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Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria

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Abstract

There are two basic types of bacterial communication systems—those in which the signal is directed solely at other organisms and those in which the signal is sensed by the producing organism as well. The former are involved primarily in conjugation; the latter in adaptation to the environment. Gram-positive bacteria use small peptides for both types of signaling, whereas Gram-negative bacteria use homoserine lactones. Since adaptation signals are autoinducers the response is population-density-dependent and has been referred to as "quorum-sensing". Gram-negative bacteria internalize the signals which act upon an intracellular receptor, whereas Gram-positive bacteria use them as ligands for the extracellular receptor of a two-component signaling module. In both cases, the signal activates a complex adaptation response involving many genes.

Keywords: Peptide signaling; *Staphylococcus aureus*; Gram-positive bacteria; Histidine kinase

1. Introduction

Both unicellular organisms and individual cells of metazoans have evolved complex signaling mechanisms by which they respond to the environment and communicate with one another. In bacteria, these mechanisms involve the production and release of small molecules that are sensed by organisms possessing cognate sensors, each setting in motion a more or less complicated response pathway. There are two basic types of bacterial communication systems—those in which the signal is directed solely at other organisms and those in which the signal is sensed by the producing organism as well. The first is typified by the mating pheromones of the enterococci, characterized in great detail by Clewell et al. [11] and by Dunny et al. [17]. Here, a potential recipient releases a small peptide that is taken up by a potential donor, initiating a plasmid-coded response that leads to cellular aggregation and transfer of the plasmid from donor to recipient. The second is the autoinduction or quorum-sensing (QS) systems, widespread among bacteria, that generate population-wide responses to low molecular weight signaling molecules, dependent on cellular density (Table 1 and Figs. 1 and 2) [20,21,74].

In Gram-positive bacteria, the mating pheromones and most QS signals are peptides. The latter are sensed by the transmembrane receptor component of a two-component signal transduction module (TCS), thus activating an intracellular response pathway (Fig. 1A). In Gram-negative bacteria, most QS signals are N-acyl homoserine lactones, which are internalized by diffusion and bind to an intracellular receptor molecule to activate the response (Figs. 1B and 2B). It has naturally been assumed that this differentiation is a consequence of the difference in structure of the cell envelope between Gram-positive and Gram-negative bacteria; however, this is probably an oversimplification since there are important exceptions. Thus, on the one hand, *Streptomyces* species

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Table 1
Bacterial processes controlled by quorum-sensing

Processes	Bacterial species	Signaling molecules
Virulence/ competence	*S. pneumoniae*	CSP, BlpC[a]
Virulence	*E. faecalis*	GBAP, $CylL_s''$
Virulence	*S. aureus*	AIP
Competence	*B. subtilis*, *S. pneumonia*	ComX, CSF, CSP
Bacteriocin production	*L. lactis*, *S. pneumoniae*	Nisin, PlnA, CB2, CS, etc.
Bioluminescence	*V. harveyi*	HSLs, AI-2
Virulence	*V. cholerae*	CAI-1, AI-2
Biofilms/virulence	*Pseudomonas aeruginosa*	HSLs
Conjugation	*Agrobacterium*	HSLs
Plant infection	*Bradyrhizobium japonicum*	Bradyoxetin

[a] Gram-negative bacteria are shaded in gray.

use gamma-butyrolactones for signaling [78], and both types of bacteria appear to use a novel furanosyl borate diester, AI-2, as a universal signaling molecule (Fig. 2) [89]; on the other hand genomic data suggest the existence of signaling peptides in Gram-negative bacteria [54]. Intercellular signaling in bacteria has, not surprisingly, become an area of intense interest in recent years and has generated a vast body of literature, various components of which have been ably reviewed in recent years [7,87]. A widespread subsection of the signaling world, the signaling peptides of Gram-positive bacteria, is the subject of the present review, focusing mainly on the autoinducing peptides (AIPs) and their receptors in staphylococci.

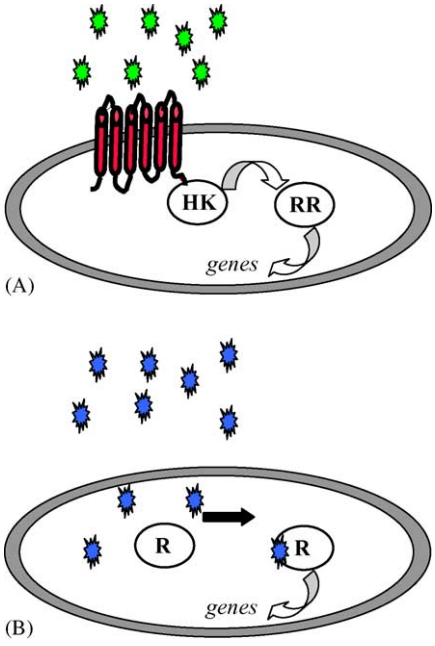


Fig. 1. Schematic of chemical signaling in bacteria. (A) Peptide signaling through receptor-histidine kinases (RHKs) in Gram-positive bacteria. The extracellular signaling molecules, shown as stars, bind to the sensor domain of the RHK, thus triggering activation via phosphorylation or dephosphorylation of the HK domain. A classic phosphorelay to or from the response regulator (RR) ensues, which controls gene expression at the level of transcription. The sensor domain of RHK's contains a variable number of transmembrane helices, with 6–8 TM helices as the standard for peptide binding. (B) Small molecule signaling through intracellular receptors in Gram-negative bacteria. An intracellular receptor protein, labeled as R, is stabilized upon binding the diffusible or actively transported signaling molecules (shown as stars). This receptor protein then binds to DNA and modulates gene expression.

2. The staphylococcal *agr* system

Analysis of the *Staphylococcus aureus* genome has revealed 17 putative two-component systems [40], all of which are presumed to control accessory functions and to play some role in cell–cell or cell–environment communication. At least four of these two-component systems, *agr*, *sae*, *arl*, and *srr* (*srh*) participate in the regulation of accessory genes involved in virulence [58]. Of these, the only one that is known to involve peptide signaling is *agr*, a ∼3 kb locus containing divergent transcription units, driven by promoters P2 and P3 (see diagram, Fig. 3). The P2 operon encodes the signaling module, a TCS of which AgrC is the receptor-histidine kinase and AgrA the response regulator, and its secreted autoinducing ligand, the AIP [35,59]. The primary function of the signaling module is to activate the two *agr* promoters; remarkably, however, the P3 transcript, RNAIII, rather than the response regulator, AgrA, is the intracellular effector of target gene regulation [32,61]. *Agr* activation leads to increased production and secretion of exoproteins, including virulence factors, and decreased production of many surface proteins (Fig. 3 and Table 2) [61,80].

As *agr* is autoinduced by an extracellular ligand, it represents a sensor of population density. Since the activating ligand is encoded within the operon, the circuit is doubly autocatalytic, resulting in a very rapid burst of activity once the autoinduction threshold has been reached.

3. Synthesis and activity of the *agr* AIP

The AIP is derived from a propeptide, AgrD, by the processing enzyme, AgrB, which removes segments from both the N- and C-terminal ends of the propeptide (G. Ji, personal communication). The N-terminal processing event appears to be a simple proteolytic cleavage, whereas C-terminal processing involves a concerted cleavage and condensation, resulting in a thioester linkage between a conserved cysteine and the C-terminal carboxyl. This thiolactone ring is the hallmark of the AgrD peptides, the only known exceptions being *Staphylococcus intermedius* and the *Enterococcus*, which have a serine instead of a cysteine [36,57] and produce AIPs with a lactone rather than a thiolactone ring. The length of the AIPs varies among different staphylococcal strains and species from 7 to 9 amino acids, with an invariant cysteine located five amino acids from the C-terminus. The C-terminus, initially established by sequencing AIPs-I and-II, is followed by a motif, DEPEVP, that is conserved, with minor variations, in over 30 different AgrDs (Fig. 4) [15], and is likely

SEX PHEROMONES

Enterococcus faecalis

```
cAD1    LFSLVLAG
cDF10   LVTLVFV
cPD1    FLVMFLSG
cAM373  AIFILAS
cOB1    VAVLVLGA
```

COMPETENCE PHEROMONES

Bacillus subtilis ComX

```
Strain 168     ADPITRQWGD
Strain RO-B-2     TNGNWVPS
CSF            ERGMT
```

Streptococcus pneumoniae

```
 CSP-1    ... GG EMRLSKFFRDFILQRKK
 CSP-2    ... GG EMRISRIILDFLFLRKK
S. gordonii
 CSP-1    ... GG DIRHRINNSIWRDIFLKRK
 CSP-1    ... GG DIRHRINNSIWRDIFLKRK
S. oralis
 CSP-1    ... GG DKRLPYFFKHLFSNRTK
 CSP-2    ... GG DWRISETIRNLIFPRRK
S. mitis
 CSP-1    ... GG EMRKPDGALFNLFRRR
 CSP-2    ... GG EMRKSNNNFFHFLRRI
```

BACTERIOCINS

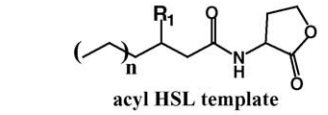
Lactobacillus plantarum

```
 PlnK       ... GG RRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK
 PlnJ       ... GG WKNFWSSLRKGFYDGEAGRAIRR
 PlnN       ... GG KNYSKTWWYKSLTLLGKVAEGTSSAWHGLG
 PlnA (AIP) ... GG KSSAYSLQMGATAIKQVKKLFKKWGW
 PlnF       ... GG VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG
 PlnE       ... GG FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR
```

S. pneumoniae

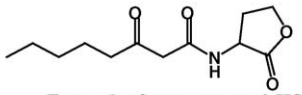
```
 BlpC-1   ... GG GLWEDLLYNINRYAHYIT
 BlpC-2   ... GG GLWEDILYSLNIIKHNNTKGLHHPIQL
 BlpC-3   ... GG GWWEELLHETILSKFKITKALELPIQL
```

(A)



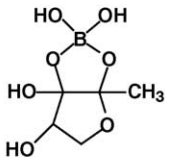
acyl HSL template

R_1 = O, OH, H n = 0, 1, 2, 3, 4, 5



Example: 3-oxo-octanoyl-HSL

(B)



(C) **AI-2**

Fig. 2. Bacterial signaling molecules. (A) Signaling peptides and bacteriocins of Gram-positive bacteria. Some of the signaling peptides and bacteriocins are N-terminally processed at a specific double glycine motif (shown as . . . GG) and secreted by unique bifunctional proteins containing a serine protease domain for processing and an ABC transporter for secretion of the peptide (see text). (B) Acyl-homoserine lactones (HSLs) of Gram-negative bacteria. A general scheme for HSL structure is shown. The acyl chains of virtually all HSLs have an even number of carbons regardless of chain length, derived from their metabolic synthesis, and some HSLs contain an unsaturated double bond in their acyl chain. A typical HSL, from *Agrobacterium tumefaciens*, is shown. (C) AI-2. Shown to trigger bioluminescence and virulence in *Vibrio harveyi* and *Vibrio cholerae*, respectively, and to serve as a universal signaling molecule in diverse species of bacteria.

to be involved in C-terminal processing. The only conserved residue in the N-terminal region of the propeptide is an absolutely conserved glycine 8 or 9 residues N-terminal to the conserved cysteine (Fig. 4). The enterococcal AIP is an 11-residue peptide with a 9-membered lactone ring (Fig. 4) [57].

Although, as noted below, most of the other known signaling peptides in Gram-positive bacteria are linear, linear peptides are, with a very few exceptions, inactive in the *agr* system. One of these exception is YSPWTNF ("RIP"), alleged to be the *agr* inhibitor produced by strain RN833 (described as a mutagenized *S. aureus* in our strain list). RIP was initially proposed to have been derived by mutation from YKPITN, an internal sequence in the N-terminal region of ribosomal protein L2 (aka RAP), which is claimed to be the *agr* activator [4,37]. Subsequently, it was found that RN833 is actually a native strain of *Staphylococcus warneri* [60], producing a typical AgrD-derived AIP, YSPCTNFF [60]. Balaban et al. subsequently have claimed that YSPWTNF (RIP) is encoded in the RN833 genome, by the sequence TATTCGCCGTGGACCAATTTTTGA, in addition to the true *agrD* peptide [60]. In fact, YSPXTNF, the actual sequence determined by Balaban et al., for the RN833 *agr* inhibitor, with "X" being indeterminate [4], corresponds very well to the true RN833 AIP, YSPCTNFF, a typical cyclic thiolactone peptide, except that it lacks the second phenylalanine. Additionally, RIP contains a tryptophan arbitrarily inserted in place of the genetically determined cysteine. Several laboratories have independently synthesized YSPWTNF and have been unable to demonstrate *agr* inhibition by this peptide at concentrations up to 50 μM (about 2000-fold higher than the IC_{50} of native AIPs [53]; T. Muir, personal communication, M. Booth, personal communication, J. Larrick, personal communication). Nevertheless, YSPWTNF (synthetic "RIP") and related linear peptides have been reported to block subcutaneous *S. aureus* abscesses in mice, albeit at extraordinarily high concentrations, between 2 and 10 mM [3,24], approximately 10,000

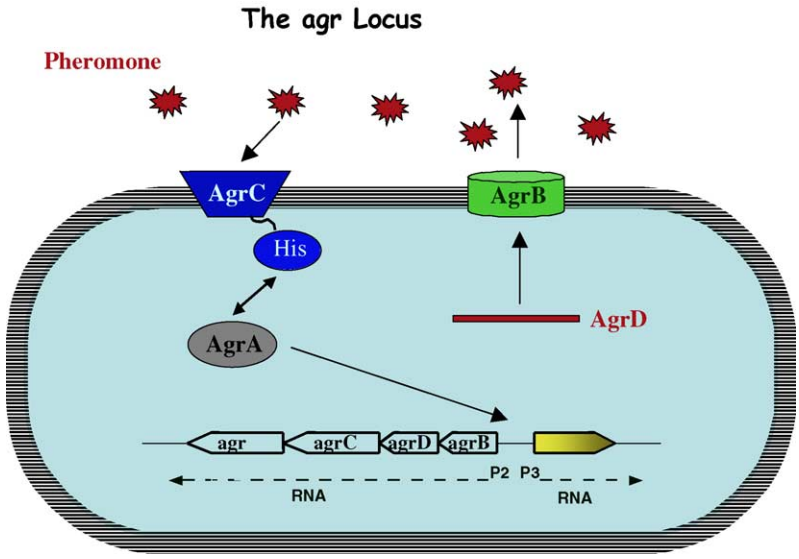


Fig. 3. The agr locus. The various components are shown, indicating their functions in the signaling network. This signaling system is a doubly auto-catalytic circuit, as AgrC activation induces transcription of more receptor, along with more of the propeptide inducer, AgrD, processed by AgrB to form the pheromone AIPs. The end result of the pathway is activation of RNAIII transcription by the response regulator, AgrA, which leads to downstream activation and repression of virulence-associated factors via transcriptional and translational regulation by this unique RNA molecule [6].

Table 2
Important virulence proteins of *S. aureus*

Protein	Pathogenic action	*Agr* regulation
Superantigens		
Enterotoxin A	Food poisoning, TSS	–
Enterotoxin B	Food poisoning, TSS	+
Enterotoxin C	Food poisoning, TSS	+
Enterotoxin D	Food poisoning, TSS	+
Enterotoxin E	Food poisoning, TSS	–
TSST-1	Toxic shock syndrome	+
Exfoliatin A (ETA)	Scalded skin syndrome	+
Exfoliatin B (ETB)	Scalded skin syndrome	+
Cytotoxins		
α-Hemolysin	Hemolysis, necrosis	+
β-Hemolysin	Hemolysis, necrosis	+
δ-Hemolysin	?	+
γ-Hemolysin	Hemolysis, necrosis	+
Leukocidin	Leukolysis	+
Enzymes		
Proteases	Spread, nutrition	+
Nucleases	Spread, nutrition	–
Lipases	Spread, nutrition	+
Hyaluronidase	Spread, nutrition	–
Esterases	Inactivation of toxic	
Fatty acids	+	
Surface proteins		
Protein A	Anti-phagocytosis	+
Coagulase	?	+
Clumping factors	?	–
Fibronectin binding proteins	Adhesion	+

+: synthesis changes in response to activation of agr signaling, with decreased production of surface factors and increased production of the other listed virulence factors; –: no agr effect.[58].

to 100,000-fold greater than the effective concentration of a native inhibitory AIP in the same model [52]. These peptides were pre-incubated with the bacteria, never injected separately, nor tested for general detrimental effects on the bacteria or on organisms other than *S. aureus*. Moreover, RIP has been reported to interfere with biofilm formation, at similar high doses, in an *agr*-independent manner [5]. It is, therefore, suggested that the properties of RIP represent non-specific detrimental effects of a random peptide at high doses, which have nothing to do with *agr*.

We have developed an efficient method for the chemical synthesis of the thiolactone AIPs [50,52]. This method uses unprotected peptides in solution or on resin and relies on a chemoselective transthioesterification reaction that proceeds quantitatively and without epimerization of the C-terminal amino acid [12]. Other published methods for the synthesis of cyclic lactone or thiolactone peptides depend on reactions involving cyclization of protected peptides in solution [53]. These methods may result in racemization of the C-terminal amino acid, which could affect the in vitro behavior of the peptide, especially if this is not discerned and proper purification not performed. Although substitution of D-methionine for the C-terminal L-methionine of AIP-I had no effect [53] (see below), substitution of D- for L-amino acids at most other positions substantially reduced the activity of AIP-I (Table 3). The possible effect on the AIP of racemization of the C-terminal carboxyl has not been fully addressed. Therefore, it is our view that any AIP synthesized by cyclization of protected peptides in solution with activated C-terminal carboxyl groups should be characterized to establish the stereochemical configuration of the C-terminal amino acid.

```
SaurI(RN6390)    MNTLFNLFFD-FITGILKNIGNIAA YSTCDFIM[1] DEVEV-PKELTQLHE-------
SaurIVRN4850)    MNTLLNIFFD-FITGVLKNIGNVAS YSTCYFIM[1] DEVEV-PKELTQLHE-------
SaurIII(RN3984) MKKLLNKVIE-LLVDFFNSIGYRAAY INCDFLL[1] DEAEV-PKELTQLHE-------
SaurII(502A)     MNTLVNMFFD-FIIKLAKAIGIVG GVNACSSLF[1] DEPKV-PAELTNLYDK------
SarI              MN-LLNSFFSFFAKKFFELIGTVAG VNPCGGWF[2] DEPEV-PEELTKYSE-------
SarcI             MMK-LVNLLLSSTTSILQMVGNRQK AKTCTVLY[2] DEPEV--KELTQELEK------
SarcII            MMK--DNLLLSSTTSILQMVGNRSK TKTCTVLY[2] DEPEV--KELIQELEK------
ScapI           MIMNSLFNLIFKFFTVIFEFIGFVAG ANPCQLYY[2] DEPEV-PEELSKLYE-------
ScapII          MIMDALFNLIFKFFTVIFEFIGFVAG ANPCALYY[2] DEPEV-PDELSKLYE-------
ScaprI           MMQII-NLLFKVITAVFEKIGFIAG YSTCSYYF[2] DEPEV-PKELLEIYKK------
ScaprII       MKMMQIF-DLLFKVISAVFEKIGFLAG YRTCNTYF[2] DEPEV-PKELFETYQK------
Scarn        MNFNMDIL-NGIFKFFAFIFEQIGNIAK YNPCVGYF[2] DEPEV-PSELLDEQK-------
Sconc             MHIFESIINLFVKF-FSVLGAISG GKVCSAYF[2] DEPEV-PKEIKDLYK-------
Sconu             MNIFESIINLFAKF-FAFIGTISS VKPCTGFA[2] DEPEI-PKELTDLYK-------
SepiII           MEIIFNLFIKFFTTI-LEFIGTVAG DSVCASYF[1] DEPEV-PEELTKLYE-------
SepiA086         MENIFNLFIKFFTTI-LEFIGTVAG DSVCASYF[2] DEPEV-PEELTKL---------
SepiI             MNLLGGLLLKLFSNFMAVIGSAAK YNPCASYL[2] DEPQV-PEELTKLDE-------
SepiIII          MNKLLGGLLLKIFSNFMAVIGNASK YNPCSNYL[2] DEPQVLPEELTKLDE-------
SepiIV           MNKLLGGLLLKIFSNFMAVIGNAAK YNPCANYL[2] DEPQVLPEELTKLDE-------
Sint              MRILEVLFNLITN-LFQSIGTFA RIPTSTGFF[1] DEPEI-PAELLEEEK-------
SlugI            MN-LLSGLFTKGISAIFEFIGNFSAQ DICNAYF[1] DEPEV-PQELIDLQRKQLIESV
SlugII           MN-LLSGLFTKGISVIFEFIGNFSVQ DMCNGYF[2] DEPEV-PQELIDLHRN------
SsimI             MDLLNGIFKLFA-FIFEKIGNLAK YNPCLGFL[2] DEPTV-PKELLEEDK-------
SsimII            MELLNGIFKLFA-FIFEKIGNLAK YYPCFGFL[2] DESEV-PQELLDEDK-------
SgaI              MNILDSLLNLATKF-FSALGASVG ARPCGGFF[2] DEPEV-PAEITELHK-------
SxyI              MNIFESILNLFAKF-FSVLGVMAG AKPCGGFF[2] DEPEV-PSEITKLYE-------
Swar(RN833)       MEFLVNLFFKFFTSIMEFVGFVAG YSPCTNFF[1] DEPEV-PSELTKIYES------
RIP (RN833)                              YSPWTNF
Efaec                          DGVGTKPRLN QNSPNIFGQWM[1] GQTEKPKKNIEK
[1]AIP amino acid sequence determined by direct sequencing, mass spectroscopy, or in vitro
synthesis. [2]Predicted.
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Fig. 4. Lineup of predicted AgrD propeptide sequences from various staphylococci and the *E. faecalis* pro-AIP (GBAP). Sequences were aligned visually. Predicted AIPs are in boldface and are set between spaces. Those whose sequence has been confirmed by in vitro synthesis or by mass spectroscopy are highlighted in gray. Abbreviations: Saur, *S. aureus*; Sarc, *S. auricularis*; Sarl, *S. arletta*; Scap, *S. capitis*; Scapr, *S. capri*; Scarn, *S. carnosus*; Sconc, *S. cohneii cohneii*; Sconu, *S. cohneii urealyticum*; Sepi, *S. epidermidis*; Sgal, *S. gallinarum*; Sint, *S. intermedius*; Slug, *S. lugdunensis*; Ssim, *S. simulans*; Swar, *S. warneri*; Sxyl, *S. xylosus*; Efaec, *E. faecalis*.

The sequence of the AIPs is highly variable, resulting in at least four specificity groups of strains within *S. aureus*, referred to as *agr*-I–IV, and many more (>25) in other staphylococci (Fig. 4) [15,33,34,64]. A group is defined as the collection of strains that produce the same AIP. The *agrB*, *D*, and *C* regions vary in concert to maintain the specificity of AIP processing and function [34], and this specificity results in four different sets of matching components in *S. aureus*, designated AIP-I–IV, AgrB-I–IV, and AgrC-I–IV, respectively. Remarkably, there is extensive cross-communication at the level of ligand-mediated signaling, as most AIPs inhibit the activation of heterologous receptors [34]. This inhibition is a form of bacterial interference that does not result in growth inhibition but rather in the blockage of accessory gene functions, presumably resulting in an advantage for the strain producing the most abundant and/or most potent AIP. The only exception to mutual cross-inhibition among the *S. aureus agr* groups is activation rather than inhibition of group I by AIP-IV, presumably owing to the strong similarity between the two AIPs [33]. This activity was confirmed both with bacterial supernatants and synthetic AIPs on groups I and IV cells [50]. McDowell et al. [53], however, found that AIP-IV, synthesized via cyclization of a protected peptide in solution, was an inhibitor of group I. This difference remains unexplained, although it is important to note that their synthetic AIP-IV was not tested for whether it could activate group IV cells, i.e. AgrC-IV.

Extensive structure–activity relationship (SAR) studies have been performed on the AIPs [48–50,52,53,63–65]. Note that the 5-amino acyl residue ring common to all of the known functional AIPs is referred to as the macrocycle; residues contained within the macrocycle are referred to as endocyclic, residues outside of the macrocycle (the linear "tail") are referred to as exocyclic. The SAR studies have shown (Figs. 2 and 5) and Table 3:

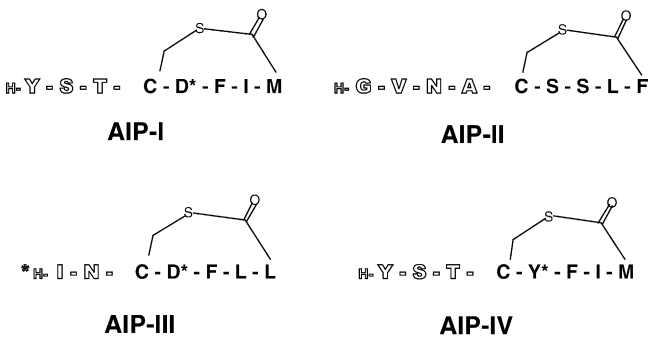

Fig. 5. Composition and key determinants of the *S. aureus* AIPs. Standard single letter codes for amino acids are used. The sulfur atom of the cysteine and the carbonyl contributed from the C-terminal amino acid are shown in a thioester linkage, which closes the macrocycle. Exocyclic (tail) residues are represented by outlined text. Residues that are critical for receptor activation are marked with symbol asterisk. The N-terminus of AIP-III is marked to reflect the fact that additional amino acids on the N-terminus abolish receptor activation, while the same does not hold true for AIP-I.

Table 3
Structure–activity analysis of AIPs-I and -II

Residue	Substitution 1[a]	Effects	Substitution 2[a]	Effects	Further substitution	Effects
AIP-I						
Y	A	Decreases activation	D-Tyr	Markedly decreased activation and no self-inhibition	Tail deletion (trAIP-I)[b]	Partial agonist of C-I and cross-inhibitor of C-II, -III, and -IV[b]
S	A	No difference from AIP-I	D-Ser	C-I self-inhibition		
T	A	Decreases activation	D-Thr	Markedly decreased activation and no self-inhibition		
C	S	Inhibits C-II, -III, and -IV	D-Cys	C-I self-inhibition	DAPA[a]	Inhibits C-II, -III, and -IV, weakly activates C-I
D	A[b]	Universal inhibitor	D-Asp	Markedly decreased activation and no self-inhibition	Y[b]	Converts to AIP-IV
					F[b]	Same activity as AIP-IV
					N[b]	Same activity as AIP-I, but also now activates C-III
F	A	Virtually inactive	D-Phe	No difference from AIP-I		
I	A	Virtually inactive	D-Ile	Markedly decreased activation and no self-inhibition		
M	A	Virtually inactive	D-Met	No difference from AIP-I	Nor-leu[a]	Inactive
					I[b]	Same as AIP-I, except much more potent activation of C-IV
					Met-sulfoxide	Inactive
AIP-II[b]						
G	A	Activation unchanged Increases cross-inhibition			Tail deletion (TrAIP-II)	Universal inhibitor
V	A	Decreases activation Increases cross-inhibition				
N	A	Universal inhibitor	L[c]	Universal inhibitor		
			D[c]	Universal inhibitor		
A	A	No difference from AIP-II				
C	S	Inhibits C-I, -III, and -IV	DAPA	Inhibits C-I, -III, and -IV, weakly activates C-II		
S	A	Decreases activation Increases cross-inhibition				
S	A	Increases activation and cross-inhibition				
L	A	Inactive				
F	A	Inactive				

All peptides are cyclized via the cysteine (or substituted) side-chain to the C-terminal carboxyl group. Single letter amino acid codes are used throughout, DAPA: diaminopropionic acid. None of the D-substituted AIP-I analogs were tested for cross-inhibition of C-II, -III or -IV.

[a] [53].

[b] [50,52].

[c] Unpublished results.

1. The interaction between activating (agonist) and inhibiting (antagonist) AIPs is strictly competitive [49], ruling out an earlier hypothesis that the AIP might make a stable covalent bond with the receptor [52].
2. The macrocycle is required for agonist activity, as all linear AIPs have thus far been inactive as agonists.
3. The nature of the linkage forming the macrocycle, normally comprised of a thioester bond, is critical for certain aspects of biological activity, as the lactam and lactone analogs of AIP-I and AIP-II are potent cross-group inhibitors but activate their cognate receptors only at very high concentrations [49,53]. This suggests that the AIP may have to adapt a particular conformation for receptor binding leading to activation but not for receptor binding leading to cross-inhibition. There are several possible explanations of why it might be thermodynamically more costly for the AIP lactams and lactones to assume an activating conformation. First, the lactam analogs have an amide proton as a hydrogen bond donor that does not exist in the thiolactone peptides. This could result in a different hydrogen bonding network. Second, the activation energy barrier for rotation around an amide linkage is

much higher than for rotation around a thioester linkage, thus incurring a thermodynamic penalty when the lactam analogs bind to AgrC. In the case of the lactone analog, there are subtle electronic and stereochemical differences between sulfur and oxygen in the context of a thiolactone versus a lactone linkage, which could collectively account for the difference in activity between them. Some of these differences are: (1) sulfur is less polar than oxygen but more polarizable; (2) sulfur has a larger radius than oxygen, 2.0 A versus 1.72 A; and (3) the bond angle around a sulfur atom is generally more acute than around an oxygen atom. A more detailed structural study of the AIP should help to resolve some of these issues.

An enduring question is why evolution has favored the use of a thioester linkage in the AIPs, which has not been described in any other receptor-ligand based signaling system. If the thioester linkage is not involved in acylation of the receptor, then other theories must be invoked to explain its presence. One possibility is that enzymatic formation of these peptides by the protein, AgrB, utilizes the relatively reactive thiolate anion of the cysteine side chain for cleavage of the pro-peptide sequence. The staphylococcal AgrBs cannot process an AgrD peptide containing a serine in place of the conserved cysteine (G. Ji and RPN, unpublished data). However, at least two enzymes have evolved, one in *S. intermedius* and one in *Enterococcus faecalis*, that can process serine-containing pro-peptides, generating AIP lactone peptides [36,57]. Alternatively, it is possible that the cyclization of the AIPs provides some level of protease resistance, but that the inherent lability of the thioester linkage at physiological pH nevertheless provides a built-in time limitation for the signal. This might allow the bacteria to down-regulate the energy-draining expression of the virulon in settings where virulence genes are not adaptive.

4. The AIP-II tail is necessary for AgrC-II activation but not for AgrC-I inhibition [52], and substitution of the exocyclic asparagine for alanine at position 3 in the AIP-II tail converts the peptide from an agonist to an antagonist of AgrC-II activation [49]. Removal of the AIP-II tail from an analog, trAIP-II, results in a peptide that is a universal inhibitor of *agr*. However, the "tailless" derivative of AIP-I is a partial agonist of AgrC-I [50].
5. Substitution of the endocyclic aspartate or tyrosine by alanine at position 5 in AIP-I or -IV, respectively, converts them from agonists into global antagonists of *agr* activation [50,53].
6. The overall composition of the known AIPs reveals a strong gradient of increasing hydrophobicity from N to C-terminus. The two C-terminal residues are, with a few exceptions, limited to the bulky hydrophobic amino acids, L, I, Y, and F, and occasionally others including methionine and tryptophan. Substitution of these by alanine eliminates all activity, suggesting that these residues are critical for receptor binding in a non-group-specific manner [52,53] (Wright et al. in preparation). The C-terminal methionine of AIP-I has been substituted by a variety of amino acids. Substituents with methionine sulfoxide, norleucine, serine, glutamate, lysine, and proline [53] had no activity, which suggested to McDowell et al. that the thioether side chain of methionine is required for activity. It is more likely, however, that AIP-I activity is based upon the above rule, as an isoleucine-containing derivative was fully active [50]. It is more difficult to explain the full activity of the D-methionine substituent reported by McDowell et al. [53] since there would be a major change in the stereochemistry of the side-chain. Since the structure of the AIP binding site in the receptor is not known, it is conceivable that D-methionine is acceptable as a bulky hydrophobic residue despite its stereochemistry. Further work is clearly required including more detailed pharmacological studies of these analogs in connection with the characterization of the ligand binding pocket in AgrC-I. These studies should help to explain the varied functionality of the other amino acid substituents (Table 3), including the alanine scan and the other D-amino acid replacements.
7. The linear tail and macrocycle interact minimally, if at all, in solution, as determined by two-dimensional ^{1}H NMR [47,50]. Nevertheless, the presence and composition of the tail has profound effects on the function of the AIP [47,48,50].
8. While AIP agonist activity is very sensitive to amino acid side chain and structural changes, cross-inhibition by the AIPs seems to be more tolerant of sequence and structural diversity, as the AIP-II lactam and several alanine-substituted analogs have potent antagonist activity [52,53]. This observation has led to the suggestion that cross-inhibition by the AIPs involves a binding interaction that is different from that of agonist AIP binding, even though the inhibition is competitive [49] (illustrated schematically in Fig. 6).
9. An AIP chimera, constructed by fusing the AIP-II tail to the AIP-I macrocycle, possessing both of the residues in AIP-I and -II critical for agonist activity, did not activate and instead universally inhibited AgrC activation [50]. This means that single residues evidently cannot, per se, determine the function of an AIP. Perhaps the entire peptide is involved in achieving the spatial configuration required for activation, but not for inhibition; alternatively particular residues in the tail and macrocycle must match. In the chimeric AIPs, the entire peptide or its critical residues must not be presented to the receptor in the activation-specific orientation (Fig. 6). It is relevant in this context that the opposite chimera, which lacks both of the critical residues; was also a universal inhibitor.

An overall conclusion from these studies is that the AIP-receptor interaction is subtle and complex, cannot readily be deciphered by simple amino acid substitution studies, and is in need of detailed structural analysis.

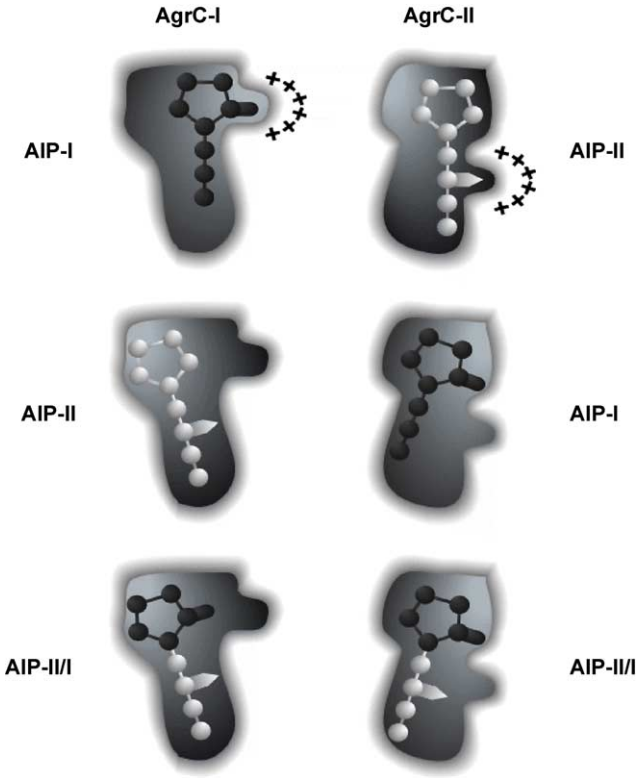

Fig. 6. Model of receptor binding modes: AIP-I, AIP-II, and the chimeric peptide, AIP-II/I, are depicted schematically as bound to the ligand binding pocket of AgrC-I and -II. The key endocyclic residue (D5) of AIP-I and the key exocyclic residue (N3) of AIP-II insert into hypothetical grooves of AgrC-I and -II, respectively, triggering receptor activation, as illustrated by +++++. Cross-inhibition most likely involves different orientations of binding to the heterologous receptor, as depicted by the tilt of the ligands in the binding pockets of AgrC-I and -II. The chimeric peptide, AIP-II/I, does not activate and instead inhibits AgrC-I and -II because it also binds in a different orientation, thus preventing the key residues from activating their respective receptor. This is a highly schematic model for the sole purpose of illustrating different modes of binding, and there is currently no information available concerning the ligand binding pocket within the sensor domain of AgrC.

4. Rationally designed antagonists of receptor-ligand interactions—the *S. aureus* AIPs

Agr mutants in *S. aureus* are greatly attenuated for virulence in several animal models of infection [1,8,9,22,23,80], thus suggesting that blockade of *agr* signaling in vivo might have therapeutic utility. Toward this end, the availability of naturally occurring peptide-based *agr* antagonists (vide supra) has opened the door to peptidomimetic and/or small molecule drug discovery efforts, much akin to what has been done so successfully in the pharmaceutical industry for the inhibition of G-protein coupled receptor and other hormonally-based signaling pathways [31]. In fact, the *agr* system is the only bacterial system described thus far where interference with quorum-sensing by the addition of an exogenous substance has been shown to attenuate virulence in an animal model of infection [52] (R. Jin and RPN, unpublished data). In these studies, a subcutaneous abscess mouse model of *S. aureus* infection was used to demonstrate that co-injection of AIP-II with the bacteria or subsequent injection of the AIP at the same site, in nanomolar concentrations, greatly attenuated abscess formation by *agr* group I *S. aureus*. Substitutions of key amino acids or removal of the linear "tail" of AIP-II (see above and Table 3) resulted in derivatives that inhibited *agr* activation for all four *S. aureus* groups (i.e. universal *agr* inhibitors) [48,50]. Ongoing studies in the authors' laboratories are focused on the testing of alanine substitutions in AIPs-I, -II, and -III and their lactone or lactam derivatives for activities in vivo and on the design and/or discovery of other peptidomimetic or small molecule antagonists of *agr* signaling that can be tested in animal models of infection.

5. AgrB and its possible functions

The mechanism by which the AIPs are processed from the propeptide, AgrD, and subsequently secreted has recently been addressed [72,90]. One group overexpressed AgrB and AgrD in *Staphylococcus epidermidis* and showed that the concentration of AIP produced was not greater than that seen in wild-type strains, ~20 nM. This result led to the suggestion that processing or secretion of the AIPs might be controlled by an overriding regulator or might require some other rate-limiting factor besides AgrB or AgrD [72]. A His-tagged cleavage product of AgrD that was derived either from cleavage at the N- or C-terminus of the AIP by AgrB was detected [90]; unfortunately, the resolution of SDS-PAGE analysis did not allow distinction between the two possibilities. Finally, it has been proposed that processing of AgrD by AgrB might proceed through an acyl-enzyme intermediate resolved by nucleophilic attack by the sulfhydryl of the cysteine side-chain in the AIP [90].

Saenz et al. [72] demonstrated that AgrB is a membrane protein, consistent with hydropathy analysis. Zhang et al. utilized a His-tagged version of AgrB expressed in *S. aureus* and *E. coli*, which allowed them to confirm the membrane localization of AgrB, and, using PhoA fusion analysis, to derive a transmembrane topology model of AgrB (Fig. 7). This model is consistent with some, but not all, of the computer-predicted transmembrane helices [90]; in fact, it predicts two transmembrane helices that are only twelve amino acids in length, which is not normally long enough to span the membrane, separated by a region whose localization is ambiguous. Perhaps this region of ambiguous topology is involved in transporting the peptide. As shown in Fig. 7, there is a conserved cysteine in AgrB that might play a role in forming a thioester acyl-enzyme intermediate.

6. AgrC, the AIP receptor

AgrC was inferred by sequence analysis to be a histidine-protein kinase, was shown by *phoA* fusions to have a polytopic transmembrane N-terminal domain [46], and by pull-

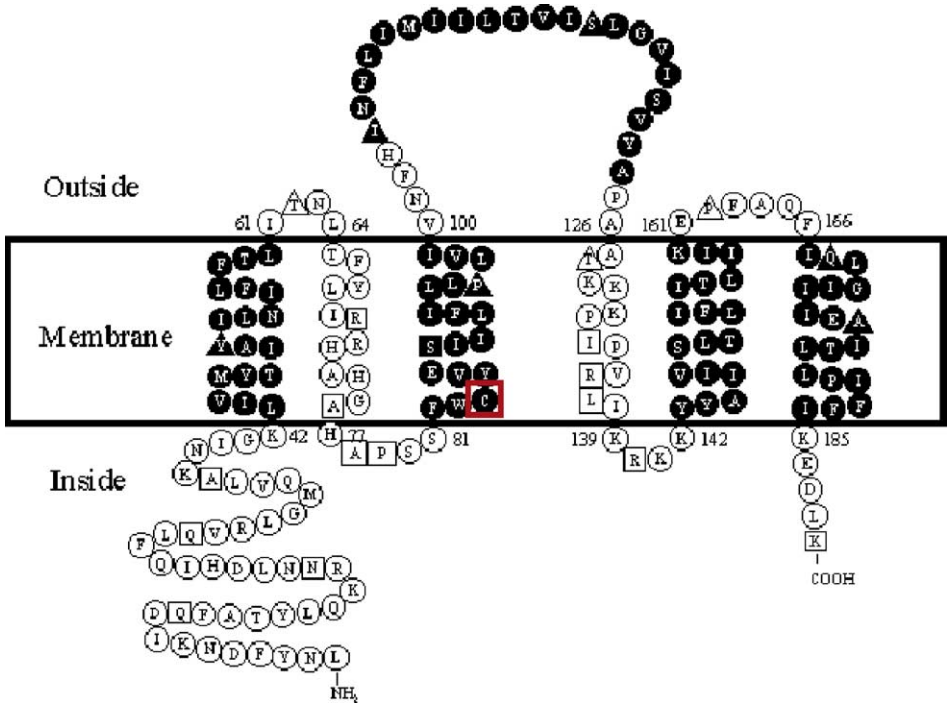

Fig. 7. AgrB topology. The transmembrane topology of AgrB-I is shown, with the cysteine residue boxed that is speculated to be of importance in AgrD processing. The location of the AgrB–PhoA fusion points are indicated with triangles and rectangles: triangle, high PhoA activities; rectangle, low PhoA activities. This figure has been reproduced from Zhang et al. [90]. The predicted transmembrane α-helices by TopPred II, TMHMM, and DAS analyses are indicated by filled shapes.

down studies to be the only cellular protein capable of binding the AIP [35], thus confirming that AgrC is the receptor of the *agr* TCS. Bioinformatics analysis of the cytoplasmic histidine protein kinase (HPK) domain has revealed with high certainty (*E*-value 4e-50 using SMART and PFAM search tools) strong similarity to the histidine-ATPase motif, commonly found in HPKs of known structure such as EnvZ [79] and PhoQ [51]. AgrC, like other transmembrane histidine kinases [18], probably functions as a preformed dimer: computer analysis has revealed that the linker connecting the sensor domain to the HPK domain of AgrC has high helical propensity and the potential to form a coiled coil, followed by a helix-turn-helix (Fig. 8). This is consistent with structural studies on the *E. coli* osmosensor EnvZ, which has a four-helix bundle dimerization motif formed from within the linker region [83] (see model – Fig. 9).

It is unclear, however, whether *agr* activation follows the classical TCS paradigm. Thus, our original report that AgrC is phosphorylated in response to the AIP [46] has recently been re-examined by Guangyong Ji, who finds AgrC to be at least partially phosphorylated in the absence of any AIP (G. Ji, personal communication). Additionally, the isolated HPK domain is constitutively autophosphorylated (unpublished data), as is typical for HPKs.

Predictably, group specificity resides in the N-terminal transmembrane domain of AgrC, as demonstrated by switching the two domains between AgrCs of different groups [50]. And switching the proximal and distal halves of the N-terminal (receptor) domain of the AgrCs has localized the specific recognition of AIPs-I and -IV to the distal (C-terminal) subdomain [88] suggesting that the single amino acid that differs between these two AIPs (aspartate vs. tyrosine at position 5) makes a specific contact in this region of the receptor. These experiments have demonstrated that the proximal subdomain possesses additional binding determinants that increase the potency of ligand binding and/or receptor activation (Wright et al., unpublished data). Although this is consistent with previous data suggesting that the last extracellular loop of the receptor domain confers AIP-dependent phosphorylation activity [46], more recent results suggest that this loop may not affect the constitutive phosphorylation observed with the isolated histidine kinase domain (unpublished data). Other chimeras constructed by switching within the N-terminal receptor domain, have given results that are very difficult to explain on the basis of the classical "lock-and-key" model for intermolecular interactions. In particular, the I–III[1] and IV–III chimeras could not be inhibited but rather were activated by a variety of AIPs, including some that are strong inhibitors of all other *S. aureus* AgrCs. These results have given rise to a model in which interaction with the receptor involves two distinct events—first, the peptide enters a hydrophobic pocket of the receptor in a non-sequence-specific manner, requiring the two bulky C-terminal residues of the AIP. Next, it makes one or more specific hydrophilic con-

[1] Chimeras of this type are represented by the AgrC group of the promoter–proximal subdomain followed by that of the promoter–distal subdomain.

AgrC-I Protein Sequence: two putative helices underlined

1-VELLNSYNFVLFVLTQMILMFTIPAIISGIKYSKLDYFFIIVISTLSLFLFKMFDS
ASLIILTSFIIIMYFVKIKWYSILLIMTSQIILYCANYMYIVIYAYITKISDSIFVIF
PSFFVVYVTISILFSYIINRVLKKISTPYLILNKGFLIVISTILLLTFSLFFFYSQIN
SDEAKVIRQYSFIFIGITIFLSILTFVISQFLLKEMKYKRNQEEIETYYEYTLKIE
AINNEMRKFRHDYVNILTTLSEYIREDDMPG**LRDYFNKNIVPMKDNLQMNAIKLNGI**E
NLKVREIKGLITAKILRAQEMNIPISIEIPDEVSSINLNMIDLSRSIGIILDNAIEAS
TEIDDPIIRVAFIESENSVTFIVMNKCADDIPRIHELFQESFSTKGEGRGLGLSTLKE
IADNADNVLLDTIIENGFFYSKS-425

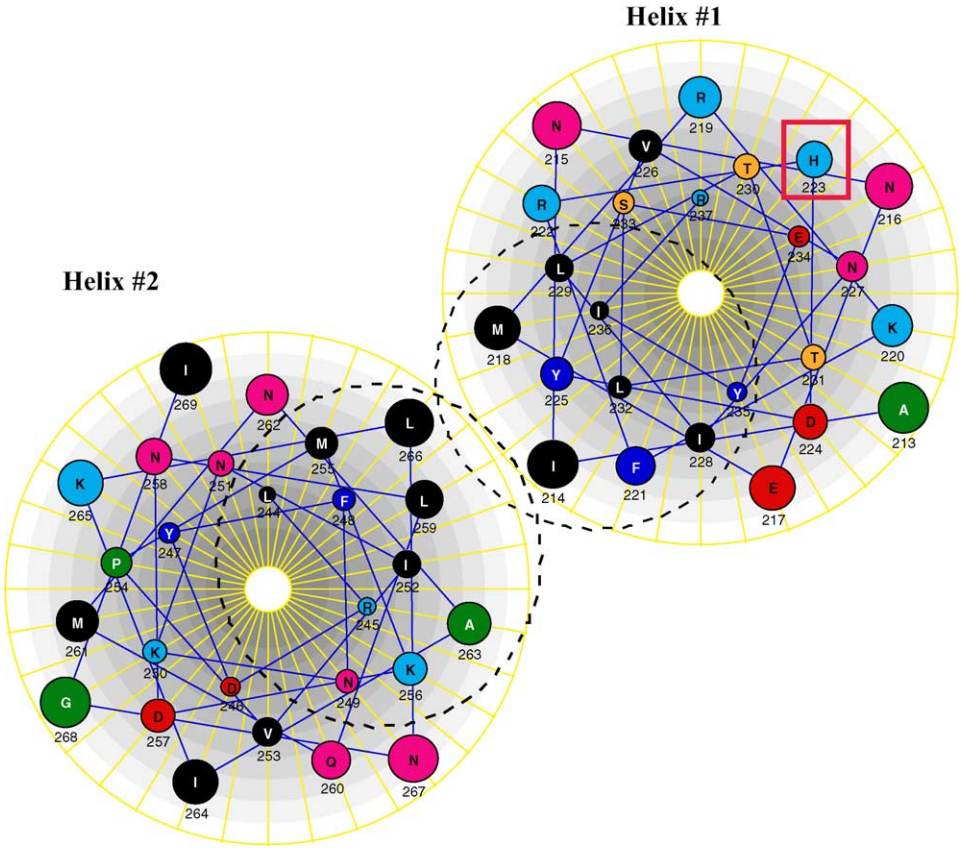

Fig. 8. Helical wheel analysis for the linker region of AgrC. The amino acid sequence of AgrC-I is shown, with portions of the linker connecting the sensor domain to the histidine kinase underlined. These portions are depicted with helical wheel analysis, showing the possible hydrophobic packing occurring in the formation of a four-helix bundle in an AgrC dimer, as indicated by the dotted circles. The critical and conserved histidine residue predicted to be phosphorylated is boxed.

tacts with specific sites in the receptor, leading to activation or inhibition. The broadening of specificity in the I–III and IV–III chimeras could then represent a situation in which the receptor is configured to be poised for activation by the binding of a hydrophobic AIP without the need for any specific activating contacts.

7. Peptide signaling in *Enterococcus faecalis*

7.1. Conjugative pheromones (see review by Clewell et al. [11])

Plasmid-mediated conjugation in enterococci involves a unique peptide-based signaling mechanism. Enterococci secrete 5 or more small hydrophobic peptides, each representing a portion of the signal peptide of a lipoprotein of unknown function [10]. Each of these peptides is recognized by a plasmid-coded receptor that is specific for a particular pheromone. Potential donor cells contain a plasmid-coded peptide that is a competitive inhibitor of the inducing peptide, and also contain a protein that down regulates synthesis of the inducing pheromone, which is therefore, present in plasmid-containing cells at a much lower level than in plasmid-negatives. The inducing pheromone is internalized by the oligopeptide permease and interacts with intracellular proteins and at least one RNA, initiating the expression of a complex genetic pathway that activates the synthesis of two key proteins, one responsible for surface exclusion—which prevents the organism from acting as a conjugative recipient—and a second that encodes an adhesin known as AS (aggregation substance), which is responsible

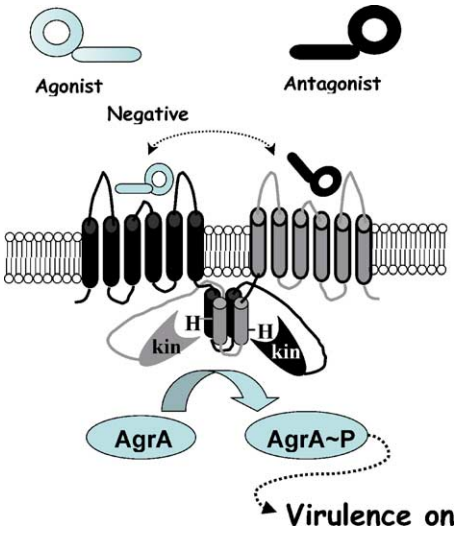

Fig. 9. Model of AgrC. The receptor is shown as a proposed dimer, mediated via a cytoplasmic four-helical bundle and possibly via interaction between the sensor domains, which themselves each have six transmembrane helices. The pharmacological data suggest possible allosteric negative cooperativity between the binding sites in a receptor dimer [49].

for attaching the organisms to suitable recipients, by means of specific receptors, ultimately leading to transfer of the plasmid from donor to recipient. Remarkably, AS is also an adhesin for mammalian cells and, is therefore, an important virulence factor [75]. It has been suggested that activation of the AS-synthetic pathway may involve mammalian peptides similar to the inducing pheromones [44].

7.2. Autoinduction of virulence

There are at least nine putative two-component systems found in the genome of *E. faecalis* [43], one of which, the *fsr* quorum-sensing system [68,69], is homologous to the *agr* system in *S. aureus*. The *fsr* locus encodes a receptor-histidine kinase, FsrC, a response regulator, FsrA, a putative processing enzyme, FsrB, homologous to AgrB, and an AgrD-like propeptide. It has been shown that all three genes in the *fsr* operon are important for the production of virulence factors, such as gelatinase and a serine protease, and that mutation of these genes results in attenuated virulence in a mouse peritonitis model and in a relatively new *Cenorhabditis elegans* killing model [55,76]. In contrast to AgrD, which is processed to generate the staphylococcal AIP, the propeptide from which the *E. faecalis* AIP (also referred to as GBAP—see Fig. 4) is derived is the C-terminal end of the putative processing enzyme, FsrB [56,57]. However, there is ∼19% sequence identity between FsrB and *S. aureus* AgrB-I–IV, and the propeptides in both systems are cleaved internally to release AIPs with new N- and C-termini. Furthermore, both AIPs contain a cyclic structure, formed from the condensation of the α-carboxyl group of the peptide with the nucleophilic side-chain of an amino acid located proximally in the AIP. For the *E. faecalis* AIP, the nucleophile is the hydroxyl group on a serine nine amino acids from the C-terminus, thus forming a peptide with a nine-amino acid lactone ring.

8. Cell–cell signaling in *Streptococcus pneumoniae* and *Bacillus subtilis*

The first studies hinting at the existence of pheromone-like signaling in bacteria related to the control of competence for transformation in *Streptococcus pneumoniae* [82]. Many years later, the signaling molecule that controlled competence development was characterized as an unmodified heptadecapeptide (competence stimulating peptide, CSP-1) [27] and the receptor, ComD, subsequently identified as the receptor component of a TCS module [28]. A later study identified a new set of *S. pneumoniae* strains making a different heptadecapeptide, CSP-2, differing from CSP-1 at eight residues (see Fig. 2) [67]. Further work demonstrated that many different pherotypes of CSP exist within many different streptococcal species, including *S. gordonii*, *S. oralis*, *S. mitis*, and *S. mutans* (see Fig. 2) [29,45]. The peptides of different pherotypes, unlike those of the *agr* groups, are inactive in heterologous combinations and do not cross-inhibit. As the competence regulon of *S. pneumoniae* includes a number of genes involved in pathogenesis, the QS system inducing competence has a key role in virulence [41]; furthermore, at least 13 other TCS have been characterized in *S. pneumoniae*, so that two-component signaling generally plays a significant role in pathogenesis [81]. Most recently, it has been shown that CSP signaling via the receptor, ComD, stimulates not only competence but also coordinated DNA release by donor cells within the same population, thus demonstrating the elegance of quorum-sensing mediated genetic exchange [77].

Natural genetic competence in *Bacillus subtilis* is also controlled by quorum-sensing. The signaling peptide ComX is detected by ComP, the HPK component of the ComAP TCS, which then triggers the expression of many genes required for competence development [84]. There are in fact at least 29 kinase-regulator pairs in the *B. subtilis* genome, and all of these have been studied to various extents by DNA microarray analysis [62]. In the case of ComP–ComA, novel genes were identified that are controlled by this system, which will require further study. This is similar to the *S. aureus* agr system, where a similar study yielded many new targets of regulation, including genes encoding cytoplasmic as well as extracellular proteins [16]. Recently, the ComX decapeptide has been shown to be isoprenylated on a conserved tryptophan, and this modification varies in different strains, thus giving rise to at least four species-specific pherotype groups in *B. subtilis* and other Gram-positive soil bacilli, some of which show moderate cross-inhibition [2]. These groups vary not only in ComX but also in the processing enzyme, ComQ, and in the sensor domain of ComP [85,86]. Indeed, the organization of the *comAP* operon is identical to that of the *agr*-P2 operon (Fig. 10), though the two share no sequence similarity be-

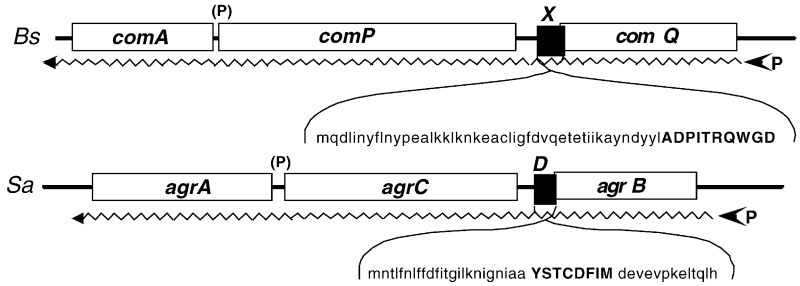

Fig. 10. The *B. subtilis comAP* and *S. aureus agr* operons have an identical organization. The operons for *B. subtilis* (Bs) and *S. aureus* (Sa) are depicted, with genes aligned according to similar functions, including *comQ* and *agrB*, *comX* and *agrD*, *comP* and *agrC*, and *comA* and *agrA*. The amino acid sequences of the proteins, ComX and AgrD, are shown, with the AIPs in boldface. The direction of transcription is indicated by an arrow and zigzag line representing the transcript. P: promoter.

yond that of the conserved motifs in the signaling proteins. A remarkable finding that allowed the characterization of the ComX modification was that expression of ComQ and ComX in *E. coli* was sufficient for pheromone production and secretion [85]; such results have not thus far been obtained for *S. aureus* AIPs. Lastly, the development of competence is further modulated by another peptide, an unmodified pentapeptide, CSF, which does not interact with a receptor at the cell surface but rather is actively transported into the cell by the oligopeptide permease Opp, where it inhibits the dephosphorylation of ComA∼P by RapC [42]. This peptide represents the five C-terminal amino acids of a 40 amino acid pre-pro-peptide, PhrC, which is exported as an 11–15 amino acid pro-peptide after signal peptide cleavage and finally processed extracellularly, probably by a protease. CSF has several different activities depending on its intracellular concentration. Thus, at lower concentrations, it activates competence by prolonging the activation phase of the TCS whereas at higher concentrations it inhibits competence and activates sporulation [42,66].

9. Quorum-sensing based control of bacteriocin production

The production in a cell-density dependent manner of antimicrobial compounds (bacteriocins) by lactic acid bacteria is well-established and extensive (for excellent reviews on the subject, see [19,70]). One example of this is nisin, the prototype of a large class of bacteriocins, the lantibiotics [25,30,73], which undergo extensive post-translational modifications, with ring structures formed by dehydration of cysteine in reaction with serine or threonine residues to form thioether-containing amino acids known as lanthionines. Genetic analysis has revealed that nisin production is stimulated in a cell-density dependent fashion by nisin itself. However, the antibacterial activity and the signaling activity of the peptide are distinct, depending on different amino acid residues, although ring formation in the peptide is required for both activities [38,39].

In most other instances, the signaling molecules that switch on bacteriocin production are similar to but distinct from the bacteriocins themselves. For example, in *Lactobacillus plantarum*, the QS plantaricin system encodes no less than five unrelated bacteriocins, all induced by the autoregulatory PlnA peptide, also unrelated (see Fig. 2) [14]. Similarly in *S. pneumoniae*, bacteriocin production is controlled by a processed and secreted linear peptide, BlpC or also named SpiP (see Fig. 2), that signals through a classic two-component signaling cascade [13]. There are at least three pherotypes of BlpC, with corresponding amino acid changes in the respective receptor-histidine kinases, particularly in the putative transmembrane helices II and V [13,71]. An interesting variation on this theme is seen with a different lactic acid bacterium, *Carnobacterium piscicola*, in which several bacteriocin operons are controlled by a TCS,*cbnKR*, which responds either to one of the bacteriocins, CB2 or to a different peptide, CS, which is evidently not a bacteriocin (Kleerebezem M., 2001 #6275). Most of the signaling peptides and bacteriocins in lactobacilli and streptococci are N-terminally processed at a specific double glycine motif and secreted by unique bifunctional proteins containing a serine protease domain for processing and an ABC transporter for secretion of the peptide [14].

Another example of quorum-sensing based control of toxin expression is illustrated by the regulation of cytolysin production in *E. faecalis* [26]. The synthesis of cytolysin is elaborate, involving post-translational modification, proteolytic cleavage, secretion, an additional step of extracellular proteolytic cleavage of two subunits, and then their dimerization to produce the mature product. One of these subunits, $CylL_s''$, induces transcription of the structural genes for both cytolysin subunits; furthermore, this transcriptional activity is dependent on the amount of $CylL_s''$ added, suggesting that cell-density dependent accumulation of one subunit signals the production of both subunits. Not only is the signaling molecule itself rather unique, but so too is the signal-sensing apparatus, which is not a classic two-component histidine-kinase/response regulator pair, but rather an apparent transmembrane protein of unknown function, CylR1, and a DNA-binding protein, CylR2 with an apparent helix-turn-helix motif.

10. Conclusions

We have only just begun to understand communication in the bacterial world. For example, there are at least 17, 9, 13, and 29 putative histidine kinase-response regulator pairs in *S. aureus*, *E. faecalis*, *S. pneumoniae*, and *B. subtilis*, respectively, and unknown numbers in other bacteria, for which the ligands have been identified in very few. Some signaling molecules will also undoubtedly act not through receptor-histidine kinases but rather through other uncharacterized proteins, as has already been seen with the case of cytolysin induction in *E. faecalis* [26]. Furthermore, it is becoming apparent that bacteria grow in communities (notably biofilms), therefore requiring extensive forms of intra- and inter-species communication. We have only just begun to understand some of the languages of a few members of the bacterial world. It is obvious that studying chemical communication among bacteria will surely further our understanding of the extraordinary diversity that surrounds us and may lead to new therapies against bacterial infections.

Acknowledgments

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