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Cell density control of staphylococcal virulence mediated by an octapeptide pheromone

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ABSTRACT **Some bacterial pathogens elaborate and secrete virulence factors in response to environmental signals, others in response to a specific host product, and still others in response to no discernible cue. In this study, we have demonstrated that the synthesis of *Staphylococcus aureus* virulence factors is controlled by a density-sensing system that utilizes an octapeptide produced by the organism itself. The octapeptide activates expression of the *agr* locus, a global regulator of the virulence response. This response involves the reciprocal regulation of genes encoding surface proteins and those encoding secreted virulence factors. As cells enter the postexponential phase, surface protein genes are repressed by *agr* and secretory protein genes are subsequently activated. The intracellular *agr* effector is a regulatory RNA, RNAIII, whose transcription is activated by an *agr*-encoded signal transduction system for which the octapeptide is the ligand.**

Studies of genetic regulation in bacteria have revealed a number of basic mechanisms, including the control of gene expression through specific DNA binding proteins, control of tandem genes (operons) by single regulatory gene products, and coordinate control of families of unlinked genes by common regulators. The latter, known as global regulation, is involved in responses to various environmental influences such as heat, radiation, osmolarity, pH, O_2 tension, starvation, etc., which are regarded as stress responses and are commonly initiated by means of two-component signal transduction pathways. Among the genes frequently controlled by such global regulators are bacterial virulence genes (1, 2).

The Gram-positive bacterium *Staphylococcus aureus* is an important pathogen, causing a variety of diseases, such as endocarditis, septic arthritis, and toxic shock syndrome. Staphylococcal pathogenesis primarily involves the production and 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. The expression of most of these virulence factors is regulated by at least one well-characterized global regulator, *agr* (3–5). As cells enter the postexponential phase, surface protein genes are repressed by *agr* and secretory protein genes are subsequently activated (6). The *agr* locus consists of two divergent transcription units, RNAII and RNAIII, controlled by two promoters, P2 and P3, respectively (6–8). The P3 transcript, RNAIII, is the actual effector of the exoprotein response (6, 7, 9) and incidentally encodes δ-hemolysin, which is not involved in regulation (5). RNAIII acts primarily at the level of target gene transcription and independently regulates the translation of at least one or two of the exoproteins (7). The P2 operon contains four genes—*agrA, agrB, agrC*, and *agrD*—which are required for the activation of RNAIII transcription (7, 8). The predicted gene product of *agrA* resembles the response regulators of the classical bacterial two-component transduction systems and that of *agrC* resembles the histidine phosphokinase signal transducers (8–11).

In a previous report from this laboratory, it has been shown that many strains of *S. aureus* produce a factor that accumulates during growth and is responsible for activating the *agr* response, whereas at least one strain, an exoprotein-defective mutant, produces a substance that inhibits activation of the *agr* response (12). The earlier data seemed to suggest that the activating factor is an ≈38-kDa protein, whereas the inhibitor is a small peptide. In the present study, we have identified the factor as a modified octapeptide, shown that this peptide is synthesized by two of the *agr* P2 genes, *agrB* and *agrD*, and demonstrated that AgrC is its receptor. We have also confirmed the existence and peptidic nature of the inhibitor (these latter studies will be described at a latter date). The earlier impression that the activator is a protein is best explained by the presence of an anomalously migrating protein that copurified with and obscured the true octapeptide activator. Present studies, therefore, indicate that the *S. aureus* virulence response is regulated by a density-sensing system that is analogous to density-sensing regulatory systems in other bacteria that utilize homoserine lactones as autoinducers. The *S. aureus* system, however, is organized identically with the competence-inducing *comAP* operon of *Bacillus subtilis* (13), which also uses a peptide as autoinducer.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *S. aureus* strains are derivatives of NTCC8325 and are listed in Table 1. RN6390B is our standard wild-type laboratory strain and is an *agr*$^+$ variant of RN450, which was originally isolated by UV curing 8325 of three prophages (14) and later found to have a partial *agr* defect (7). RN6911 is a derivative of RN6390B in which the *agr* locus has been replaced by *tetM* (7).

Cells were grown in CYGP broth (15), supplemented with antibiotics when necessary, with shaking at 37°C. Overnight cultures of *S. aureus* on GL plates (15) were routinely used as inocula and for plating. Cell growth was monitored with either 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}. Wherever needed, antibiotics (5 μg/ml) were added: chloramphenicol, erythromycin, and tetracycline. 2-(2-Carboxyphenyl)benzoyl-6-aminopenicillanic acid (CBAP) (5 μg/ml) was used to induce genes under the control of *blaZ* promoter.

Construction of Plasmids. Plasmids used in this study are listed in Table 1. Plasmid pRN6852 was constructed by cloning a polymerase chain reaction (PCR) product containing the

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Abbreviations: MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; CBAP, 2-(2-carboxyphenyl)benzoyl-6-aminopenicillanic acid.

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Table 1. *S. aureus* strains and plasmids

Strain or plasmid	Genotype or description	Ref. or source
Strain		
RN6390B	*agr*$^+$ laboratory strain	7
RN6911	RN6390B Δ*agr* 1057-4546::*tetM*	7
RN7270	RN6911 *hla*::*ermC*	7
RN7668	RN7270(pI524)	This study
Plasmid		
pRN5548	pRN5543 with pI258 *bla* promoter	7
pRN6672	*agrBCD*-promoters P2 and P3-RNAIII cloned into pSK265	8
pRN6683	*agr* RNAIII-*blaZ* fusion	8
pRN6718	Internal deletion in *agrB* from pRN6672	8
pRN6720	Internal deletion in *agrD* from pRN6672	8
pRN6724	Partial 3′ deletion in *agrC* from pRN6672	8
pRN6735	*rnaIII* determinant cloned into pRN5548	7
pRN6753	Promoter P1-*agrA* cloned into pRN6641	8
pRN6852	*agrABCD* in pRN5548	This study
pRN6911	*agrBD* in pRN5548	This study
pRN6912	*agrB* in pRN5548	This study
pRN6913	*agrD* in pRN5548	This study
pRN6917	*agrAC* in pRN5548	This study
pRN6918	*agrC* in pRN5548	This study

entire *agr* P2 operon into the polylinker site of plasmid pRN5548 (7). In the resulting construct, the P2 operon is under the control of *S. aureus* plasmid pI258 β-lactamase promoter (P_{bla}). This promoter is repressed by BlaI, a standard repressor encoded by coresident plasmid pI524. Plasmid pRN6911, containing *agrB, agrD*, and part of *agrC*, was constructed by digesting pRN6852 DNA with *Eco*RI and *Bst*NI, blunting with DNA polymerase I large fragment (Klenow), and religating. pRN6912, containing *agrB* and part of *agrD*, was constructed by similarly removing the smaller *Eco*RI–*Sca* I fragment from pRN6852. pRN6913, containing *agrD, agrB* with an in-frame deletion, and part of *agrC*, was constructed by first digesting pRN6852 DNA with *Sph* I and *Xba* I, blunting with Klenow, and religating to remove a *Pst* I site from the polylinker region of plasmid pRN5548. Then the resulting plasmid DNA was digested with *Pst* I and *Bsi*KHAI and religated. pRN6917, containing *agrA, agrC*, and part of *agrD*, was made by removing an *Xba* I–*Sca* I fragment from pRN6852, as above. pRN6918, containing *agrC* and part of *agrA*, was constructed by removing an *Eco*RI–*Bst*EII fragment from pRN6917, as above. Fig. 2*A* is a map of the *agr* P2 operon showing the open reading frames and sites used in these constructs. Transformation of plasmid DNA was done by the protoplast method (16) as modified for *S. aureus* (15).

Preparation of Activator. *S. aureus* RN6390B (*agr*$^+$) was grown in CYGP broth at 37°C for 6 hr starting at a cell density of 2×10^9 cells per ml. Cells were removed by centrifugation at 4°C. The supernatant was filtered (0.22-μm filer, Gelman), boiled for 10 min, centrifuged, and filtered with a Centricon 3 filter (Amicon) with 3-kDa cutoff. The filtrate was stored at −80°C and used as a source of activator.

Activator Assay. RN6390B containing the *agr* P3-*blaZ* fusion plasmid, pRN6683 (8), was grown in CYGP broth (starting at 7.5×10^7 cells per ml) at 37°C to 4.5×10^8 cells per ml. To 45 μl of cells, 5 μl of the activator preparation was added. The mixture was incubated at 37°C with shaking in a THERMOmax microplate reader for 55 min. β-Lactamase activity was measured by the nitrocefin method (17) modified as follows. Culture samples were diluted with CYGP broth plus 5 mM sodium azide to a final volume of 50 μl and transferred to a microtiter plate. Nitrocefin solution (50 μl of 132 μg/ml in 0.1 M sodium phosphate buffer, pH 5.8) was added and the plate was incubated with shaking at 37°C. The microtiter reader was set to read $\varepsilon_{490nm} - \varepsilon_{650nm}$ and to calculate the initial reaction rates as $\Delta\varepsilon \times 10^3$ per min. For these experiments, an increase in $\varepsilon_{490nm} - \varepsilon_{650nm}$ of 0.001 per min is defined as 1 unit of β-lactamase activity.

Northern Blot Hybridization. Northern blot DNA·RNA hybridization was carried out as described (18). ^{32}P-labeled RNAIII probes were prepared by PCR using oligonucleotide primers corresponding to nt 1259–1280 and nt 1558–1571 of RNAIII (7), respectively, and pRN6735 (see Table 1) DNA as template. In labeling reactions, dATP concentration was reduced to 2 μM and the reaction mixture contained 50 μCi of [α-^{32}P]dATP (Amersham; 1 Ci = 37 GBq).

Purification and Analysis of Activator Pheromone. The activator pheromone was purified from a 6-hr supernatant of *S. aureus* strain RN7668(pRN6911) (see Fig. 2*A*) grown in tryptophan assay medium (Difco) plus 50 μg of L-tryptophan per ml and 5 μg of CBAP per ml starting at 2×10^9 cells per ml. Before use, the medium was dialyzed with a 2-kDa cutoff membrane, discarding the contents of the membrane sac. Strain RN7668 is a derivative of the *agr*-null strain, RN6911, containing plasmid pI524, which encodes the *bla* repressor. After 6 hr of growth, cells were removed by centrifugation and the culture supernatant was filtered (0.22-μm filter), boiled for 10 min, lyophilized, and resuspended in 2.5% acetonitrile/0.1% trifluoroacetic acid (1/40 volume of the culture supernatant). This material (3 ml) was loaded onto an HPLC C_{18} column in 2.5% acetonitrile/0.1% trifluoroacetic acid and eluted with an acetonitrile gradient (16–48%) at 0.27% acetonitrile per min. The collected fractions (1.5 ml per fraction) were lyophilized and suspended in 0.1 ml of 20 mM Tris·HCl buffer (pH 7.5). Fractions with activator pheromone activity were pooled and filtered through a Centricon 3 filter with 3-kDa cutoff. The filtrate (1 ml) was rerun on the HPLC C_{18} column and eluted with an acetonitrile gradient at 0.2% acetonitrile per min over the interest range (20–32%) The activator peptide, eluting at an acetonitrile concentration of about 28.5%, was analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (19) and its amino acid sequence was determined by a Procise Edman Sequencer (Perkin–Elmer). MALDI-MS was performed using a linear time-of-flight mass spectrometer with a nitrogen laser

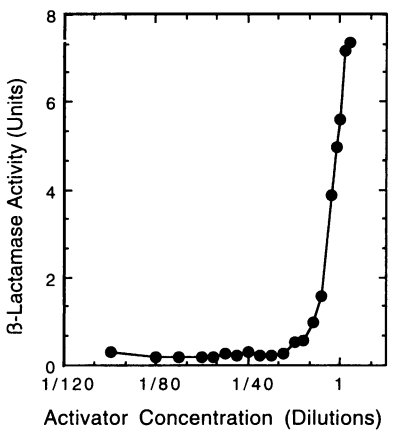

FIG. 1. Concentration dependence of pheromone activity. Activator pheromone activity was determined by using as an assay the induction of an RNAIII-*blaZ* transcriptional fusion in cells at low density. Activator pheromone was prepared from a 6-hr culture supernatant of RN6390B, diluted with CYGP medium and assayed as described in the text.

ion source custom-built at New York University. The matrix used was α-cyano-4-hydroxycinnamic acid and the sample was prepared using the dried droplet method. A peptide with the same amino acid sequence as the native peptide was synthesized commercially (Yale University, New Haven, CT) and analyzed by MALDI-MS.

RESULTS

Concentration Dependence of Activator Pheromone on *agr* Activation. Postexponential-phase culture supernatants of typical *S. aureus* strains, such as RN6390B, contain a substance that, when added to early exponential cultures, causes the immediate activation of *agr* RNAII (data not shown) and RNAIII transcription (12). Although this substance accumulates gradually during the growth of the culture (12), the response exhibits a sharp concentration-dependent activation threshold, as shown in Fig. 1. This type of kinetics represents a cell density-sensing mechanism similar to that observed with *Pseudomonas, Vibrio, Agrobacteria*, and other bacterial species, which use homoserine lactones as autoinducers (20–22).

Role of *agr* P2 Operon in Activator Pheromone Production and Activation of *agr* Locus. In contrast to the activity of RN6390B supernatant, that of an *agr*-null derivative, RN6911, had no detectable activity (see Fig. 2*A*). Accordingly, different *agr* subfragments were cloned into vector, pRN5548, under the

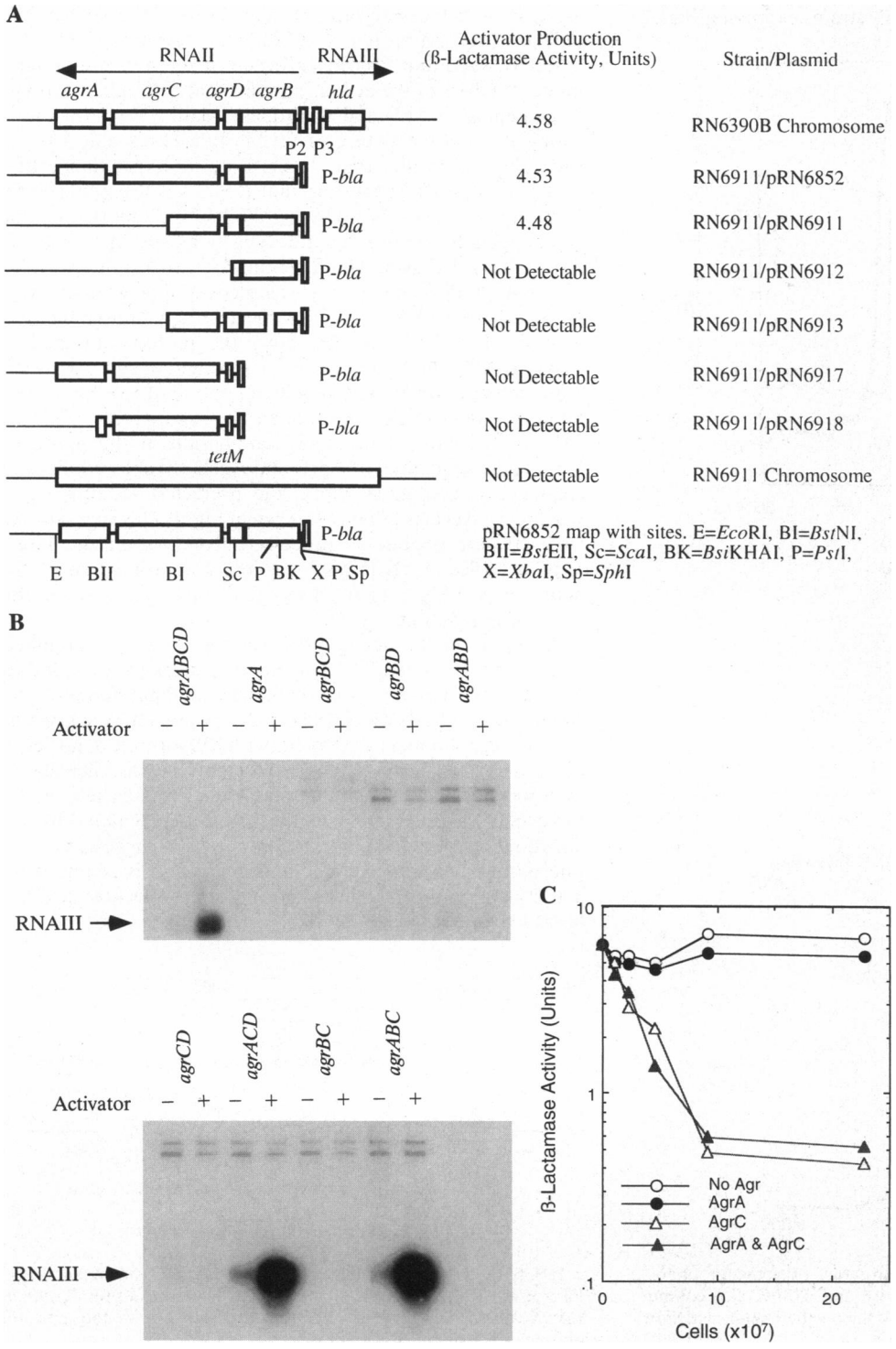

FIG. 2. Role of the *agr* P2 operon. Various deletions and subclones of the four P2 operon genes were tested for their effects on production of and response to the activator. (*A*) RN6911 derivatives containing different combinations of the *agr* P2 genes cloned under the control of *blaZ* promoter were induced with 5 μg of CBAP per ml for 2 hr and the resulting supernatants were tested for activator pheromone activity. (*B*) RN6911 strains containing derivatives of the cloned *agr* locus with in frame deletions affecting different genes of the P2 operon were tested for their response to the activator. Low-density cultures were incubated at 37°C for 30 min in the presence (+) or absence (−) of a standard quantity of the activator preparation used as in *Materials and Methods*. Whole cell lysates were prepared from these cultures and analyzed by Northern blot hybridization using an RNAIII-specific probe. RN6390B was used as agr^+ strain and labeled as *agrABCD*. RN6911(pRN6753) contains only *agrA*. For all others, the *agr* genes were cloned into plasmids (see Table 1) and labeled as follows: *agrBCD*, pRN6672; *agrBD*, pRN6724; *agrABD*, pRN6724/pRN6753; *agrCD*, pRN6718; *agrACD*, pRN6718/pRN6753; *agrBC*, pRN6720; and *agrABC*, pRN6720/pRN6753. (*C*) RN6911 strains carrying different *agr* genes cloned under the control of *blaZ* promoter (see *A*) were induced with CBAP, washed with 20 mM Tris·HCl buffer (pH 7.5), incubated for 1 min in the presence of a standard amount of the activator preparation (see text), and then centrifuged to remove the cells. The resulting supernatants were assayed for activity.

control of staphylococcal β-lactamase promoter and transferred to the *agr*-null strain. As seen in Fig. 2*A*, only strains expressing *agrB* and *agrD* produced the activator, indicating that the activating substance was either encoded or regulated by these two genes.

Since the other two gene products of the *agr* P2 operon, AgrC and AgrA, are thought to comprise a signal transduction system (8, 9), we next tested a series of similar subclones for their ability to respond to the activator. These tests, also performed with the *agr*-null strain, utilized a plasmid expressing various *agr* P2 genes and the *agr* effector, RNAIII, under the control of its native promoter, P3, as the indicator of *agr* activity. Fig. 2*B* shows the results of a Northern blot hybridization analysis of RNA prepared from strains containing the indicated subclones. As expected, only strains expressing AgrA and AgrC responded to the activator, suggesting that the *agr*-determined signal transduction pathway is the activation conduit. AgrC, which resembles the histidine protein kinase component of the classic bacterial two-component signal transduction systems (10, 11), was predicted to be the receptor and therefore to bind the activator. Fig. 2*C* shows the results of an experiment confirming this prediction. In this experiment, cells expressing different combinations of the *agr* P2 operon genes were incubated briefly with an activator preparation and then centrifuged to remove the cells. Residual activator activity in the resulting supernatant was assayed as described in *Materials and Methods.* As can be seen in Fig. 2*C*, cells expressing AgrC removed the activator activity, whereas cells not expressing AgrC did not. It is formally possible that AgrC is or induces an enzyme that destroys the activator or induces a second protein that binds the activator.

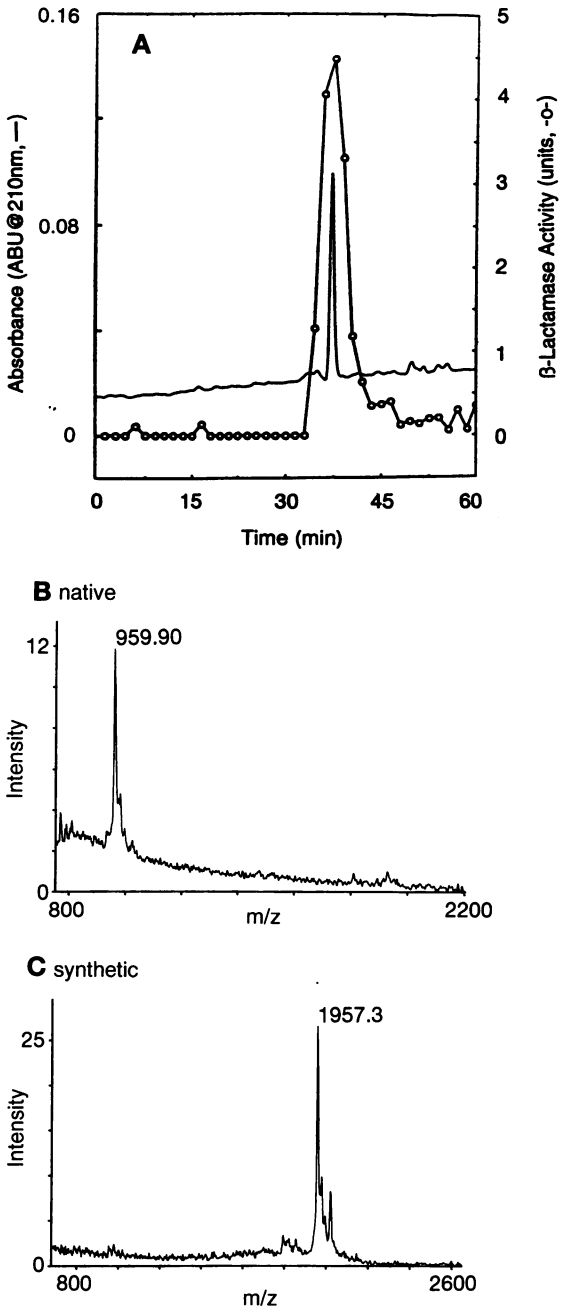

FIG. 3. Purification and mass spectroscopy of activator pheromone. (*A*) Activator pheromone activity and HPLC C_{18} column profile. (*B* and *C*) MALDI-MS analysis of the purified native activator pheromone (*B*) and its synthetic analog (*C*).

Purification and Characterization of the Activator Pheromone. Analysis of the activating substance revealed sensitivity to proteinase K (12) and to Pronase (data not shown), indicating its peptidic nature. This substance was able to pass through an ultrafilter that retains material larger than 3 kDa (data not shown), suggesting that it is a small peptide. Purification was accomplished by first boiling the supernatant and centrifuging to remove denatured proteins and then fractionating it by HPLC using a C_{18} column. After two cycles of HPLC chromatography, the activity was recovered as a single, well-defined peak suitable for mass spectroscopy and amino acid analysis (Fig. 3*A*). Fig. 3*B* shows the pattern obtained by MALDI-MS, which gave a molecular weight of 960 ± 1 atomic mass units (amu), consistent with a peptide of 8 or 9 residues. Sequence determination revealed the octapeptide YSTCDFIM (single-letter code) and examination of the predicted amino acid sequences of AgrB and AgrD (8) showed that this sequence is contained within the predicted 46 amino acid residues of AgrD product, as shown in Fig. 4. This suggests that the activator peptide is processed from within the larger peptide; since AgrB is also required for production of the activator (see Fig. 2*A*), it is likely that AgrB is involved in this processing reaction.

A sample of the octapeptide was then prepared commercially (Yale University). The synthetic material had no detectable activity (data not shown) and had an apparent molecular weight by MALDI-MS of 1957 ± 2 amu (Fig. 3*C*), suggesting that it was a dimmer. Treatment with dithiothreitol, however, did not generate any activity (data not shown). The native material occurred as a monomer (see Fig. 3*B*) and had a molecular weight 18 amu smaller than that calculated for the above-mentioned octapeptide. These results suggests that the pheromone contains a cyclic anhydride that is required for activity and prevents dimerization by intermolecular disulfide bond formation.

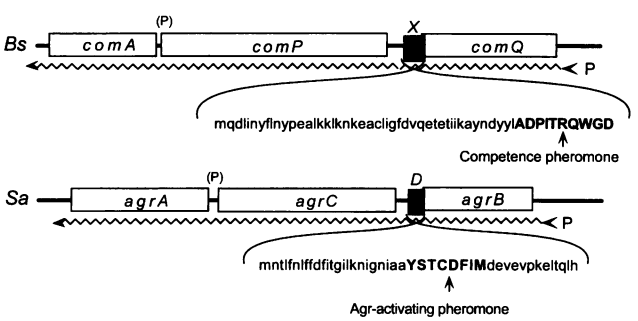

FIG. 4. Comparison of the *B. subtilis com AP* (13) and *S. aureus agr* P2 operons (8). The predicted amino acid sequences of the *B. subtilis* ComX and *S. aureus* AgrD peptides are shown with the activator pheromone sequences in bold type.

DISCUSSION

These results demonstrate that the *agr* P2 operon is, in a sense, doubly autocatalytic: (*i*) it encodes its own activator in the form of a modified octapeptide that is the ligand for the signal receptor also encoded by the same operon; (*ii*) the primary function of the signal transduction pathway is the activation of its own promoter (as well as the divergent P3 promoter), which up-regulates production of the activator as well as of the signal transduction proteins. The net effect of this dual activation is the rapid expression of RNAIII at very high levels (RNAIII can sometimes be visualized directly on agarose gels stained with ethidium bromide). We suppose that RNAIII is not highly active and therefore that a high concentration is required.

The density-sensing feature of this system is revealed by the gradual accumulation of activator activity during growth coupled with the striking activation threshold seen in Fig. 1. Once this threshold has been crossed the doubly autocatalytic nature of the regulatory circuit generates a rapid burst of activator synthesis as well as causing a rapid response of RNAIII. The nature of the threshold phenomenon remains to be determined. One possibility is that a certain fraction (or number) of the AgrC receptors needs to be bound by the ligand in order to activate the internal components of the signaling pathway. Another is that the activating form of the receptor is oligomeric and that ligand binding is responsible for oligomerization, as is seen with many eukaryotic signal receptors.

In an earlier report from this laboratory (12) demonstrating the existence of an *agr*-activating factor in staphylococcal culture supernatant, the data were more consistent with a protein of ≈38 kDa than with a small peptide. This material fractionated anomalously on a gel filtration column, eluting with an apparent molecular mass of ≈1 kDa; on SDS/PAGE, however, this particular fraction showed a single protein with an apparent molecular size of 38 kDa. We believe that this material represents an anomalously migrating protein and that the octapeptide was present in the 1-kDa gel filtration fraction, accounting for the activity, but was not seen on the SDS/PAGE.

A search of the protein database (Protein Identification Resource) did not reveal significant sequence similarity between AgrB, AgrD, or the octapeptide and any other proteins or peptides. However, comparison of the *agr* P2 operon with the *comAP* operon of *B. subtilis* revealed a striking organizational similarity, as shown in Fig. 4. The *comAP* operon is required for the postexponential-phase induction of competence for DNA transformation. ComA and ComP comprise a two-component signal transduction system. ComX is processed to generate a nonapeptide that is posttranslationally modified to form an activating pheromone, whose structure has not yet been determined (13). The synthetic nonapeptide was inactive. ComQ is required for production (processing?) of the nonapeptide and ComQ and ComX are translationally coupled, as are AgrB and AgrD. This identity of genetic organization implies coancestry, suggesting that the same density-sensing module has been applied to the induction of competence by *B. subtilis* and to the global regulation of virulence by *S. aureus*. Although our report demonstrates control of a multifactorial virulence response by a peptide-mediated density sensor, we predict that versions of this density-sensing module or cassette will be found widely among Gram-positive bacteria.

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