Regulation of *Staphylococcus aureus* Pathogenesis via Target of RNAIII-activating Protein (TRAP)*

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In culture, the bacteria produce toxic exomolecules only when in higher densities, at the post-exponential phase of growth. In the early exponential phase, when in lower densities, the bacteria express surface molecules, such as fibronectin-binding proteins and fibronogen-binding protein, that allow the bacteria to adhere to and colonize host cells. The ability of the bacteria to switch between expression of surface adhesion molecules and toxin exomolecules (1) is regulated primarily by an RNA molecule termed RNAIII (6–8). It is hypothesized that RNAIII enables the bacteria to adhere to host cells when in low numbers but to disengage and spread when too crowded, thus allowing dissemination and establishment of the infection.

RNAIII is encoded by the *agr* locus (6) and regulates at least 15 genes coding for potential virulence factors. *agr* mutants are nonpathogenic and show a decreased synthesis of extracellular toxins and enzymes, such as α-, β-, and δ-hemolysin, leucocidin, lipase, hyaluronate lyase, and proteases, and at the same time an increased synthesis of adhesion molecules, coagulase, and protein A (2, 9). The *agr* locus contains two divergent transcription units, RNAII and RNAIII, driven by the promoters P2 and P3, both of which are active only from the mid-exponential phase of growth (9). RNAII contains four open reading frames: *agrA*, *agrB*, *agrC*, and *agrD*. The *agrA* and *agrC* genes encode a classical two-component signal transduction pathway composed of the AgrC signal receptor and the AgrA response regulator. The *agrD* gene product is a prepeptide that is processed and secreted through AgrB, which is an integral membrane protein. The resultant mature autoinducing peptide (AIP)1 (10) is the ligand that binds to and activates the phosphorylation of AgrC (11), which in turn is thought to phosphorylate AgrA, leading to up-regulation of RNAII synthesis (12).

The synthesis of RNAIII is regulated by a quorum sensing mechanism (13). Molecules produced and secreted by the bacteria (autoinducers) accumulate, and when they reach a threshold concentration, RNAIII is synthesized. The autoinducers of RNAII that have been described to date are the RNAII-activating protein (RAP)1 (14–16) and the *agr*-encoded AIPs (10, 17, 18). RAP is a ~38-kDa protein containing the NH₂-terminal sequence IKKYKPKTN (16). The AIPs are octapeptides encoded by the *agr*, are processed from Agrp, and activate RNAII by inducing the phosphorylation of their receptor AgrC. Interestingly, AIPs produced by some *S. aureus* strains inhibit the expression of *agr* in other strains, and the

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* Staphylococcus aureus can cause disease through the production of toxins. Toxin production is autoinduced by the protein RNAIII-activating protein (RAP) and by the autoinducing peptide (AIP), and is inhibited by RNAIII-inhibiting peptide (RIP) and by inhibitory AIPs. RAP has been shown to be a useful vaccine target site, and RIP and inhibitory AIPs as therapeutic molecules to prevent and suppress *S. aureus* infections. Development of therapeutic strategies based on these molecules has been hindered by a lack of knowledge of the molecular mechanisms by which they activate or inhibit virulence. Here, we show that RAP specifically induces the phosphorylation of a novel 21-kDa protein, whereas RIP inhibits its phosphorylation. This protein was termed target of RAP (TRAP). The synthesis of the virulence regulatory molecule, RNAIII, is not activated by RAP in the *trap* mutant strain, suggesting that RAP activates RNAIII synthesis via TRAP. Phosphoamino acid analysis shows that TRAP is histidine-phosphorylated, suggesting that TRAP may be a sensor of RAP. AIPs up-regulate the synthesis of RNAIII also in *trap* mutant strains, suggesting that TRAP and AIPs activate RNAIII synthesis via distinct signal transduction pathways. Furthermore, TRAP phosphorylation is down-regulated in the presence of AIP, suggesting that a network of signal transduction pathways regulate *S. aureus* pathogenesis.

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1 The abbreviations used are: AIP, autoinducing peptide; bp, base pair(s); HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PFB, phosphate-free buffer; RAP, RNAIII-activating protein; RIP, RNAIII-inhibiting peptide; TRAP, target of RAP; TRAP-P, phosphorylated TRAP.
aureus (15). RNAIII synthesis can be inhibited by AIPs of nonself (10, 18) and by RNAIII-inhibiting peptide (RIP) (14–16, 20). The RIP is produced by coagulase negative staphylococcus (suggested to be Staphylococcus xylosus) (16, 19) and has the sequence YSP (15). Although the synthetic potential of inhibiting RNAIII synthesis was confirmed by Mayville et al. (10), who demonstrated that peptides (AIPs) that inhibit RNAIII synthesis in vitro do in fact inhibit S. aureus infections in vivo.

The potential therapeutic efficacy of peptides (AIPs) in preventing S. aureus infections in vivo has been shown to prevent S. aureus SD cellulitis in mice (15). Synthetic RIP has been shown to prevent keratitis (tested in rabbits against S. aureus 8325-4), osteomyelitis (tested in rabbits against S. aureus MS), mastitis (tested in cows against S. aureus Newburd 305, AE-1, and environmental infections), and septic arthritis (tested in mice against S. aureus LS-1) (20). These findings strongly evidence the potential value of RIP as a therapeutic agent. The therapeutic potential of inhibiting RNAIII synthesis was confirmed by Mayville et al. (10), who demonstrated that peptides (AIPs) that inhibit RNAIII synthesis in vitro do in fact inhibit S. aureus infections in vivo.

Because of the sequence similarity between the NH2-terminal sequence of RIP and tested for their activity, 50 μl of 10× active fraction was applied to 450 μl of early exponential cells (containing about 2 × 109 cells) as described below.

In Vivo Phosphorylation Assays—S. aureus RN6390B (4 ml) was grown in CY broth supplemented with β-glycerophosphate (21) at 37 °C with shaking from early exponential phase of growth.

Preparation of AIP and RIP—To partially purify AIP from RN6390B and RIP from RN8332 post-exponential supernatants, cells were grown to the post-exponential phase of growth. Growth culture was centrifuged at 6000 × g for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22 μm filter to remove residual cells. The supernatant was lyophilized and resuspended in water to one-tenth of the original volume.

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times indicated. Cells were collected by centrifugation for 2 min at 12,000 × g, supernatants were removed, and cells were washed once in PBS to remove unincorporated 32P. Radiolabeled cells were resuspended in 20 μl of 50 μg/ml lysostaphin in 10 mM Tris, pH 7.2, 1 mM EDTA, 20% sucrose and incubated for 10 min at room temperature. Cells were collected by centrifugation (2 min at 12,000 × g), and RNA was purified as described below. If cells were incubated with synthetic RIP, the amount of peptide used was 10 μg/6 × 10^6 cells.

Detection of RNAIII and TRAP Transcript: RNA Purification and Northern Blotting—Equal number of cells were resuspended in 50 μl of PBS, pH 7.5. Fractions containing high cpm in comparison with the rest of the fractions were selected, separated by 15% SDS-PAGE, and gel autoradiographed. The intensity of the band determined using a quantitative analysis program (Molecular Analyst).

Phosphorylated TRAP (TRAP-P) Purification—A cell pellet of 500 μl of early exponential S. aureus cells (grown as described above) was resuspended in 30 ml of PFB, 560 μCi of 32P, and 3 ml of RAP and grown for 1 h with shaking at 37 °C. Cells were collected by centrifugation, washed in PBS, resuspended in 2.5 ml of water containing 100 μM lysostaphin (Sigma), and incubated on ice for 20 min. 7.5 ml of water was added, and cells were further incubated on ice for 20 min and disrupted by extensive sonication (Sonic Dismembrator, Fisher Scientific, microtip probe) (three times for 10 s each time on ice). Sonicated material was centrifuged (10 min at 12,000 × g), and soluble material was collected. Soluble material was concentrated by lyophilization (Savant, SpeedVac Plus SC110A) to 800 μl. Material was fractionated on a gel filtration HPLC column (Bio-Sil SEC-125 300 × 7.8 mm, Bio-Rad) in 1 mM phosphate-buffered saline, pH 8.0, at a flow rate of 1 ml/min. 1-ml fractions were collected and tested for radioactivity in a scintillation counter (Beckman, LS6000 multipurpose scintillation counter). Samples containing high cpm in comparison with the rest of the fractions were selected, separated by 15% SDS-PAGE, and gel autoradiographed. The fraction containing the radiolabeled 21-kDa protein (phosphorylated TRAP) was used to further purify TRAP by anion exchange chromatography (HPLC SynchroFloc Q2000, Keystone Scientific, Inc.) in 0.1 × PBS, pH 7.5. Bound material was eluted by a salt gradient of 0–1 M NaCl in 0.1 × PBS, pH 7.5. Fractions containing high cpm were separated by 15% SDS-PAGE and gel autoradiographed to determine the fraction containing TRAP. Phosphorylated TRAP eluted at 0.75 M NaCl. The 21-kDa radioactive band was cut and submitted for amino acid sequencing (see below).

Amino Acid Sequence Analysis—The anion exchange TRAP-containing fraction was applied to SDS-PAGE, and the gel was stained in Coomassie and autoradiographed. The protein band corresponding to phosphorylation was cut and NH2-terminally sequenced or subjected to tryptic digestion for acquiring internal sequences. Specifically, the gel was dried, rehydrated in 50 mM ammonium bicarbonate, pH 7.8, and incubated with 0.5 μg of trypsin overnight at 37 °C. Peptides were extracted by 70% acetonitrile/5% formic acid and fractionated on a C18
that the trap containing the disrupted gene was amplified by PCR, employing primers that are homologous to the plasmid region (universal reverse primer 5'-GTG-GTCTCCATCTCTTCTAG-3' and 3'-GTGG-TAATGACTAGTTTATCATCGT-3'). A DNA fragment of 500 bp was generated using the above primers, indicating the integration of the plasmid and disruption of trap. The S. aureus strain RN4220 cells (a restriction-deficient derivative of strain 8325-4 and termed S. Del-Cardayre), which is a shuttle vector between S. aureus S. aureus and other gram-positive bacteria, was used to generate transformants. CRISPR-Cas9-mediated genome editing was performed in these strains.

**RESULTS**

**RAP Activates and RIP Inhibits RNAIII Synthesis and the Phosphorylation of a 21-kDa Protein—Early exponential wild-type S. aureus cells were incubated for 40 min in the presence of RAP, synthetic RIP (Genemed Synthesis, Inc. CA), or PBS only as a control. Cells were collected, RNA purified, and Northern blotted, and membranes were incubated with radiolabeled RNAIII-specific DNA as a probe. As previously demonstrated (14, 16) and as shown in Fig. 1, RAP activates and RIP inhibits RNAIII synthesis. The pathway by which the autoinducer RAP activates and the peptide RIP inhibits RNAIII synthesis was not known, but it was hypothesized that RAP and RIP interact with the same receptor, one as an agonist (RAP), the other as an antagonist (RIP). Therefore, it seemed reasonable to assume that, like other quorum sensing molecules, they would regulate a bacterial two component system by phospho-

**Phosphoamino Acid Analysis—**Purified radiolabeled TRAP-P was applied to SDS-PAGE. Slices of acrylamide, containing labeled TRAP-P, were excised and submerged in 3 N KOH at 105 °C for 5 h. The resulting hydrolysate was diluted 25-fold with water containing internal standards of phosphoserine and phosphotyrosine. Phosphoamino acids were separated by ion-exchange chromatography (24). O-Phthalaldehyde was added to the eluate, and the resulting fluorescence was detected on-line (24). Radioactivity was quantified by liquid scintillation counting. Phospholysine and phosphohistidine were synthesized as described previously (24). All other standards were purchased from Sigma. In this system, phosphoarginine and phosphoproline elute before phosphoserine, phosphothreonine elutes close to phosphoserine, and phosphohistidine elutes between phosphoserine and phosphotyrosine (24).

**HPLC (Vydac, 4.6 x 25 cm) in 0.1% TFA. Peptides were eluted on a 115-min gradient of 0–80% acetonitrile. Peptides were collected and amino acid sequence determined using Edman degradation chemistry (ABI 477 sequencer, Protein Structure Laboratory, UC Davis). Sequences were compared with the S. aureus Genome Sequencing Project data base, and the sequence of TRAP was obtained. Primers corresponding to the 5’- and 3’-ends of the gene were constructed, trap was amplified by PCR, and the DNA sequence was confirmed.

Inactivation of trap—An internal 317-bp fragment (73–390) of the trap gene was amplified by PCR using the following primers: 1) 5'-CGCGGGATCCCTCCGCTCTCCGCTCCGCTC-3' (containing the BamHI site), and 2) 5'-CGCGGAAGCTTTCTAAAGTCTTCGTATG-3' (containing the HindIII site). The amplified PCR fragment was cloned into the BamHI/HindIII sites of the pAUL-A vector (kindly provided by S. Del-Cardayre), which is a shuttle vector between S. aureus and Escherichia coli. This plasmid contains an erythromycin resistance marker and carries a temperature-sensitive mutation at the origin of replication, and therefore it is capable of replication in Escherichia coli and transformants were grown on NYE agar (23) in the presence of 10 μg/ml erythromycin at the restrictive temperature of 42 °C overnight. Colonies were excised and submerged in 3N KOH at 105 °C for 5 h. The resulting hydrolysate was diluted 25-fold with water containing internal standards of phosphoserine and phosphotyrosine. Phosphoamino acids were separated by ion-exchange chromatography (24). O-Phthalaldehyde was added to the eluate, and the resulting fluorescence was detected on-line (24). Radioactivity was quantified by liquid scintillation counting. Phospholysine and phosphohistidine were synthesized as described previously (24). All other standards were purchased from Sigma. In this system, phosphoarginine and phosphoproline elute before phosphoserine, phosphothreonine elutes close to phosphoserine, and phosphohistidine elutes between phosphoserine and phosphotyrosine (24).

**Regulation of Pathogenesis**

The phenotype of S. aureus, YG1, had a similar phenotype to that of OU20.2

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**Fig. 4. A and B, amino acid sequence of TRAP (A) and DNA sequence of trap (B). C, secondary structure prediction of TRAP generated by the PHD package (27). Cylinders and arrows denote a-helix and b-sheet, respectively.**
Regulation of Pathogenesis

RAP Does Not Activate RNAIII Synthesis in the trap Mutant Strain—The trap gene was inactivated by gene disruption. An S. aureus trap gene was cloned into pAUL (Fig. 5A), a temperature-sensitive shuttle vector (kindly provided by S. Del Cardayre), and plasmid was used to transform S. aureus RN4220 cells. Transformants were analyzed for integration of the plasmid into the chromosome at the trap site via a Campbell insertion process. The analysis was

RAP in the trap mutant strain. RNA was purified, equal amounts of RNA (10\(^{-9}\) cells/ml) were applied to SDS-PAGE, and the gel was autoradiographed. Lane 1, trap\(^-\); Lane 2, trap\(^+\). The synthesis of RNAIII is not activated by RAP in the trap mutant strain. Early exponential S. aureus trap\(^-\) and trap\(^+\) cells were in vivo phosphorylated in the presence of RAP. After 40 min, cells were collected, total cell homogenate was applied to SDS-PAGE, and the gel was autoradiographed. Lane 1, trap\(^-\). Lane 2, trap\(^+\). C, the synthesis of RNAIII is not activated by RAP in the trap mutant strain. Early exponential S. aureus trap\(^-\) and trap\(^+\) cells were incubated for 40 min with PBS (RAP\(^-\)) or with RAP (RAP\(^+\)). RNA was purified, equal amounts of RNA (10 \(\mu\)g) were applied to the gel, and the gel was Northern blotted. RNAIII was detected using radiolabeled RNAIII-specific DNA as a probe, and the membrane was autoradiographed.

Early exponential (1 to 3 h, respectively. Lanes 1–4, the RNAIII and TRAP transcript was tested by Northern blotting, and the membrane was autoradiographed. As shown in Fig. 2, RAP activates and RIP inhibits TRAP (Fig. 3, the higher the amount of RIP present, the lower the amount of TRAP phosphorylation, suggesting that RIP competes with RAP on the phosphorylation of TRAP.

To determine whether RAP induces the synthesis of TRAP, cells were incubated in phosphate-free buffer supplemented with radiolabeled orthophosphate, together with RAP in PBS, with PBS, or with RIP (native or synthetic). After a 40-min incubation period, the cells were collected by centrifugation and treated with lysostaphin followed by the addition of sample buffer; without boiling, total cell homogenate was applied to both 7.5 and 15% SDS-PAGE, and the gel was stained in Coomassie or autoradiographed. As shown in Fig. 2, RAP activates and RIP inhibits the specific phosphorylation of a 21-kDa protein that we termed TRAP. Endogenous RAP is produced as the cells grow (14), probably contributing to the positive signal in the control PBS group (Fig. 2, lane 1). To determine whether RIP competes with RAP on TRAP phosphorylation, cells were incubated with RAP together with increasing amounts of native RIP and in vivo phosphorylation assays were carried out. As demonstrated in Fig. 3, the higher the amount of RIP present, the lower the amount of TRAP phosphorylation, suggesting that RIP competes with RAP on the phosphorylation of TRAP.

To determine whether RAP induces the synthesis of TRAP and not only its phosphorylation, the in vivo phosphorylation assays were carried out in the presence of 100 \(\mu\)g/ml chloramphenicol, to inhibit potential translation processes. The results of these experiments (not shown) indicate that RAP activates TRAP phosphorylation also in the presence of chloramphenicol, suggesting that RAP activates TRAP phosphorylation and not synthesis.

Structure of TRAP—To purify TRAP, wild type early exponential S. aureus cells were in vivo phosphorylated, cells were disrupted by extensive sonication, and soluble material containing TRAP-P was fractionated on an HPLC gel filtration column. Positive fractions (determined by peak radioactivity and confirmed by separating a sample by SDS-PAGE) were applied to an HPLC anion exchange column, and bound material was eluted by a 0–1 M NaCl gradient. The positive fraction containing TRAP-P eluted at \(\sim\)0.75 M NaCl.

To determine the amino acid sequence of TRAP, purified TRAP was internally digested by trypsin, and peptide digests were amino acid sequenced. Acquired sequences were compared with the S. aureus data base, and the sequence of TRAP was determined to be a 167-amino acid polypeptide (Fig. 4) (GenBank\textsuperscript{TM} accession number AF202641). The sequence of TRAP (Fig. 4, A and B) is unique to S. aureus and shows no significant sequence homology to known proteins or genes but for 5’-end of the Bacillus subtilis penicillin-binding protein gene (\(\text{pbpF}\)), with which it shares 28% identity (26). Two-dimensional proton NMR spectra (not shown) reveal a folded protein made up of both \(\alpha\)-helices and \(\beta\)-sheet secondary structure elements, in agreement with sequence and threading analysis (Fig. 4C) generated by the PHD package and threading analysis (27).

RAP in the trap mutant strain. RNA was purified, equal amounts of RNA (10 \(\mu\)g) were applied to the gel, and the gel was Northern blotted. RNAIII was detected using radiolabeled RNAIII-specific DNA as a probe, and the membrane was autoradiographed.

Early exponential wild type S. aureus cells were grown for 1–4 h. Equal number of cells were collected, RNA was extracted, the RNAIII and TRAP transcript was tested by Northern blotting, and the membrane was autoradiographed. Lanes 1–4, cells were grown for 1–4 h, respectively.

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done by PCR, employing primers that are homologous to the vector and to the 5’ sequences of the pre-trap gene not present on the plasmid construct. A DNA fragment of about 500 bp was generated, indicating the integration of the plasmid and the disruption of trap, resulting in a trap$^{-}$ mutant strain (S. aureus OU20). Sequence analysis of OU20 indicated the replacement of the 3’-end of the trap gene (from nt 390) with pAUL DNA. S. aureus OU20 was in vivo phosphorylated in the presence of RAP, total cell homogenate was applied to SDS-PAGE, and TRAP-P was detected by autoradiography. As shown in Fig. 5B, RAP activated the phosphorylation of TRAP in the trap$^{-}$ strain (lane 1), but it did not activate phosphorylation in the trap$^{-}$OU20 strain (lane 2), suggesting that in fact the trap gene was disrupted.

To test whether RAP activates the synthesis of RNAIII via TRAP, early exponential S. aureus trap$^{-}$ and trap$^{+}$ cells were incubated together with RAP; after 40 min, cells were collected, RNA was extracted, and RNAIII was analyzed by Northern blotting using radiolabeled RNAIII-specific DNA as a probe. As shown in Fig. 5C, although RNAIII synthesis was activated by RAP in the parent trap$^{+}$ strain, it was not activated in the trap$^{-}$ strain, suggesting that the presence of an intact trap gene is necessary for RAP to activate RNAIII synthesis. To test whether RNAIII can be synthesized in the absence of TRAP during bacterial growth, trap$^{-}$ and trap$^{+}$ cells were grown for several hours from the early exponential phase of growth, and RNAIII and TRAP tested by Northern blotting. As shown in Fig. 5D, RNAIII synthesis was greatly reduced in the trap$^{-}$ mutant strain but was not abolished. These results suggest that trap is important for the activation of RNAIII synthesis but that the synthesis of RNAIII can nevertheless be activated at a later stage in the absence of TRAP, possibly by alternate pathways, such as sar (28, 29). As also shown in Fig. 5D, trap transcription is in fact absent in the trap$^{-}$ strain and is constitutive in the trap$^{+}$ strain. Of note is the fact that the translation of TRAP also appears to be constitutive in the wild type trap$^{+}$ strain (data not shown). The fact that trap is constitutively transcribed and translated while its phosphorylation is regulated further supports our results indicating that RAP regulates TRAP phosphorylation and not synthesis.

**TRAP Is Histidine-phosphorylated—**Two-component systems act through phosphorylation of the substrate domain of the sensor protein and subsequent transfer of the phosphate to an aspartate residue in the regulator protein. The initial phosphorylation is catalyzed by a protein histidine kinase domain in the sensor protein and results in an N-phosphorylated histidine residue, which is stable in alkaline conditions but not in acidic conditions (24).

To test whether TRAP may be histidine-phosphorylated, we tested the sensitivity of phosphorylated TRAP to acidic and basic conditions. Phosphorylated TRAP was incubated at pH ranging from 1 to 10 for 10 min at room temperature. The mixture was then applied to SDS-PAGE, and the gel was autoradiographed. As shown in Fig. 6A, phosphorylation of TRAP was found to be stable at pH greater than 8.0 but labile at lower pH values, consistent with a possible N phosphorylation of a histidine. Phosphoamino acid analysis indicates that in fact TRAP-P contains phosphohistidine. Purified radiolabeled TRAP-P was applied to SDS-PAGE. The gel band containing phosphorylated TRAP-P was subjected to alkaline hydrolysis followed by chromatography (24). The labeled phosphoamino acid eluted at the position of phosphohistidine, which is distinct from phosphoarginine, phosphosine, phosphothreonine, phosphoserine, or phosphotyrosine (Fig. 6B). Histidine phosphorylation indicates that TRAP may in fact be a sensor of RAP.

**Regulation of TRAP Phosphorylation—**RNAIII is produced only from the mid-expontential phase of growth, whereas TRAP is continuously transcribed (Fig. 5D). If RAP activates RNAIII via TRAP phosphorylation, it seemed reasonable to assume that TRAP phosphorylation and RNAIII synthesis should be coupled. To determine when TRAP is phosphorylated during bacterial growth, wild type S. aureus were grown from early to late logarithmic phase of growth in the presence of $^{32}$P. Cells were collected at time intervals and assayed both for TRAP phosphorylation and for RNAIII synthesis. As shown in Fig. 7, A and B, peak phosphorylation of TRAP was reached at the mid-expontential phase of growth. Peak phosphorylation of TRAP directly correlates with RNAIII synthesis, supporting...
our hypothesis that RAP regulates RNAIII synthesis via TRAP phosphorylation.

As shown in Fig. 7, A and B, TRAP reached its peak phosphorylation by the mid-exponential phase of growth but was dephosphorylated by the late logarithmic phase of growth. The RNAIII gene, on the other hand, once activated, remained up-regulated throughout growth (Fig. 7B). The fact that TRAP was dephosphorylated by late log indicates that TRAP phosphorylation is necessary only for the induction of the RNAIII gene but not for its ongoing transcription.

![Figure 7](image_url)

**A** Wild type *S. aureus* log phase

| lag | early | mid | late |
|-----|-------|-----|------|
|     |       |     | -46  |
|     |       |     | -30  |
|     |       |     | -21.5|
|     |       |     | -14.3|

**B** TRAP phosphorylation versus RNAIII and cell number (cell #).

**C** TRAP is phosphorylated in *agr*-null strains. Mutant *agr*-null *S. aureus* cells RN6911 were grown from early to late log phase of growth in PFB together with 32P. Cells were collected, the total cell homogenate was applied to SDS-PAGE, and the gel was autoradiographed.

**AIP Activates RNAIII Synthesis but Inhibits TRAP Phosphorylation**—RNAIII production has been shown to be autoinduced also by AIP, an octapeptide encoded by the *agr* itself. AIP activates RNAIII synthesis by inducing the phosphorylation of a two-component system, also encoded by the *agr*. Specifically, once the *agr* is activated in the mid-exponential phase of growth, an octapeptide is produced (processed from AgrD), inducing the phosphorylation of a 46-kDa protein, AgrC (11), which is hypothesized to phosphorylate AgrA (12), leading to up-regulation of RNAIII synthesis. To determine the interac-
Our work demonstrates that the autoinducer of virulence RAP activates and the inhibitor of virulence RIP inhibits the phosphorylation of a 21-kDa protein termed TRAP. Amino acid sequence analysis of TRAP indicates that the 167 amino acid polypeptide is unique to *S. aureus*. RAP does not activate RNAIII synthesis in a *S. aureus* strain containing a disrupted *trap*, suggesting that an intact *trap* gene is necessary for the activation of RNAIII synthesis by RAP. Phosphoamino acid analysis of TRAP-P indicates that TRAP is histidine-phosphorylated, indicating that TRAP may be a sensor of RAP. Secondary structure predictions suggest it, however, to be globular – and may be a sensor of RAP. Second-

**DISCUSSION**

The phosphorylation of TRAP is activated by RAP and inhibited by AIP. A. wild type early exponential *S. aureus* cells were incubated for 1 h in PFB, containing no RAP, with post-exponential supernatant (total, containing 20\% RAP and 80\% AIP (16, 19)), with AIP (containing no RAP), or with PBS as a control. Cells were collected and applied to SDS-PAGE, and the gel was autoradiographed. The autoradiogram was scanned, and the density of the bands was determined. B. in parallel, cells (in CY) were incubated in the presence of RAP, PBS, AIP, and total supernatant for 40 min, and cells were assayed for RNAIII by Northern blotting. The density of the bands was determined, and results are presented as percentage of maximum RNAIII observed (% of max). C. in vivo phosphorylation: RAP/AIP competition experiment. Wild type early exponential cells in PFB and 32P (450 µl) were incubated with 50 µl of RAP (40×, >10) that was partially purified from post-exponential supernatants (lane 1), together with 25 µl of AIP and 25 µl of CY (lane 2) or with 50 µl of AIP (lane 3). This gave estimated RAP:AIP ratios of 1:0 (lane 1), 1:0.5 (lane 2), and 1:1 (lane 3). After 40 min, cells were collected and applied to SDS-PAGE, and the gel was autoradiographed, and the density of the bands was determined. D. RNAIII synthesis is activated by AIP but not by RAP in a *trap* mutant strain. Early exponential (1 × 10^5 cells/ml) *S. aureus* *trap*^−^ and *trap*^+^ cells were grown for 40 min in the presence of RAP, PBS, or AIP. Equal numbers of cells were collected, RNA was extracted, RNAIII was tested by Northern blotting, and the membrane was autoradiographed.

TRAP reaches its peak phosphorylation by the mid-exponential phase of growth but is dephosphorylated by late logarithmic phase of growth. The gene for RNAIII, on the other hand, once activated, remains up-regulated throughout. The fact that TRAP is dephosphorylated by late log indicates that TRAP...
phosphorylation is necessary only for the induction of the RNAIII gene but not for its ongoing transcription. TRAP is phosphorylated also in the agr-null strain. As in the wild type, peak phosphorylation is reached at the mid-exponential phase of growth. However, unlike in the wild type strain, TRAP is not dephosphorylated by late log, suggesting that the agr itself, once activated in the mid-exponential phase, produces, or regulates the production of, a factor, which down-regulates TRAP phosphorylation.

One of the factors produced by the agr is the octapeptide AIP that also activates RNAIII synthesis. However, the agr locus is temporally regulated, and therefore the AIP is only produced from the mid-exponential phase of growth (17). We show here that whereas RAP activates RNAIII synthesis as well as TRAP phosphorylation, the AIP activates RNAIII synthesis but inhibits TRAP phosphorylation. Furthermore, whereas RAP does not activate RNAIII synthesis in a trap- strain, AIP does up-regulate RNAIII synthesis in a trap- strain, suggesting that RAP and AIP activate RNAIII synthesis via different signal transduction pathways. The fact that TRAP phosphorylation is down-regulated in the presence of the AIP may explain why TRAP is dephosphorylated at the mid-exponential phase of growth, coinciding with AIP production. The interplay between RAP and AIP may explain why TRAP is dephosphorylated at the mid-exponential phase of growth, coinciding with AIP production.

In summary, we propose (Fig. 9) that autoinduction of virulence occurs in a two-step process. As the colony multiplies, the autoinducer RAP accumulates and induces the phosphorylation of its target molecule TRAP, resulting in up-regulation of agr to produce RNAII. Once agr is activated (in the mid-exponential phase of growth), AIP and its receptor AgrC are produced. AIP up-regulates the phosphorylation of its receptor, AgrC (11), leading to phosphorylation of AgrA, up to regulation of RNAIII synthesis (9), and to down-regulation of TRAP phosphorylation. Production of RNAIII, in parallel with up-regulation of sar and sae, causes the expression of toxic exomolecules and the suppression of adhesion molecules (C) (6, 32, 33), resulting in dissemination and in disease.

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Regulation of *Staphylococcus aureus* Pathogenesis via Target of RNAIII-activating Protein (TRAP)
Naomi Balaban, Tzipora Goldkorn, Yael Gov, Miriam Hirshberg, Nir Koyfman, Harry R. Matthews, Rachael T. Nhan, Baljit Singh and Orit Uziel

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Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the RIM101 pathway.

Teresa M. Lamb, Wenjie Xu, Aviva Diamond, and Aaron P. Mitchell

Pages 1853 and 1854: We reported in Tables IV and V that a *vma4-lacZ* fusion gene showed alkaline-induced, Rim101p-dependent expression. Subsequent analysis showed that these strains carried an *arn4-lacZ* fusion gene rather than the *vma4-lacZ* fusion gene. Therefore, our conclusion that VMA4 expression responds to pH and to Rim101p is in error.

Regulation of *Staphylococcus aureus* pathogenesis via target of RNAIII-activating protein (TRAP).

Naomi Balaban, Tzipora Goldkorn, Yael Gov, Miriam Hirshberg, Nir Koyfman, Harry R. Matthews, Rachael T. Nhan, Baljit Singh, and Orit Uziel

Page 2661: The left column, line 8 from the bottom, should read: “... universal reverse primer 5'-AACAGCTATGACC-ATG-3'.”

Page 2662, Fig. 5: The arrow of Ery is drawn backward. Instead of pointing clockwise, it should point counterclockwise.

These changes do not affect the conclusions of the paper.
Additions and Corrections

Vol. 276 (2001) 2658–2667

Regulation of Staphylococcus aureus pathogenesis via target of RNAIII-activating protein (TRAP).

Naomi Balaban, Tzipora Goldkorn, Yael Gov, Miriam Hirshberg, Nir Koyfman, Harry R. Matthews, Rachael T. Nhan, Baljit Singh, and Orit Uziel

Page 2658, top right: The DOI of the Paper in Press (PIP) as shown on the print version is in error. The DOI should read: 10.1074/jbc.M005446200.

Page 2658, right column: The GenBank™ accession number appeared in the PIP version but was deleted in the final print/online version. The sentence ending 5 lines from the bottom of the page should read: NH2-terminal sequence IKKYKPITN (GenBank™ accession number AF205220) (16).

Vol. 275 (2000) 20814–20821

The high resolution crystal structure of yeast hexokinase PII with the correct primary sequence provides new insights into its mechanism of action.

Paula R. Kuser, Sandra Krauchenco, Octávio A. C. Antunes, and Igor Polikarpov

The Protein Data Bank accession numbers for the structures included in this paper were omitted. The PDB code is 1IG8 and the RCSB code is RCSB013246.

Vol. 276 (2001) 8904–8909

Dinucleotides as growth-promoting extracellular mediators. Presence of dinucleoside diphosphates Ap2A, Ap2G, and Gp2G in releasable granules of platelets.

Joachim Jankowski, Joost Hagemann, Martin Tepel, Markus van der Giet, Nina Stephan, Lars Henning, Ioanna Gouni-Berthold, Agapios Sachinidis, Walter Zidek, and Hartmut Schlüter

Dr. Gouni-Berthold’s name was printed incorrectly. The corrected version is shown above.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Mechanisms of hepatic very low density lipoprotein overproduction in insulin resistance. Evidence for enhanced lipoprotein assembly, reduced intracellular apoB degradation, and increased microsomal triglyceride transfer protein in a fructose-fed hamster model.

Changiz Taghibiglou, André Carpentier, Stephen C. Van Iderstine, Biao Chen, Debbie Rudy, Andrea Aiton, Gary F. Lewis, and Khosrow Adeli

Page 8423, Fig. 9: The wrong figure was published although the legend is correct. The correct figure is shown below.

**Fig. 9. Intracellular distribution of nascent apoB-containing lipoproteins in microsomal lumen of control and fructose-fed hepatocytes.** Cultured primary hamster hepatocytes were pulsed for 45 min with [35S]methionine, and the radioactivity was chased for 0 or 1 h. Labeled cells were then subjected to homogenization and fractionation of microsomes. Luminal lipoproteins were extracted from microsomes by carbonate treatment and were separated from the membrane fraction by centrifugation followed by fractionation on a sucrose-gradient. After centrifugation, gradient fractions were collected and immunoprecipitated with an anti-hamster apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography, and apoB radioactivity was quantitated by cutting and scintillation counting of the apoB-100 band. A, luminal lipoproteins in control hepatocytes at 0 and 1 h chase; B, luminal lipoproteins in fructose-fed hepatocytes at 0 and 1 h chase.

**Data Interpretation:** The interpretation of the data in Fig. 9 does not significantly change with this correction. However, an important observation also reported previously should be emphasized further. As observed in the corrected Fig. 9, microsomes from control cells had a significant amount of dense apoB-containing lipoproteins (HDL density), at both 0 and 1 h, which were absent in hepatocytes from fructose-fed hamsters. The absence of HDL-dense apoB particles suggests the stimulated state of VLDL assembly and secretion in hepatocytes of fructose-fed hamsters.