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Autocrine regulation of toxin synthesis by *Staphylococcus aureus*

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ABSTRACT ***Staphylococcus aureus* is a major human pathogen causing diseases which range from minor skin infection to endocarditis and toxic shock syndrome. The pathogenesis of *S. aureus* is due primarily to the production of toxic exoproteins, whose synthesis is controlled by a global regulatory system, *agr*. We show here that *agr* is autoinduced by a proteinaceous factor produced and secreted by the bacteria and that it is inhibited by a peptide produced by an exoprotein-deficient *S. aureus* mutant strain. The inhibitor, RIP, competes with the activator, RAP, and may be a mutational derivative. Our results suggest two possible approaches, independent of antibiotics, to the control of *S. aureus* infections. RIP may prove useful as a direct inhibitor of virulence and RAP as a vaccine against the expression of *agr*-induced virulence factors; either could interfere with the ability of the bacteria to establish and maintain an infection.**

During exponential growth of bacteria, interactive regulatory systems are coordinated to ensure a rapid and efficient growth rate and constant cell composition. As the culture ages and growth stops, new regulatory systems are activated that reorganize cell metabolism for long-term survival under unfavorable conditions. It is during this postexponential phase that most exoproteins are synthesized (1, 2). In Gram-positive bacteria, many of these are enzymes that degrade biopolymers, presumably to exploit environmental sources of nutrition. In pathogenic bacteria, many of the exoproteins are virulence factors that facilitate the survival, multiplication, and spread of the organism in infected tissue.

Postexponential-phase gene expression, including that of virulence factors, is often governed by global regulatory systems in which common regulators control the activities of a number of unlinked genes in response to certain environmental signals. Environmental signals, such as heat, radiation, osmolarity, pH, O_2 tension, and starvation have been shown to control the expression of virulence determinants in bacteria (3). Studies in our laboratory on *Staphylococcus aureus* have shown that the postexponential phase regulation of the expression of virulence factors and other exoproteins involves at least one well-characterized global regulator, *agr* (4, 5). The *agr* locus consists of two divergent transcription units driven by promoters *P2* and *P3*. The *P2* transcript includes four open reading frames referred to as *agrA*, *-B*, *-C*, and *-D*, all four of which are required for the *agr* response (6). The peptides predicted for *agrA* and *agrC* resemble the response regulators and signal transducers of the two component bacterial signal transduction systems (6, 7). The primary function of these four genes is to activate the two promoters; the *P3* transcript, RNAIII, however, is the actual effector of the exoprotein response (6, 8, 9). RNAIII activates transcription of secretory protein genes and represses transcription of surface protein genes (6, 8, 10) by an unknown mechanism that probably involves other factors. It also regulates translation of several of the exoproteins, probably via complementary base pairing (8).

Here we report staphylococcal autocrine factors that activate or inhibit the *agr* response by inducing or repressing the synthesis of RNAIII. Most strains produce and secrete an activator, which may be a protein of ≈38 kDa. One exoprotein-deficient (Exp^-) mutant, RN833, produces and secretes an inhibitor, a peptide which competes with the activator and may be a mutational derivative.

METHODS

Bacterial Strains. The *S. aureus* strains used in this study were RN6390B, a wild-type agr^+ strain (our standard laboratory strain); RN7111 and RN7112, spontaneous Exp^- mutants; RN8471, a wild-type agr^+ strain; and RN8470, a sar^- mutant (24). RN833 is a variant of a nitrosoguanidine-induced nuclease-deficient mutant of a clinical strain, Foggi (11), now listed as RN831. RN833 has changed during storage at −80°C (since 1968), as it originally produced α-hemolysin but no longer does so. It has been producing an inhibitor of *agr* since we first tested it in 1993. All strains were grown in CY broth (12).

RNAIII Transcription During the Growth of RN6390B. Time-course experiments were performed in 500-ml Klett flasks containing 50 ml of CY broth (12). Wild-type *S. aureus* was grown in CY broth with shaking at 37°C, starting in early exponential phase at ≈3.5×10^8 cells per ml. Growth was monitored turbidimetrically with a Klett–Summerson photoelectric colorimeter read at 540 nm. Samples containing an equal number of cells were removed at the indicated times, and whole-cell lysates were analyzed by Northern blotting with radiolabeled RNAIII-specific DNA as a probe (see below).

Activation of *agr*. Wild-type *S. aureus* RN6390B was grown in CY broth with shaking at 37°C, starting at 5×10^7 cells per ml. When the cells reached the density of about 3.5×10^8 cells per ml (early exponential phase), concentrated supernatants or column fractions were added (to a final dilution of 1×, unless otherwise indicated) and growth continued. Samples containing an equal number of cells were removed at the indicated times, and whole-cell lysates were analyzed by Northern blotting with radiolabeled RNAIII-specific DNA as a probe (see below). Note that all fractions tested for their ability to activate *agr* were concentrated by lyophilization before being added to the cells.

Whole-Cell Lysates and Northern Blotting. Equal numbers of cells (5×10^8) were collected at the indicated times and whole-cell lysates were prepared as described (13). In brief, the cells were treated for 10 min with lysostaphin (100 μg/ml) in TES buffer [(20% (wt/vol) sucrose/20 mM Tris pH 7.6/10 mM EDTA/50 mM NaCl] and then vigorously shaken for 10 min with proteinase K (10 μg/ml; Sigma) in the presence of 1% SDS. For Northern blotting, (10), 10 μl of cell extract was electrophoresed through a 0.66 M formaldehyde/1% agarose gel in Mops buffer (14). Nucleic acids were transferred to a nitrocellulose membrane (Amersham) with a VacuGene apparatus (Pharmacia) in 20× SSC (1× SSC is 0.15 M NaCl/

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Abbreviations: Exp^-, exoprotein-deficient; PIU, PhosphorImager units × 10^{-3}.

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0.015 M sodium citrate, pH 7) and the membrane was baked at 80°C under vacuum for 1 hr. The membrane was preincubated for 1 hr at 57°C in 2 × Denhardt's solution (1× is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.01 M EDTA, pH 8/0.2% SDS/5× SSC with sonicated and heat-denatured salmon sperm DNA (100 μg/ml) and then hybridized overnight with ^{32}P-labeled DNA probe in prehybridization solution supplemented with 10% (wt/vol) dextran (Sigma). ^{32}P-labeled DNA probes were prepared by PCR, as described for RNAIII (8). The blot was exposed to a storage phosphor screen (Molecular Dynamics) and the relative duplex yields were measured with IMAGE QUANT software and are presented in PhosphorImager units × 10^{-3} (PIU).

Isolation and Purification of RAP and RIP. RN6390B and RN833 were grown in CY broth (12) for 6 hr from early exponential phase of growth (5 × 10^8 cells per ml) to late postexponential phase. The cells were removed by centrifugation at 5000 × *g*, culture supernatant was filtered to remove all remaining cells, and filtered supernatant was concentrated by lyophilization and suspended to 10× in water. To purify RAP, RN6390B supernatant was boiled for 10 min and centrifuged for 10 min at 14,000 × *g* and a 1-ml sample of the supernatant was applied to an HPLC gel filtration column (Bio-Rad, Bio-Sil SEC 150-5) in the presence of M9 salts (14) at a flow rate of 1 ml/min.

To purify RIP, 10× concentrated postexponential culture supernatant of RN833 was passed through a 3-kDa-cutoff membrane (Amicon), and the flowthrough was applied to a C_{18} reverse-phase HPLC column (Rainin) and eluted with a 0–75% acetonitrile gradient in 0.1% trifluoracetic acid at a flow rate of 1 ml/min.

RESULTS

Activation of *agr*. *agr* is temporally regulated, as the production of RNAIII is low in early exponential phase but increases up to 40-fold later in growth (8, 10). An example of the kinetics of RNAIII synthesis is shown in Fig. 1. Given the resemblance between AgrC and the classical sensory transducing proteins of bacteria, it seemed likely that *agr* activation would be initiated by an environmental signal generated during bacterial growth. To test this hypothesis we performed an experiment in which postexponential culture supernatants were concentrated and added to early exponential cultures of *S. aureus* at a time before *agr* is normally activated. The results of this experiment (Fig. 2) were dramatic: the concentrated supernatant of postexponential culture of a wild-type (*agr*$^+$) strain caused the immediate activation of RNAIII transcription. Activity could be demonstrated in supernatants from midexponential phase (when *agr* is normally activated) but was higher in postexponential supernatants (Fig. 3), suggesting that the activating substance accumulates in the supernatant during the growth of the bacteria.

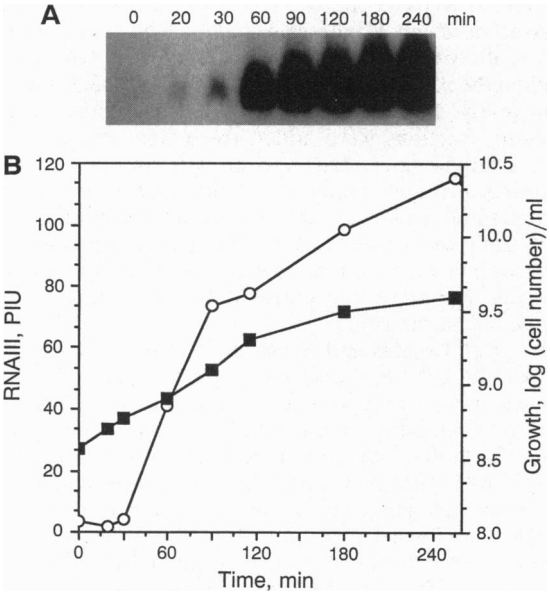

FIG. 1. Growth of *S. aureus* wild-type RN6390B and RNAIII synthesis. RN6390B cells were grown from early exponential to postexponential phase and *agr* activity was measured by analyzing RNAIII synthesis. The autoradiogram of the Northern blot is presented in *A* and its analysis and the growth curve are presented in *B* (■, growth curve; ○, RNAIII).

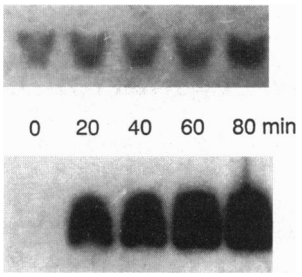

FIG. 2. Activation of *agr*. Supernatant from a 6-hr culture of RN6390B (corresponding to point T6 in Fig. 3) was concentrated 10-fold by lyophilization and added to early exponential wild-type RN6390B cells and the activity of *agr* was monitored during the subsequent growth of the bacteria by Northern blotting with RNAIII-specific DNA as a probe. (*A*) CY broth was added. (*B*) Concentrated (10×) RN6390B T6 supernatant was added (final dilution, 1×).

We ruled out low pH as a possible activating factor because adjusting the postexponential culture supernatant to pH 7.4 (from pH 5.5) did not change its activity (data not shown). To

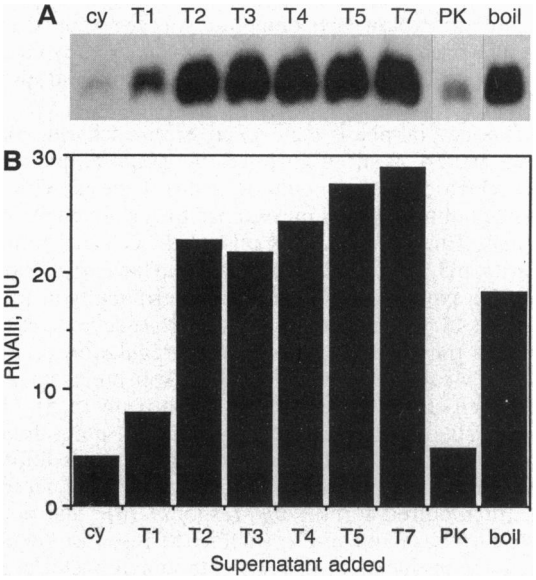

FIG. 3. Characterization of the *agr*-activating factor. A wild-type RN6390B culture was grown from midexponential (T1, 6 × 10^8 cells per ml) to postexponential (T7, 4.7 × 10^9 cells per ml) phase of growth, and supernatants were collected hourly (T1–T7). The supernatants were added to early exponential wild-type RN6390B cells and *agr* activity was analyzed 30 min later by Northern blotting. Culture supernatants tested: cy, CY broth; T1–T7, concentrated supernatants of a wild-type RN6390B collected hourly (see above); PK, T7 adjusted to pH 7.5 and treated with proteinase K (1 mg/mg) for 1 hr at 37°C followed by the addition of 1mM phenylmethylsulfonyl fluoride; boil, T7 was boiled for 10 min and centrifuged for 10 min at 14,000 × *g* to remove precipitate. The autoradiogram is presented in *A* and its analysis in *B*.

show that the *agr* induction is caused by a proteinaceous substance, we treated the concentrated culture supernatant with proteinase K and indeed found that the RNAIII-activating effect was eliminated (Fig. 3). Henceforth, we refer to this material as RAP (RNAIII activating protein). We also tested the effect of heat and found that boiling the culture supernatant for 10 min caused partial inactivation (Fig. 3). The size of the activating molecule was estimated to be around 30 kDa, as the activity was retained when the culture supernatant was dialyzed with a 14-kDa-cutoff membrane but was partially lost when passed through a 30-kDa-cutoff membrane (data not shown).

Purification of RAP, the *agr* Activator. To purify RAP, concentrated postexponential wild-type culture supernatants were boiled for 10 min to eliminate heat-labile proteins, centrifuged, and applied to an HPLC gel filtration column. A single fraction, eluted with an apparent molecular weight of 1000, contained the RAP activity (Fig. 4*A*). By SDS/PAGE and silver staining, this fraction was shown to be specifically enriched for a protein of about 38 kDa (Fig. 4*B*). The discrepancy between the elution time and the apparent size of the molecule on SDS/PAGE may be due to binding of this molecule to the matrix of the column (16) which delayed its elution. Alternatively, the active factor could be a small peptide that is eluted with an anomalously migrating protein.

Mutant *S. aureus* Exp⁻ Strain Producing an Inhibitor of *agr*. To search for mutants defective in RAP production, we tested a number of independently isolated Exp⁻ mutants of *S. aureus* and found a single mutant, RN833, that did not produce RAP. RN833, which is a variant of a nitrosoguanidine-induced nuclease-deficient mutant derived from strain Foggi (11), had a profound defect in exoprotein synthesis, and produced a molecule that totally blocks the normal activation of *agr* in RN6390B (Fig. 5*A*). Its inhibitory effect was detected even when the culture supernatants are diluted up to 1:100 (Fig. 5*B*).

The inhibitory activity found in RN833 postexponential supernatant passed through a membrane with a cutoff of 3 kDa (Amicon) and was slightly sensitive to treatment with proteinase K and boiling (data not shown), suggesting that the inhibitory factor may be a peptide. This substance is henceforth referred to as RIP (RNAIII inhibitory peptide).

Purification of RIP, the *agr* Inhibitor. To purify the inhibitor, a postexponential culture supernatant of RN833 was passed through a 3-kDa-cutoff membrane, and the low molecular weight fraction was applied to a reverse-phase C_{18} HPLC column and eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid. One fraction, which was eluted at 35% acetonitrile, contained the inhibitory activity (Fig. 6*A*). This fraction was applied again to the same column and eluted with acetonitrile in 0.1% trifluoroacetic acid. This elution yielded one major peak (Fig. 6*B*) which contained the inhibitory activity. Peptide sequence analysis performed on the peak fraction (by the Howard Hughes Medical Institute, Harvard Medical School) indicated the presence of a pentapeptide.

Competition Between RAP and RIP for RNAIII Transcription. Experiments were done to try to detect RAP in RN833 culture supernatants that were dialyzed with a 14-kDa-cutoff membrane and RIP in RN6390B culture supernatants that were run through a 3-kDa-cutoff membrane. The activator was not detected in the supernatants of the mutant RN833, and the inhibitor was not detected in supernatants of the wild-type RN6390B, suggesting that the inhibitor may be derived from

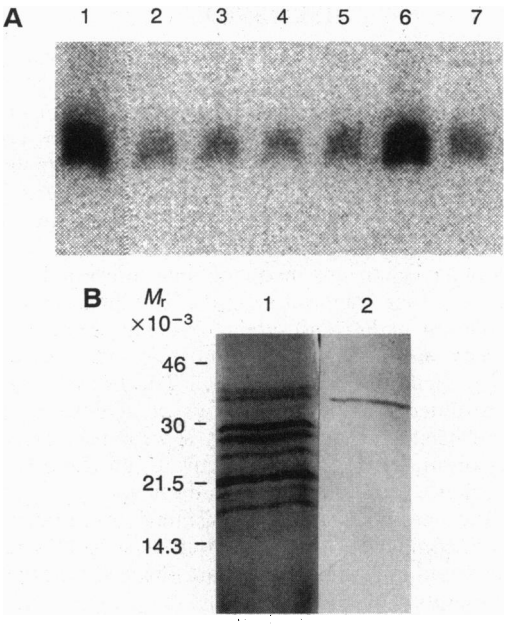

FIG. 4. Purification of RAP. (*A*) Concentrated supernatant of late-phase RN6390B (T7) was applied to an HPLC gel filtration column, and fractions were collected, lyophilized, and added to early exponential RN6390B to test for their ability to activate *agr*. Cells were analyzed for RNAIII 20 min after the addition of boiled T7 (see Fig. 3 legend; lane 1) CY broth (lane 2), or column fractions corresponding to elution volumes of 5–7, 7–10, 10–13, 13–13.5, and 14–15 ml (lanes 3–7, respectively.) (*B*) The column fractions were separated by SDS/12.5% PAGE (15) and the gel was silver stained (Bio-Rad). The numbers on the left represent the positions of molecular weight markers. Lanes: 1, column fraction corresponding to elution volumes of 7–10 ml, having no *agr*-upregulating activity but maximum protein concentration; 2, column fraction corresponding to elution volumes of 13–13.5 ml, which contained the *agr*-upregulating activity.

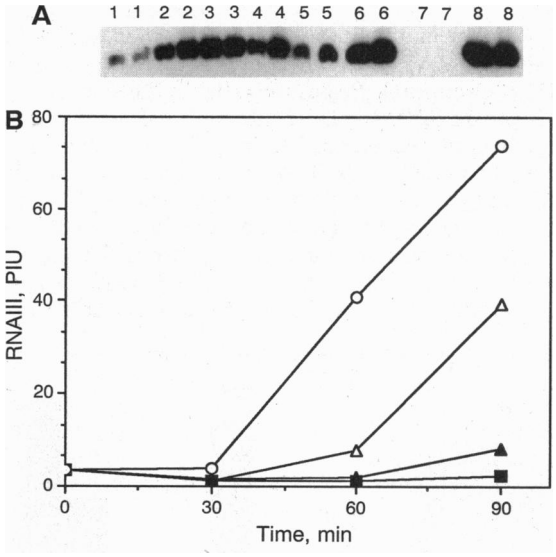

FIG. 5. Inhibition of *agr*. Postexponential culture supernatants were collected from various *S. aureus* strains and were added to early exponential wild-type RN6390B cells and the activity of *agr* was monitored 20 and 40 min later by Northern blotting, with RNAIII-specific DNA as a probe. (*A*) Exp⁻ mutants. The concentrated supernatants that were added were taken from postexponential cultures as follows: lane 1, no addition; lane 2, *S. aureus* RN7111 (spontaneous Exp⁻ mutant); lane 3, *S. aureus* RN7112 (spontaneous Exp⁻ mutant); lane 4, RN8471, *S. aureus* wild-type *agr*⁺ strain; lane 5, RN8470, a *sar*⁻ mutant (24); lane 6, RN831, a *S. aureus* wild-type *agr*⁺ strain; lane 7, RN833, a nitrosoguanidine-induced Exp⁻ mutant of RN831; lane 8, RN6390B, a *S. aureus* wild-type *agr*⁺ strain. Duplicate numbers refer to RNAIII analysis at times 20 min and 40 min, respectively, after the addition of culture supernatants. (*B*) Titration of the RN833 inhibitory activity. RN833 culture supernatant was added as above (time 0) and growth continued. Activity of *agr* was analyzed by Northern blotting at the indicated times. ○, CY broth; △, RN833 supernatant diluted 1:100; ▲, RN833 supernatant diluted 1:10; ■, RN833 supernatant diluted 1:1.

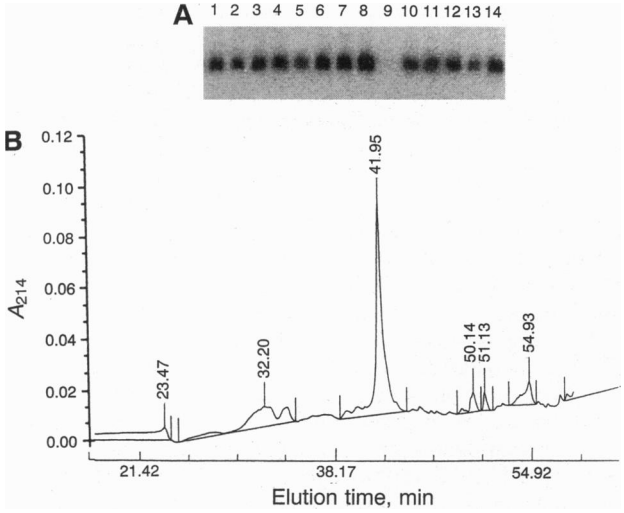

FIG. 6. Purification of RIP, the *agr* inhibitor. (*A*) Postexponential culture supernatant of RN833 was passed through a 3-kDa-cutoff membrane and the flowthrough was applied to a C_{18} reverse-phase HPLC column and eluted with increasing amounts of acetonitrile in 0.1% trifluoroacetic acid. Eluted fractions were lyophilized, redissolved in water to 10×, and added to an early exponential *S. aureus* wild-type RN6390B culture. The activation of *agr* was tested by Northern blotting 40 min after the addition of the following: lanes 1–12, column fractions collected from the column with increasing amounts of acetonitrile; lanes 13 and 14, no addition, at times 0 and 40, respectively. The autoradiogram is presented. (*B*) column fraction 9 was reapplied to the C_{18} reverse-phase column and the absorbance at 214 nm was measured.

the activator by mutation. Studies were carried out to determine whether RAP and RIP compete for the activation of RNAIII transcription. Postexponential culture supernatants of wild-type RN6390B cells (containing the activator) were applied to early exponential RN6390B cells together with increasing amounts of RN833 postexponential culture supernatants (containing the inhibitor). The results (Fig. 7) indicate that the presence of the inhibitor decreased the level of activation seen with the activator alone, suggesting that RAP and RIP may bind to the same receptor, RAP serving as an agonist and RIP serving as an antagonist. It is also possible that the

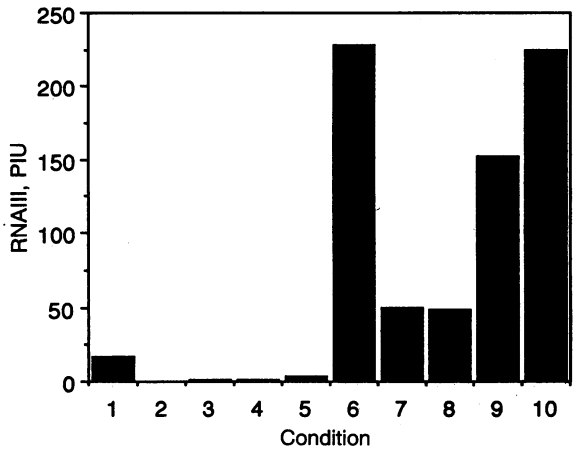

FIG. 7. Competition between the activator (RAP) and the inhibitor (RIP) for the activation of *agr* in early exponential RN6390B cells. The activation of *agr* was tested by Northern blotting 40 min after the addition of the following: bar 1, CY broth; bar 2, RN833 postexponential culture supernatant (T6) diluted 1:1; bar 3, T6 diluted 1:2; bar 4, T6 diluted 1:10; bar 5, T6 diluted 1:100; bar 6, culture supernatant of postexponential wild-type RN6390B; bars 7–10, as for bar 6 but with decreasing amounts of the RN833 culture supernatant (1:1, 1:2, 1:10, and 1:100).

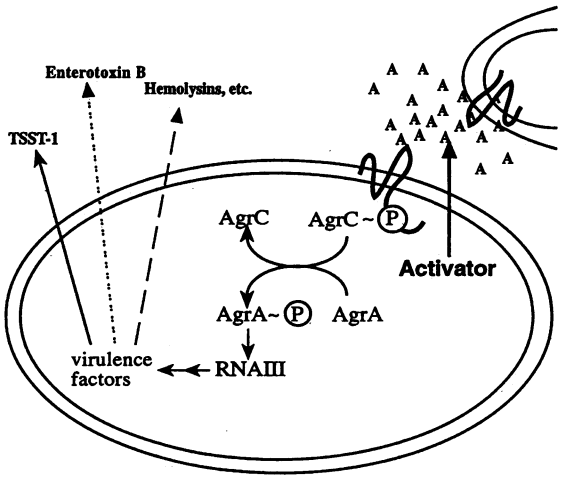

FIG. 8. A schematic diagram showing the proposed mechanism of *agr* activation and its effect on the synthesis of virulence factors. The activator RAP (A), produced and secreted by the bacteria, binds to its putative receptor AgrC (resembling the signal transducer of the two component bacterial signal transduction system), which then phosphorylates AgrA (resembling the response regulator of the two component bacterial signal transduction system) (6). Transcription of RNAIII is then initiated, which in turn regulates the expression of virulence factors (8).

activator and the inhibitor bind to different receptors, activating different signal transduction pathways which in turn activate or inhibit the *agr* response.

DISCUSSION

While attention has formerly been focused on environmental signals which activate regulatory systems for virulence factors in bacteria (3), we show here that a factor, RAP, probably a protein, produced and secreted by the bacteria, is an activator of the *agr* regulatory system in *S. aureus*. Our results indicate that the bacterium is not merely a passive responder to environmental changes but an active participant in activating its own virulence regulatory systems (Fig. 8).

Regulatory mechanisms involving autoinducers have been described for other bacterial systems, including competence and sporulation in *Bacillus subtilis* (18, 19), competence in *Streptococcus pneumoniae* (20), conjugation in *Enterococcus faecalis* (17), luciferase production by *Vibrio fischerii* (21), and elastase production by *Pseudomonas aeruginosa* (22). Although the elastase inducer affects a virulence factor, the possibility of an autoinducer that activates an extensive multigenic virulence regulon appears to be novel.

Due to the increase in bacterial resistance to antibiotics, new strategies to combat bacterial infections are urgently needed. Preliminary studies (unpublished work) have shown that sera containing antibodies directed against the activator block the activation of *agr*. It is therefore possible that blocking the expression of virulence factors by RIP or by antibodies directed against RAP or its receptor will interfere with the ability of *S. aureus* to establish and maintain an infection. The possibility of controlling bacterial infections by interfering with the expression of virulence is a novel approach (23). Suppression of virulence would not kill the bacteria but rather interfere with their pathogenicity and may consequently provide less selective pressure for new resistant strains to emerge.

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