) and not by Group II supernatant or by *agr*-null

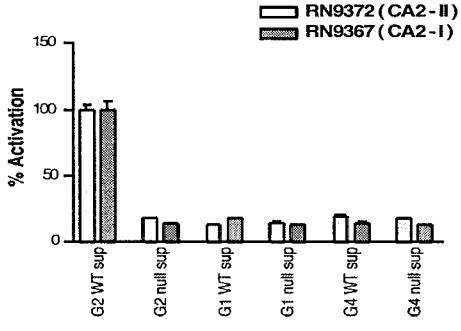

Fig. 3. A representative example of activation of RN9372 (CA2-II) and RN9367 (CA2-I) by postexponential supernatants collected from Group 1, 2, and 4 $agr^{\pm}$ cells. Data were collected as β-lactamase activity (V_{init} in mOD/min) and then normalized to percentage activation.

MICROBIOLOGY

supernatants from either group (data not shown). Moreover, similar EC_{50} values for activation were obtained for both strains by using the Group I AIP (Table 2). Therefore, the group specificity of *agr* signaling does not depend on the host background. Furthermore, the conversion of an *agr*-null Group I strain, RN7206 into a Group II responsive strain, RN9367 (CA2-I), by the insertion of plasmid-encoded Group II *agrC* and *agrA* (and vice versa) suggests strongly that the group specificity of the *agr* response depends on the expression of the two-component module containing *agrC* and *agrA*.

The next question was whether cross-strain inhibition also proceeds through the AgrC/AgrA two-component cascade. If so, a Group II *agr* null strain (RN6607) reconstituted with Group I *agrC* and *agrA*, i.e., RN9366 (CA1-II), should be inhibited by Group II agr^+ supernatants or by Group II AIP. Alternatively, if inhibition were to involve a group-specific factor outside of the *agr* locus, then inhibition would depend on the host background in which AgrC and AgrA are expressed. The former alternative was shown to be correct, as very similar IC_{50}s for inhibition by the Group II AIP in RN9366 (CA1-II) and RN9222 (CA1-I) were obtained (Table 2). The same was true for inhibition of RN9372 (CA2-II) and RN9367 (CA2-I) by Group I AIP, albeit with a slight difference in the IC_{50}s (Table 2). It is apparent that inhibition by the AIP depends on which *agrC* and *agrA* genes are expressed and not on the genetic background in which they are expressed, thereby indicating that the site of inhibition must be within the Agr A-C module. Because AgrC is a variable transmembrane receptor (16) and AgrA is highly conserved, it is suggested that the inhibitors bind to and act at AgrC.

Synthesis and Testing of a Global Inhibitor of Virulence in *S. aureus*. Previous structure–activity studies have identified key regions within the Group II AIP required for differential *agr* activation and inhibition (12). Changes in the ring structure of the AIP could affect either activation or inhibition of the *agr* response or both, whereas changes in the tail region of the molecule affected only activation. We reasoned that a truncated AIP analog containing only the thiolactone ring structure and lacking the activating tail might be able to bind its own AgrC receptor, because of conservation of the critical thiolactone moiety, but would not have the tail necessary for activation. This analog would therefore be a good inhibitor not only in a crossgroup manner but also within the same group. Converting a receptor agonist into an antagonist of its autologous group as well as an inhibitor of heterologous groups would represent a global inhibitor of virulence in *S. aureus.* This would comprise a key step in the development of an *agr*-based therapeutic.

A truncated Group II thiolactone peptide was synthesized by using the transthioesterification approach (12) and tested for its ability to activate and inhibit the *agr* response in Group I–IV strains. The Group I, II, and IV experiments used the β-lactamase reporter assay, whereas the Group III strain was tested by an HPLC assay described below. The truncated Group II AIP had no detectable activation activity for the four groups. Furthermore, the peptide was an inhibitor in the nanomolar range of the *agr* response in all four groups (Table 2), indicating that deletion of the tail region of the peptide does indeed convert the AIP from an agonist into an antagonist of self activation as well as retaining intergroup inhibition.

The β-lactamase reporter assay could not be used for Group III, because of the production of β-lactamase by all of the available strains. However, we were able to demonstrate inhibition of Group III *S. aureus* by the full-length and truncated Group II AIP by HPLC analysis of δ−toxin production (13). δ−Toxin is a ≈25-aa hemolytic peptide encoded by RNAIII, and the level of this translation product is proportional to the level of RNAIII in the cell (13). Early exponential-phase cultures were treated with increasing concentrations of truncated Group II AIP and then allowed to grow for 9 h. Supernatants were collected and the amounts of δ−toxin quantitated. Inhibition by the full-length Group II AIP was demonstrated with an IC_{50} value of 17 ± 13 nM. A representative curve for inhibition by the truncated Group II AIP is shown in Fig. 4, with an IC_{50} value of 10 ± 1 nM (Table 2).

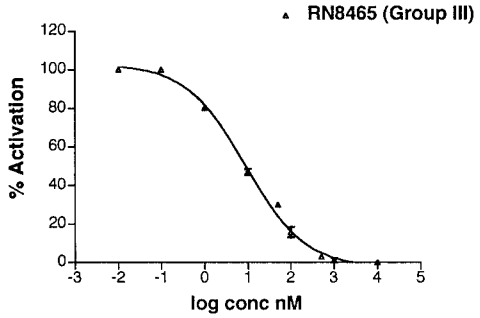

Fig. 4. The Group III strain, RN8465, was grown in the presence of differing concentrations of the truncated Group II analog. A representative inhibition curve derived from reversed-phase HPLC analysis of δ−toxin levels is shown. Quantitation of δ−toxin levels was obtained by integration of peak area from the HPLC trace.

The truncated thiolactone peptide is therefore a potent inhibitor for all four *agr* specificity groups of *S. aureus.* We have also begun to test the activity of AIPs in alternative staphylococcal species by using HPLC analysis of δ−toxin production. Our preliminary results thus far show only weak inhibition (in the micromolar range) of *S. epidermidis* strain RN2375 by the full-length and truncated group II AIPs. However, strong inhibition (in the nanomolar range) by the truncated group II AIP was seen with the *S. warnerii* strain RN3178, with an IC_{50} value of 20 ± 12 nM.

Discussion

Two-component systems are the major means by which bacteria sense a large variety of environmental stimuli. In most cases, the activating ligands for the receptors are unknown (17). The *agr* system is one of a few cases where the ligands have been chemically characterized, as thiolactone peptides (AIPs), and the system is unique in that AIPs from heterologous strains are naturally occurring inhibitors of *agr* signaling. In this study, we have begun to analyze the mechanism by which naturally occurring AIP sequence variants inhibit *agr* activation. Although the *agr* locus is conserved throughout the staphylococci, the *agr* autoinducing peptides and their cognate receptors have radically diverged, generating at least four autoinduction specificity groups in *S. aureus*. In general, the AIP activates *agr* expression in strains belonging to the same group as the producing organism and inhibits *agr* expression in organisms belonging to any other group. It is very possible that this divergence causes intergroup interference, leading to environmental isolation, and is at least partially responsible for speciation within the staphylococci. Similar divergence within the structurally similar *comAP* locus, required for competence in *Bacilli*, is also thought to be responsible for speciation in these organisms (18).

Structure–activity analysis has revealed two key features of the AIP–receptor interaction in *S. aureus* that are critical for the understanding of the present results: (*i*) Derivative peptides of the Group II AIP with a lactone or lactam replacing the essential thiolactone bond showed substantial loss of intragroup *agr* activation activity (at least 100-fold less potent) but retention of cross-inhibition activity. These derivatives, however, did not inhibit activation of the cognate receptor by the native thiolactone peptide (did not self inhibit). (*ii*) Substitution of any amino acid residue within the C-terminal thiolactone ring affected

either activation and/or inhibition activity, whereas substitution of any of the four residues in the N-terminal linear section of the peptide decreased or eliminated activation but did not decrease inhibition. We have previously hypothesized that activation and inhibition by native AIPs must therefore involve different mechanisms—either different modes of interaction with the AIP receptor, AgrC, or interaction with cellular components other than AgrC, e.g., AgrB.

This latter possibility was clearly ruled out by means of plasmid constructs containing a β-lactamase reporter driven by the (conserved) *agr* P3 promoter and including the P2 driven *agrA + C* modules from different specificity groups. Placement of these plasmids in *agr*-null derivatives of the *agr* specificity group corresponding either to that of the cloned module or to that of other groups enabled the direct *in vivo* analysis of AIP-specific activation/inhibition in the absence of AgrB or of any endogenous AIP. Because these tests demonstrated the predicted cross-group inhibition, qualitatively in parallel with previous results obtained with the endogenous *agr* loci intact, it is clear that AgrB is not the site of inhibition. Since these tests also demonstrated that crossgroup inhibition occurs independently of host background, and with similar dose responses, it is also clear that the only determinant of *agr* group specificity is the AgrA + C module. Although we have not tested AgrA and C independently, AgrA is highly conserved among *agr* specificity groups within and between species, whereas the receptor portion of AgrC is highly variable; thus there is little doubt that AgrC is the key determinant. Although there are certainly other cellular factors involved in *agr* signaling, our results indicate that none of these determines group specificity.

The assay method used in this study is different from that previously used (12), in enabling an assessment of AIP activity in the absence of skewing effects of the endogenous peptide. The values obtained here are a more accurate reflection of the correct physiological values, because we can precisely deliver and quantify the amount of AIP in the system. The inhibition studies for the *agr*-null Group I and II strains reconstituted with the AgrA + C modules were performed with activator at 100 nM, which was determined to be a saturating but not oversaturating dose of activator. This generated maximal activation against which to test various concentrations of inhibiting heterologous AIPs. This differs from the natural situation, where the concentration of the endogenously produced peptide appears to be less than 100 nM, on the basis of comparison of the measured IC_{50} values with those of a previous study by using endogenously produced peptide as the source of activator (12).

The apparent difference in the roles of the ring and tail moieties of the Group II AIP could be interpreted to mean that the ring structure is responsible for recognition of and binding to the putative ligand pocket of the receptor, and that the tail region is responsible for initiating the signaling process. It was reasoned that removal of the tail region might render a ligand that should be unable to activate AgrC signaling but should be a strong inhibitor of its cognate AgrC as well as of the heterologous AgrCs. This was confirmed by using a synthetic Group II AIP lacking the tail but retaining the thiolactone structure. This peptide inhibited not only the Group II receptor but also those of Groups I, III, and IV (which, based on our current understanding of *agr* grouping, represents global *agr* inhibition in *S. aureus*). Furthermore, the truncated analog weakly inhibited δ−toxin production in the *S. epidermidis* strain, RN2375, but strongly inhibited δ−toxin production in the *S. warnerii* strain, RN3178. These results suggest that the truncated thiolactone Group II AIP may serve as the model for a global inhibitor of staphylococcal *agr* activation, which may have significant clinical utility.

The observation that an AIP agonist of the *agr* response (i.e., the intragroup AIP) can be converted into an antagonist of that response by removing the tail (i.e., the truncated AIP) provides further compelling evidence that AgrC is the molecular locus of activation and inhibition. These results also suggest that other less dramatic modifications in the AIP tail might lead to the generation of global inhibitors of the *agr* response. Several questions remain to be answered in this system. For example, we have yet to fully account for why the lactam and lactone Group II AIP analogs are potent intergroup inhibitors but are not potent self activators or inhibitors. Clearly, this points to the thiolactone linkage being an important determinant of self activation or inhibition. We have previously speculated that the acylating nature of the thioester group might play a role in these processes and, although the current study does not address this issue specifically, it does identify AgrC as the likely target of such a modification, should it take place. Future studies will now address this issue, as well as whether activation and inhibition occur within the same binding pocket of AgrC.

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