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Transcriptional Profiling of Target of RNAIII-Activating Protein, a Master Regulator of Staphylococcal Virulence

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***Staphylococcus aureus* is a gram-positive bacterium that is part of the normal healthy flora but that can become virulent and cause infections by producing biofilms and toxins. The production of virulence factors is regulated by cell-cell communication (quorum sensing) through the histidine phosphorylation of target of RNAIII-activating protein (TRAP), which is a 21-kDa protein that is highly conserved among staphylococci. Using microarray analysis, we show here that the expression and phosphorylation of TRAP upregulate the expression of most, if not all, toxins known to date, as well as their global regulator *agr*. In addition, we show here that the expression and phosphorylation of TRAP are also necessary for the expression of genes known to be necessary for the survival of the bacteria in a biofilm, like *arc*, *pyr*, and *ure*. TRAP is thus demonstrated to be a master regulator of staphylococcal pathogenesis.**

Staphylococcus aureus is a gram-positive bacterium that is part of the normal flora of the skin, but it can become pathogenic and cause fatal diseases once it forms a biofilm and/or produces toxins (18, 25). Biofilm formation and toxin production are regulated by a quorum-sensing mechanism, where molecules produced and secreted by the bacteria (autoinducers) reach a threshold concentration and activate signal transduction pathways, leading to activation of the genes that encode virulence factors (22, 27, 32, 35, 37).

To date, two staphylococcal quorum-sensing systems (SQS) have been described. SQS 1 consists of the autoinducer RNAIII-activating protein (RAP) and its target molecule, target of RNAIII-activating protein (TRAP) (4, 5, 20, 21). SQS 2 consists of the molecules encoded by *agr* (28, 29). The bacteria secrete RAP, a 33-kDa protein, as they multiply (23); when RAP reaches a threshold concentration (in the mid-exponential phase of growth), RAP induces the histidine phosphorylation of its target molecule TRAP (5, 20). The phosphorylation of TRAP leads, in an as-yet-unknown mechanism, to the synthesis of SQS 2, which is composed of the products of the *agr* system. *agr* encodes two divergently transcribed transcripts, RNAII and RNAIII (28, 29). RNAII encodes AgrA, AgrC, AgrD, and AgrB, where AgrD is a propeptide that yields an autoinducing peptide (AIP) that is processed and secreted with the aid of AgrB. Once *agr* is activated and AIP is secreted, AIP induces the phosphorylation of its receptor AgrC, leading to the production of the regulatory RNA molecule termed RNAIII (28). RNAIII upregulates the production of numerous secreted toxins (28). SQS 1 and SQS 2 interact with one another because once AIP is made in the mid-exponential phase of growth, it indirectly downregulates the phosphorylation of TRAP (5). The interplay between the phosphorylation of TRAP and AgrC by their respective autoinducers, RAP or AIP, regulates the expression of adhesion molecules or toxins

Like typical sensors of classical two component systems, TRAP is histidine phosphorylated in the presence of RAP (5, 20); immunoelectron microscopy and Western blotting studies indicate that it is membrane associated (N. Balaban, unpublished data). However, unlike classical sensors, TRAP does not contain a kinase or a transmembrane domain. In addition, TRAP is phosphorylated on three conserved histidine residues and not just one (20). It is therefore suggested that TRAP is a nonclassical signal transducer and that it may be bound to the membrane through other proteins.

TRAP has been demonstrated to be a key molecule regulating pathogenesis because when TRAP expression is inhibited (by mutagenesis) or when TRAP phosphorylation is suppressed (by mutagenesis or by inhibitory peptides), bacteria do not form a biofilm, do not produce toxins, and do not cause disease (1–4, 6, 9–12, 15–17, 19, 20, 33, 36). Here, we show that the expression and phosphorylation of TRAP are necessary for the expression of multiple genes, many of which are virulence factors or their regulators.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. aureus* strains used in this study are the 8325-4 parent strain TRAP+, which is hemolytic; the TRAP− strain, which is nonhemolytic (8325-4 containing a disrupted *traP* gene and referred to as ΔTRAP); and mutant strain H66A, which is nonphosphorylated and nonhemolytic (8325-4 containing an in-frame mutation in the *traP* gene, resulting in the exchange of the conserved histidine [His-66] residue with alanine) (20). Strains were grown overnight in 10 ml of tryptic soy broth at 37°C with shaking. Mutant strains were grown also with 100 μg/ml kanamycin. Overnight cultures were used to inoculate (1:100 dilution) 5 ml of fresh tryptic soy broth medium with no antibiotics. Cultures were incubated with shaking at 37°C, and aliquots were removed at the indicated times. It is of note that mutants and parent strains have been shown to have similar growth curves (20).

Microarray. GeneChip *S. aureus* Genome Array (Affymetrix) contains sequences of *S. aureus* N315, Mu50, NCTC 8325, and COL. The array contains probe sets to >3,300 *S. aureus* open reading frames and probes to study >4,800 intergenic region sequences.

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RNA isolation and cDNA labeling. Cells were collected by centrifugation, and total RNA was isolated by using the RNeasy Protect (QIAGEN) protocol with some modifications, as follows. At the indicated times, *S. aureus* bacterial cultures were collected and immediately mixed in 2 volumes of RNA Protect for 5 min at room temperature. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. Cells were lysed by using 100 μl of lysostaphin (3 mg/ml [Sigma-Aldrich]) in TE (100 mM Tris, pH 7.2; 1 mM EDTA) for 10 min at room temperature. RLT buffer (a buffer supplied with the Qiagen kit and supplemented with mercaptoethanol and ethanol) was immediately added. The lysate was then placed on a spin column to bind total RNA. The column was washed multiple times with supplied buffer, and RNA was eluted with diethylpyrocarbonate-treated water. RNA concentration and purity were determined spectrophotometrically. Purified RNA was DNase I treated and RNAsin (RNase inhibitor; Promega). The following was carried out by Genome Explorations, Memphis, TN. cDNA was prepared by using random primers. The RNA-primer mixture (10 μg of RNA and 150 ng of primers) was incubated at 70°C for 10 min, followed by a snap freeze in a dry ice-ethanol bath for 30 s. The sample was centrifuged for 1 min. A deoxynucleoside triphosphate mixture was made (10 mM) and mixed in a final 0.5 mM concentration to the reverse transcription reaction mixture (dithiothreitol and SUPERase·In RNase Inhibitor and SuperScript II [Invitrogen Life Technologies]) was added to the denatured, cooled RNA-primer mixture. A negative control was also included, in which no reverse transcriptase was added; this sample underwent all subsequent sample preparation, hybridization, and analysis steps. The reaction mixture was incubated at 25°C for 10 min, at 37°C for 60 min, and at 42°C for 40 min. The reverse transcriptase was inactivated at 70°C for 10 min and then chilled on ice. RNA was removed by the addition of NaOH and incubation at 65°C for 30 min, followed by neutralization with HCl. cDNA was fragmented by DNase (1 U of DNase I/μg of cDNA) at 37°C for 20 min, DNase was inactivated at 98°C for 10 min, and the fragmented cDNA was applied to the terminal labeling reaction mixture. 3′-Termini labeling was carried out by using the Enzo Bioarray Terminal labeling kit with biotin-ddUTP as described by the manufacturer, with Terminal DNA Transferase. A gel shift assay was used to determine the efficiency of the labeling, which should be >90%. For this assay, labeled material was incubated with avidin prior to electrophoresis, according to the manufacturer's instructions (Enzo) and gel stained with SYBR Gold.

Oligonucleotide array hybridization and analysis (carried out by Genome Explorations). The cRNA pellet was resuspended in 10 μl of RNase-free H_2O, and 10.0 μg was fragmented by heat- and ion-mediated hydrolysis at 95°C for 35 min in 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate. The fragmented cRNA was hybridized for 16 h at 45°C to a GeneChip *S. aureus* Genome Array (Affymetrix). Arrays were washed at 25°C with 6× SSPE (0.9 M NaCl, 60 mM NaH2PO4, and 6 mM EDTA plus 0.01% Tween 20), followed by a stringent wash at 50°C with 100 mM morpholineethanesulfonic acid, 0.1 M Na^+, and 0.01% Tween 20. The arrays were then stained with phycoerythrin-conjugated streptavidin (Molecular Probes), and fluorescence intensities were determined using a laser confocal scanner (Hewlett-Packard). The scanned images were analyzed with Microarray software (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to constant target intensity (250) for all arrays used. Data analysis was conducted with Microarray Suite 5.0 (Affymetrix), following user guidelines. The signal intensity for each gene was calculated as the average intensity difference, represented by [Δ(PM − MM)/(number of probe pairs)], where PM and MM indicate perfect match and mismatch probes.

To normalize for global systematic variations that could be caused by inconsistencies in loading, each average difference value was divided by the median average difference for a given GeneChip. To identify genes that are below the detection limit of the system, the signal strengths indicative of genes with profiles at the level of noise were determined for each strain as the average signal strength of genes considered absent (via GeneChip algorithms) plus 2 standard deviations.

Northern blot analysis. Early exponential cells were grown from an optical density at 600 nm (OD_{600}) of 0.03 for 6 h (to a postexponential phase of growth) with shaking at 37°C. Cells (~200 μl) were collected by centrifugation (2 min at 12,000 × *g*) and resuspended in 20 μl of lysostaphin in TES buffer (100 μg/ml lysostaphin [Sigma-Aldrich] in 100 mM Tris [pH 7.2], 1 mM EDTA, and 20% sucrose) and incubated for 10 min at room temperature. A total of 20 μl of 2% sodium dodecyl sulfate containing proteinase K (100 μg/ml) was added and vigorously vortexed for 1 min, followed by 10-min incubation at room temperature. The sample was frozen and thawed twice. A 15-μl RNA sample was mixed with 11% deionized glyoxal, 16 mM phosphate buffer, pH 7.0, and 55% dimethyl sulfoxide (final concentrations) and incubated for 1 h at 65°C. RNA loading buffer (Ambion) was added, and the sample was applied to a 1% agarose gel in 10 mM phosphate buffer, pH 7.0, supplemented with 5 mM iodoacetic acid (Sigma-Aldrich). The gel was Northern blotted by dry transfer. Membranes were prehybridized with Rapid-Hyb (Amersham Pharmacia Biotech), followed by hybridization with a PCR-radiolabeled probe derived from the target gene (Table 1), using DNA isolated from *S. aureus* 8325-4 as a template. Membranes were autoradiographed.

TABLE 1. Sequences of forward (top) and reverse (bottom) primers used in this study

Gene	Oligonucleotides
gyrB	5′ TTATGGTGCTGGGCAAATACA 3′
	5′ CACCATGTAAACCACCAGATA 3′
hld	5′ ATGATCACAGAGATGGTA 3′
	5′ CTGAGTCCTAGGAAACTAACT 3′
agrC	5′ GTTTGATAGCGCGTCCTTAAT 3′
	5′ GAAATAATCACGTAGGCCAGG 3′
spa	5′ AGGTGTAGGTATTGCATCTGT 3′
	5′ TTTTTAGCTTCTGACAATAGG 3′
sspA	5′ TTGCTAAATCACCTTCGCCT 3′
	5′ CAGCAAACGCGTTATCTTCA 3′
hla	5′ GCAGATTCTGATATTAATATTA 3′
	5′ AATTTGTCATTTCTTCTTTTTC 3′
hlb	5′ GGAGTGATAATGATGGTGAA 3′
	5′ TTAGTTAGTTGAGCACTATT 3′
epbS	5′ TGCTGTTCCAGCACCAATAG 3′
	5′ AGAAGAAAAAGACGCCGTGA 3′
aur	5′ CCACGCCTACTTCATTCCAT 3′
	5′ GCACACGAATTAACACACGG 3′
clfB	5′ ATGGTGATTCAGCAGTAAATCC 3′
	5′ CATTATTTGGTGGTGTAACTCTT 3′
icaR	5′ AAGGATAAGATTATTGATAACGCAATAAC 3′
	5′ TTTCTTCAAAAATATGTTTAGTAGCGAATACAC 3′
icaA	5′ CCTGTATTTATGTCTATTTACTGG 3′
	5′ CTTCTCGTATTTGAGTGCAAG 3′
sdrC	5′ TAAAGCGGCAGAACATACGA 3′
	5′ GCTGTAGCGTTTTGTGGTGA 3′
sdrD	5′ AAAGGCGTTGGCAATGTAAC 3′
	5′ GCCGTTTGAATCTAATTCTTCG 3′

Two-dimensional gel electrophoresis. For preparation of cell extracts, cells were grown from OD_{600} of 0.03 for 6 h (to postexponential phase) with shaking at 37°C. Cells from 50-ml cultures were collected by centrifugation (7,000 × *g*) for 10 min at 4°C, washed twice with Tris-EDTA buffer (100 mM–10 mM), and then resuspended in Tris-EDTA buffer containing lysostaphin (100 μg/ml [Sigma-Aldrich]) and DNase I (15 U [Ambion]). After incubation for 10 min at 37°C, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was added (final concentration), and the mixture was incubated on ice for 1 h at 4°C. The lysate was then centrifuged for 30 min at 13,000 × *g* at 4°C (to remove cell debris). The supernatant was collected, and the protein concentration was determined (Bio-Rad). The proteins were precipitated overnight with 10% (wt/vol) trichloroacetic acid at 4°C. The precipitate was harvested by centrifugation (4°C; 13,000 × *g* for 10 min), washed several times with 96% (wt/vol) ethanol, and dried. The protein extracts were resolved in an appropriate volume of a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% carrier ampholytes, 2 mM tributylphosphine, and 0.0002% bromophenol blue. Protein samples (each, 500 μg) were separated on preparative two-dimensional gels with immobilized pH gradient strips (Bio-Rad) in the pH range from 4 to 7. Gels were stained with Coomassie blue G-250 (Bio-Rad). Protein spots were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry at the Maiman Institute for Proteome Research at Tel-Aviv University. Dual-channel images were produced with Delta2D software (Decodon GmbH). The resulting peptide mass fingerprints were analyzed by using the MS-Fit software, GPMAW 4.10, and compared to available genome sequences of *S. aureus*.

cDNA-PCR. The relative expression levels of *spa* (protein A), *agrC* (accessory gene regulator C), *rnaIII* (δ-hemolysin), *sspA* (staphylococcal serine protease; V8 protease), and *aur* (zinc metalloproteinase aureolysin precursor) genes were determined by cDNA-PCR. As internal standards, the relative expression levels of the *epbS* gene were used because it was shown by microarray analysis to be equal in the mutant and parent strain. Briefly, cells were grown to the postex-

TABLE 2. Genes upregulated by TRAP[a]

Affymetrix Probe set no.	N315 ORF[b]	N315 gene[c]	N315 description	Repeat 1	Repeat 2	Repeat 3	H66A
c4149s	SA1844	*agrA*	Accessory gene regulator A	4.6	4.8	2.1	3.7
c1047s	SA1842	*agrB*	Accessory gene regulator B	4.6	5.4	1.2	3.2
c10473s	SA1843	*agrC*	Accessory gene regulator C	4.3	4.7	1.8	3.6
c4148s	SAS066	*agrD*	Accessory gene regulator D	4.1	4.2	–	2.1
c4144s	SAS065	*hld*	δ-hemolysin	5.3	4.3	1.5	2.6
c1023s	SA1007	*hla*	α-Hemolysin	1.9	3.7	4.6	2
c4094s	SA1752	*hlb*	β-Hemolysin	–	4.3	4.7	4.9
c5668s	SA2209	*hlgB*	γ-hemolysin component B	1.3	3.8	1.9	4.1
c2346s	SA0144	*capA*	Capsular polysaccharide synthesis enzyme Cap5A	–	3.7	3.8	1.6
c2413s	SA0147	*capD*	Capsular polysaccharide synthesis enzyme Cap5D	–	3.8	3.9	1.5
c2479s	SA149	*capF*	Capsular polysaccharide synthesis enzyme Cap5F	–	2.7	3.7	1.2
c10088s	SA0153	*capJ*	Capsular polysaccharide synthesis enzyme Cap5J	2.6	1.9	2.3	–
c10089s	SA0154	*capK*	Capsular polysaccharide synthesis enzyme Cap5K	1.2	1.6	2.1	–
c2662s	SA0155	*capL*	Capsular polysaccharide synthesis enzyme Cap5L	–	1.2	1.8	–
c6688s	SA2463	*lip*	Triacylglycerol lipase precursor	4.6	8.3	3.4	4.9
c6266s	SA0253	*lrgB*	Holin-like protein LrgB	1	3.3		2.3
c6975s	SA0309	*geh*	Glycerol ester hydrolase	1.5	2.2	2.3	2.1
c3504s	SA2003	*hysA*	Hyaluronate lyase precursor	–	1.4	3.4	2.3
c570s3	SA0901	*sspA*	Staphylococcal serine protease (V8 protease)	6.9	7.3	5.3	6.5
c568s3	SA0900	*sspB*	Cysteine protease precursor	6.1	7	4.8	6.1
c564s3	SA0899	*sspC*	Cysteine protease	6.6	7.1	5.2	6.2
c3834s	SA1725	*staphopain*	Staphopain, cysteine proteinase	4.1	3	4.3	5
c724s5	SA0091	*plc*	1-Phosphatidylinositol phosphodiesterase	2	4.4	2.5	4.4
c6539s	SA2430	*aur*	Zinc metalloproteinase aureolysin precursor	2.8	6.7	3.7	4.4
c6531s	SA2428	*arcA*	Arginine deaminase	2.5	2.5	3.1	5.2
c6527s	SA2427	*arcB*	Ornithine transcarbamoylase	2.5	2.4	3.3	5.2
c6513s	SA2425	*arcC*	Carbamate kinase	2.7	1.4	2.4	2.5
c6509s	SA2424	*arcR*	HP, similar to transcription regulator Crp/Fnr family protein	2	1.1	2.5	2.2
c6673s	SA2458	*icaR*	Ica operon transcriptional regulator IcaR	–	1	–	–
c5023s	SA2082	*ureA*	Urease gamma subunit	–	1.8	2.1	2.2
c5029s	SA2083	*ureB*	Urease beta subunit	–	1.9	1.9	2.7
c5031s	SA2084	*ureC*	Urease alpha subunit	–	2.2	2	2.2
c9293s	SA2088	*ureD*	Urease accessory protein UreD	–	1	1.6	1.6
c5035s	SA2085	*ureE*	Urease accessory protein UreE	1.7	1.3	1.8	1.9
c5039s	SA2086	*ureF*	Urease accessory protein UreF	–	1.6	2	1.6
c5043s	SA2087	*ureG*	Urease accessory protein UreG	–	1.2	1.6	1.3
c2958s	SA0053	*radC*	Truncated HP, similar to DNA repair protein		1.5	2.4	–
c10233s	SA2285		HP, similar to accumulation to methicillin-resistant surface protein	5.2	3.3	–	4.8
c4755s	SA2006		HP, similar to MHC class II analog	2.1	6	3.4	1.9
c6626s			Serine-threonine rich antigen	1	2.4	–	1
c1063s			Phenol-soluble modulin beta 2	3	6.6	5.4	6.3
c7931s			NADH-ubiquinone oxidoreductase chain 14	–	1	1.4	–
c9065s			Lantibiotic epidermin biosynthesis protein EpiB	–	1.5	1.7	–
c9062s			Lantibiotic epidermin biosynthesis protein EpiC	–	1.6	1.8	–
c10345s	SA0171	*fad*	NAD-dependent formate dehydrogenase	2.1	5	3.2	4.3
c3949s	Sa1751	*mapW*	Cell surface protein map-w	–	1.9	2.4	3.3
c6088s	SA2318		HP, similar to L-serine dehydratase	1.1	1.8	1.7	1.5
c6092s	SA2319		HP, similar to beta-subunit of L-serine dehydratas	1.5	2.7	2.9	1.8
c1147s	SA1041	*pyrR*	Pyrimidine operon repressor chain A	1.2	1.1	–	1.3
c1151s	SA1042	*pyrP*	Uracil permease	1.5	1.7	2.4	1.3
c9991s	Sa1043	*pyrB*	Aspartate transcarbamoylase chain A	1.4	1.3	2	1.2
c1155s	Sa1044	*pyrC*	Dihydro-orotase	1.5	1.3	1.8	–
c1159s	SA1045	*carA*	Carbamoyl-phosphate synthase small chain	1.3	1	1.7	–
c1165s	SA1046	*carB*	Carbamoyl-phosphate synthase large chain	1.3	–	1.3	–
c9938s	SA1992	*lacE*	PTS system, lactose-specific IIBC component	–	1.7	–	1.9
c10378s			Lantibiotic gallidermin precursor	–	2.1	1.4	–
c7937s	SA0582		HP, similar to Na+:H+ antiport	–	1.1	1.2	–
c9259s	SA0260		HP, similar to ribose transporter RbsU	–	3.1	1.1	1.8
c6443s	SA2411		HP, similar to magnesium citrate secondary transporter	–	2.5	2.2	2
c756s5	SA0377		HP (pathogenicity island SaPIn2)	–	1.2	1.6	–
c7313s	SA0394		HP (pathogenicity island SaPIn2)	–	2.1	2.9	1.7
c767s9	SA0406		HP (pathogenicity island SaPIn2)	–	1.2	1.6	1
c10102s	SA1634		Truncated hypothetical protein (pathogenicity island SaPIn3)	–	1.2	1.1	–

[a] Log fold change of TRAP$^-$ mutant compared with parent 8325-4 TRAP$^+$ (repeats 1, 2, and 3) and log fold change of mutant His66 (H66A) compared with parent TRAP$^+$. A minus sign indicates no difference between control and mutant.

[b] Designed *S. aureus* GeneChip ORF number.

[c] Based on the published sequence of strain N315 (24).

TABLE 3. Gene downregulated by TRAP[a]

Affymetrix Probe set no.	N315 ORF[b]	N315 gene[c]	N315 description	Repeat 1	Repeat 2	Repeat 3	H66A
c7698s	SA0519	*sdrC*	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein	1	–	1.4	–
c8852s	SA0520	*sdrD*	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein	1.4	2.9	4.1	3.3
c1007s	SA2423	*clfB*	Fibrinogen-binding protein precursor	–	1.7	–	–
c1082S	SA0107	*spa*	Immunoglobulin G-binding protein A	–	2	4.4	2.2
c4116s	SA1836	*groEL*	60-kDa chaperonin (GroEL protein)	2.4	1.4	–	–
c4124s	SA1837	*groES*	10-kDa heat shock protein (GroES protein)	2.5	1.5	–	–
c2703s	SA1408	*dnaJ*	Immunoreactive heat shock protein DnaJ	1.9	1.6	–	–
c8390s	SA0728	*pgk*	Phosphoglycerate kinase 2	1	1.4	–	–
c5588s	SA2189	*nirR*	HP, similar to NirR	1.3	3.1	–	2.1
c3173s	SA1537		HP, similar to thiamine biosynthesis protein ThiI	1.5	1.5	–	–
c5246s			Glutamate permease	1.2	4.2	1.3	3.7
c9058s	SA0904		HP, probable ATL autolysin transcription regulator	–	1.4	1.1	2.5
c6262s	SA1676		HP, similar to regulatory protein (PfoS/R)	–	1.2	1.1	–
c9380s	SA2303		HP, similar to membrane-spanning protein	–	3.8	2.5	3.1
c8053s	SA0097		HP, similar to AraC/XylS family transcriptional regulator	1.4	1.6	–	1.6

[a] Log fold change of TRAP$^-$ mutant compared with parent 8325-4 TRAP$^+$ (repeats 1, 2, and 3) and log fold change of mutant His66 (H66A) compared with parent TRAP$^+$. A minus sign indicates no difference between control and mutant.
[b] Designed *S. aureus* GeneChip ORF number.
[c] Based on the published sequence of strain N315 (24).

ponential phase (from OD_{600} of 0.03 for 6 h) at 37°C. RNA was isolated as described above. DNase-treated RNA was reverse transcribed with SuperScript II as described by the manufacturer (Invitrogen Life Technologies). An equal amount (1/20) of each reaction mixture was then used as a template for PCR amplification. The sequences of the primers are shown in Table 1.

Real-time PCR. Parent *S. aureus* 8325-4 and ΔTRAP mutant cells were grown to postexponential phase (from OD_{600} of 0.03 for 6 h) at 37°C. Cells were collected and treated with lysostaphin as described above. RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions, followed by treatment with DNase I (Ambion, Inc.) at 37°C for 20 min according to the manufacturer's instructions. To verify the absence of genomic DNA, PCR was carried out using these DNase I-treated RNA samples as templates, using *hld* primers. Two micrograms of each RNA sample was used for cDNA synthesis with the ImProm-II Reverse Transcription system, according to the manufacturer's instructions (Promega). Random hexamers (Invitrogen) were used to prime the reaction. A total of 1 μl of the resulting cDNA reaction mixture was used to set up the real-time PCR, using the LightCycler fast-start DNA master SYBR Green I kit (Roche), according to the manufacturer's instructions. The transcripts for *hld*, *hla*, *clfB*, *spa*, *icaR*, *icaA*, *sdrC*, and *sdrD* were amplified using the primers shown in Table 1. The *gyrB* transcripts that are constitutively expressed were used as an internal control. To monitor specificity, the PCR products were analyzed by melting curves and agarose gel electrophoresis. The values are an average of two to three replications normalized with respect to *gyrB* expression, and the data are expressed as the ratio of cycle threshold (C_T) of ΔTRAP/parent 8325-4.

RESULTS

To investigate which genes are regulated by TRAP expression or phosphorylation, TRAP+ *S. aureus* parent strain 8325-4, the TRAP− mutant, and H66A (a TRAP mutant that acts like a TRAP− mutant because it contains alanine instead of His66) (20) were grown to the postexponential phase (from OD_{600} of 0.03 for 6 h); cells were collected; and RNA was purified and used for functional genomics experiments (microarray analysis).

Genes upregulated by TRAP. The results presented in Table 2 indicate that multiple genes are upregulated by TRAP. Many of those are virulence factors and their regulatory genes, such as *agrABCD* (encoding accessory gene regulator ABCD), *hld* (δ-hemolysin, which is encoded by RNAIII), *hla*, *hlb*, *hlgB* (α-, β-, and γ-hemolysin, respectively), *capADFJKL* (capsular polysaccharide synthesis enzyme Cap5ADFJKL), *lip* (triacylglycerol lipase precursor), *geh* (glycerol ester hydrolase), *hysA* (hyaluronate lyase precursor), *sspA* (staphylococcal serine protease [V8 protease]), *sspB* (cysteine protease precursor), *sspC* (cysteine protease), SA1725 (staphopain and cysteine proteinase), *lrgB* (holin-like proteins), *plc* (1-phosphatidylinositol phosphodiesterase), and *aur* (zinc metalloproteinase aureolysin precursor) (13, 21, 24–26, 30).

Some of the genes upregulated by TRAP are metabolic: *arcA* (encoding arginine deaminase), *arcB* (ornithine transcarbamoylase), *arcC* (carbamate kinase), *ureABC* (urease α-, β-, and γ-subunits), *ureDEFG* (urease accessory proteins), *pyrR* (pyrimidine operon repressor chain A), *pyrP* (uracil permease), *pyrB* (aspartate transcarbamoylase chain A), *pyrC* (dihydroorotase), *carA* (small-chain carbamoyl-phosphate synthase), and *carB* (large-chain carbamoyl-phosphate synthase) (7, 13).

Genes downregulated by TRAP. Table 3 shows genes that are downregulated by TRAP. Those include genes involved in cell surface or adhesion molecules such as *sdrCD* (encoding Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding proteins), *clfB* (fibrinogen-binding protein precursor), and *spa* (immunoglobulin G-binding protein A precursor). Some are involved in adaptive response, such as *groEL* (encoding a 60-kDa chaperonin), *groES* (10-kDa heat shock protein), *dnaJ* (immunoreactive heat shock protein DnaJ), and other genes

TABLE 4. Influence of *traP*, *agr*, and *sar* on virulence determinants[a]

Virulence determinant	Gene	Influence of:		
		traP	*agr*	*sarA*
Exotoxins				
Aureolysin[c]	*aur*	+	+	−
Capsular polysaccharide type 5 genes	*cap*	+	+	+
V8 protease[c]	*sspA*	+	+	−
Toxic shock syndrome toxin	*tst*	unknown	+	+
Glycerol ester hydrolase	*geh*	+	+	+
α-Hemolysin[b,e]	*hla*	+	+	+
β-Hemolysin[b]	*hlb*	+	+	+
γ-Hemolysin	*hlgB*	+	+	+
δ-Hemolysin[c,e]	*hld*	+	+	+
Hyaluronate lyase	*hysA*	+	+	Unknown
Lipase	*lip*	+	+	−
Holin-like proteins	*lrgB*	+	+	+
1-Phosphatidylinositol phosphodiesterase	*plc*	+	+	Unknown
Surface-adhesion molecules				
Protein A[c,d,e]	*spa*	−	−	−
Coagulase	*coa*	Ø	+/−	+
Fibrinogen-binding protein[e]	*sdrC*	Microarray − Real time +	Unknown	+
Fibrinogen-binding protein[e]	*sdrD*	Microarray − Real time +	Unknown	Unknown
Ica operon transcriptional regulator IcaR[e]	*icaR*	Microarray Ø Real time +/−	Ø	Ø
Intracellular adhesin A[e]	*icaA*	Microarray Ø Real time +/−	Ø	+
Clumping factor B[e]	*clfB*	Microarray +/− Real time −	Ø	+
Collagen-binding protein	*cna*	Ø	+/−	−
Fibronectin-binding protein	*fnbAB*	Ø	−	+

[a] The influence of *traP* is as determined in studies described here and the influence of *agr* and *sar* is as previously determined (7, 8, 13). Ø, not influenced; +, increased; −, decreased; +/−, marginal change; unknown, gene is absent in the examined strain or the gene array or unreported.
[b] Confirmed by Northern blotting.
[c] Confirmed by cDNA-PCR.
[d] Confirmed by two-dimensional gel electrophoresis.
[e] Confirmed by real-time PCR.

involved in various cell functions, like *pgk* (encoding phosphoglycerate kinase 2) (7, 13).

Comparison between genes regulated by TRAP and those shown by other microarray studies to be regulated by *agr* or *sar*. Table 4 compares genes regulated by TRAP and those shown by other microarray and real-time PCR studies (7, 8, 13) to be regulated by other virulence regulatory loci like *agr* or *sar*. As shown in Table 4, some of the virulence genes regulated by TRAP are also regulated by *agr*, like *hla*, *hlb*, *hlgB*, *capJ*, *geh*, *lip*, *plc*, *spa*, *sspA*, *aur*, and *hysA*. Because TRAP regulates *agr*, it is likely that TRAP regulates these genes via *agr*.

Confirmation of microarray data. Some of the microarray results were confirmed by cDNA-PCR, real-time PCR, Northern blotting, and two-dimensional gel electrophoresis. The results in Fig. 1 confirmed by cDNA-PCR that TRAP upregulates *agrC*, *hld* (RNAIII), *aur*, and *sspA* and downregulates *spa*

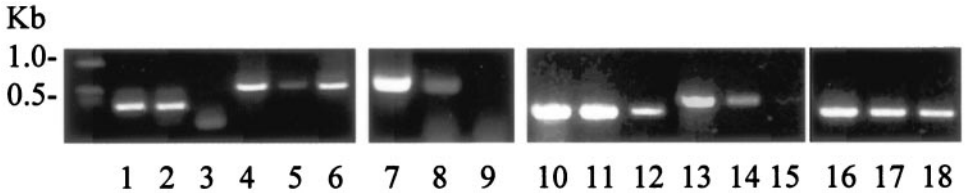

FIG. 1. cDNA-PCR analysis of *hld*, *spa*, *agrC*, *sspa*, *aur*, and *epbS*. Specific primers (Table 1) were used to amplify the various genes using cDNA as a template (or DNA as a control). Lanes 1 to 3, *rnaIII* was amplified using DNA of 8325-4 (lane 1), cDNA of 8325-4 (lane 2), or cDNA of ΔTRAP (lane 3). Lanes 4 to 6, *spa* (encoding protein A) was amplified using DNA of 8325-4 (lane 4), cDNA of 8325-4 (lane 5), or cDNA of ΔTRAP (lane 6). Lanes 7 to 9, *agrC* (encoding accessory gene regulator C) was amplified using DNA of 8325-4 (lane 7), cDNA of 8325-4 (lane 8), or cDNA of ΔTRAP (lane 9). Lanes 10 to 12, *sspA* (encoding staphylococcal serine protease [V8 protease]) was amplified using DNA of 8325-4 (lane 10), cDNA of 8325-4 (lane 11), or cDNA of ΔTRAP (lane 12). Lanes 13 to 15, *aur* (encoding aureolysin) was amplified using DNA of 8325-4 (lane 13), cDNA of 8325-4 (lane 14), or cDNA of ΔTRAP (lane 15). Lanes 16 to 18, *epbS* (encoding elastin-binding protein) was amplified using DNA of 8325-4 (lane 16), cDNA of 8325-4 (lane 17), or cDNA of ΔTRAP (lane 18).

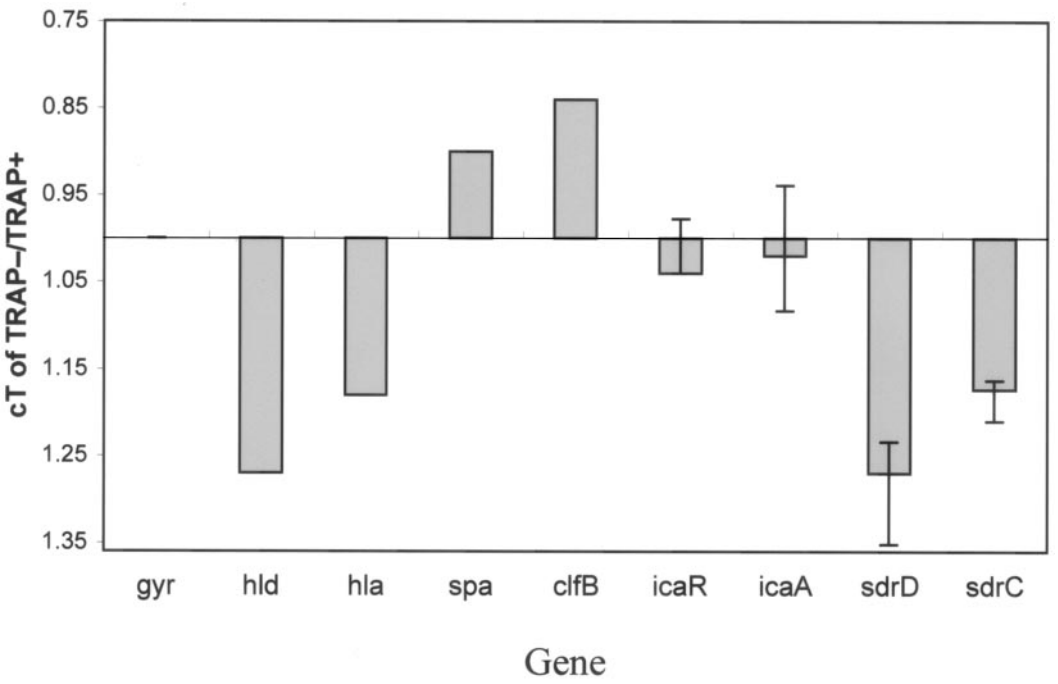

FIG. 2. Real-time PCR quantification of the expression of indicated genes in postexponential *S. aureus* TRAP+ parent strain and its corresponding TRAP– mutant. The values are an average of two to three replications normalized with respect to *gyrB* expression, and the data are expressed as the ratio of cycle (C_T) threshold of TRAP–/TRAP+. Genes with a ratio is of >1 are downregulated in the TRAP–mutant.

while not affecting the expression of the *epbS* gene. The results shown in Fig. 2 confirmed by real-time PCR that TRAP upregulates *hld* (RNAIII) and *hla*, downregulates *spa* and *clfB*, and only insignificantly affects *icaR* and *icaA*. Real-time PCR results of *sdrD* and *sdrC* are contradictory to the microarray results, where according to real-time PCR, less *sdrC* and *sdrD* are expressed in the TRAP− mutant (both microarray and real-time tested at least three times).

Figure 3 confirms by Northern blotting that TRAP upregulates *hla* and *hlb*.

Figure 4 confirms by two-dimensional gel electrophoresis that NAD-dependent formate dehydrogenase (encoded by *fdh*) is upregulated, while protein A (*spa*) and general stress 20U protein (*dps*) are downregulated by TRAP.

Regulation of genes through TRAP phosphorylation. Transcriptional profiling experiments were carried out using H66A strain that contains an intact TRAP with His 66 replaced by alanine, making that strain nonpathogenic (20). As shown in Tables 2 and 3, similar results were obtained using RNA from postexponential-phase TRAP− cells and H66A cells, suggest-

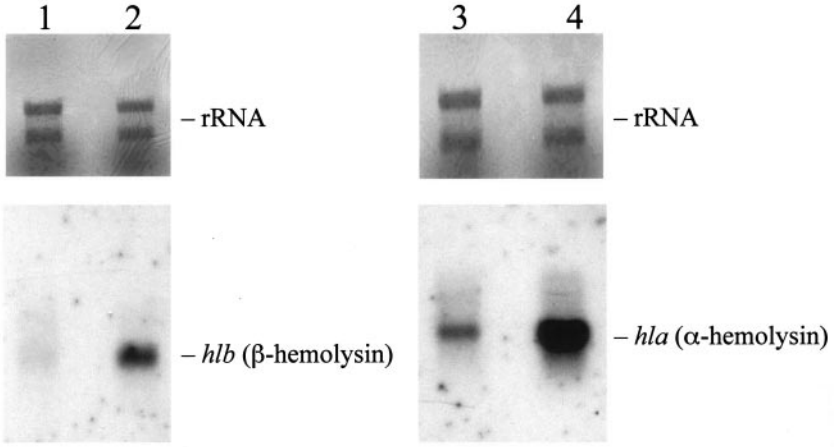

FIG. 3. Northern blot analysis of *hla* and *hlb*. Early exponential cells were grown from OD_{600} of 0.03 for 6 h (to the postexponential phase of growth). Cells were collected, Northern blotted, and membrane stained in methylene blue to visualize rRNA (top). mRNA of *hla* (α-hemolysin) and *hlb* (β-hemolysin) were detected with specific radiolabeled DNA. Membranes were autoradiographed. Lanes 1 to 2, *hlb* (β-hemolysin); lane 1, ΔTRAP; lane 2, 8325-4. Lanes 3 to 4, *hla* (α-hemolysin); lane 3, ΔTRAP; lane 4, 8325-4.

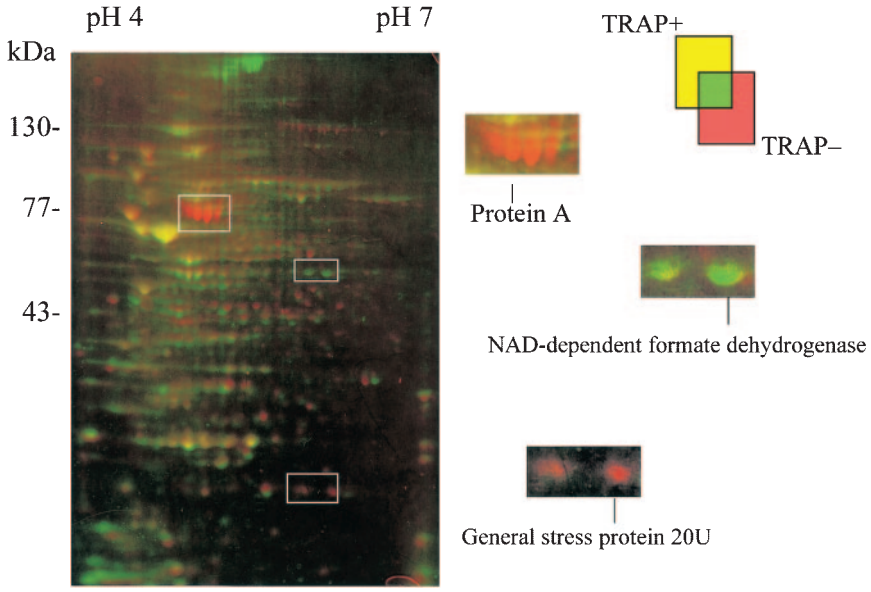

FIG. 4. Two-dimensional gel electrophoresis of postexponential wild-type *S. aureus* 8325-4 (green) and the mutant strain ΔTRAP (red) using dual-channel images which were produced with Delta2D software (Decodon GmbH). Boxes indicate representative proteins that were differentially expressed.

ing that it is the phosphorylation of TRAP that is important for the regulation of observed genes.

DISCUSSION

Regulation of virulence by TRAP. TRAP has been shown to be an important protein regulating virulence in staphylococci; when TRAP expression or phosphorylation is disrupted by mutagenesis or by inhibitory peptides, no *S. aureus*- or *Staphylococcus epidermidis*-induced disease was observed in any of the animal model systems so far tested (1–4, 6, 9–12, 15–17, 19, 20, 33, 36).

As shown here, in the absence of TRAP expression or phosphorylation, multiple virulence factors are not expressed. Those include α-, β-, γ-, and δ-hemolysins; triacylglycerol lipase precursor; glycerol ester hydrolase; hyaluronate lyase precursor; staphylococcal serine protease (V8 protease); cysteine protease precursor; cysteine protease; staphopain-cysteine proteinase; 1-phosphatidylinositol phosphodiesterase; zinc metalloproteinase aureolysin precursor; holing-like proteins; and capsular polysaccharide synthesis enzymes. These proteins have been shown to be important for establishment of the bacteria in the host and subsequent disease progression (13, 21, 24–26, 30).

The data presented here show that TRAP upregulates the *agr* locus (*agrABCD* and *hld* [transcribed by RNAIII]). Most of the virulence factors regulated by TRAP (listed above) have been shown to be regulated by the *agr* locus (13, 25, 28), and thus we assume that these are regulated by TRAP via *agr*. No information is available regarding *tst*, which encodes toxic shock syndrome toxin, as this gene is not present in strain 8325-4. Viewing the extensive list of toxins that are not expressed when the *traP* gene is disrupted can easily explain why TRAP mutants show no sign of pathogenesis whatsoever in mice, even when injected in very high numbers (20).

Regulation of genes important for biofilm formation. Cells containing TRAP that is defective in expression or phosphorylation adhere less to plastic polymers and to host cells in vitro and do not form a biofilm in vivo (1, 3, 6, 9–12, 15–17). Indeed, in the absence of TRAP expression or phosphorylation, although *agr* is suppressed as shown here (see below), no substantial upregulation of adhesion molecules is observed. The only virulence genes shown to be upregulated in the absence of TRAP were *spa*, *clfB*, and *sdr*. However, while TRAP–mutants were shown to overexpress protein A (encoded by *spa*) by every method tested, only in one of four microarray repeats was fibrinogen-binding protein (*clfB*) shown to be upregulated. This result was, however, confirmed by real-time PCR. The Ser-Asp-rich fibrinogen-binding bone sialoprotein-binding proteins (*sdrC* and *sdrD*) were shown by microarray studies to be upregulated in the TRAP− mutant, but opposite results were obtained by real-time PCR. Differences between microarray and real-time PCR results may be due to technical inherent experimental differences, choice of oligonucleotides, sensitivity, or variability in expression levels or detection (31).

Both microarray and real-time PCR data indicate that the *icaR* gene is marginally downregulated in the TRAP− mutant while not significantly affecting *icaA* expression.

Other surface proteins, such as the fibronectin-binding protein (encoded by *fnbAB*), collagen-binding protein (*cna*), elastin-binding protein (*epbS*), clumping factor A (*clfA*), extracellular fibrinogen-binding protein (*efb*), and extracellular adherence protein (*eap*) (14, 25) are not overexpressed by the TRAP mutants. Additionally, in the absence of TRAP expression or phosphorylation, the level of expression of genes required for biofilm survival (7) was reduced in *arcABC*, *ureABC*, *ureDEFG*, *pyrR*, *pyrP*, *pyrB*, *pyrC*, *carA*, and *carB*. These results explain our observation that TRAP mutants do not adhere as well as the wild type and do not form a biofilm in vitro or in vivo.

In general, our microarray results are compatible with what was observed with microarray studies using *agr* mutants (7, 8, 13), suggesting that most genes are regulated by TRAP via *agr*. The accepted notion has been that phase variation occurs once *agr* is activated at the mid-exponential phase of growth, where *agr* downregulates genes encoding adhesion molecules and upregulates genes encoding toxins (25). However, microarray studies using TRAP or *agr* mutants do not support this hypothesis and show that while multiple genes encoding exotoxins are indeed downregulated if *traP* or *agr* is disrupted, most adhesion molecules are not upregulated (7, 13). In addition, in our studies of biofilms, we have shown that under flow conditions, the volume of biofilm is significantly lower by day 4 in TRAP− mutants, while by day 1 the volume of biofilm of TRAP mutant was transiently higher (3), which is also compatible with what was observed with *agr* mutants (34).

As shown in Table 4, there is some incompatibility in reported regulation of adhesion genes by *agr* and that of *traP*. This suggests either that TRAP regulates some of the adhesion genes independently of *agr* or that there are differences in experimental approaches, use of strains, or use of different gene arrays.

To summarize, the results presented here can easily explain our observation that in the absence of TRAP expression or phosphorylation, the ability of the bacteria to produce toxins, to attach, to form a biofilm, and to survive within the host is seriously compromised; thus in the presence of TRAP inhibitors, staphylococcal diseases are prevented. That disruption of TRAP reduces both biofilm formation and exotoxin production is of major importance when considering TRAP as a target site for therapy, making TRAP a safe therapeutic target site.

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