Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation

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Staphylococcus aureus **is a major bacterial pathogen, which causes severe blood and tissue infections that frequently emerge by autoinfection with asymptomatically carried nose and skin populations. However, recent studies report that bloodstream isolates differ systematically from those found in the nose and skin, exhibiting reduced toxicity toward leukocytes. In two patients, an attenuated toxicity bloodstream infection evolved from an asymptomatically carried high-toxicity nasal strain by loss-of-function mutations in the gene encoding the transcription factor repressor of surface proteins (*rsp*). Here, we report that *rsp* knockout mutants lead to global transcriptional and proteomic reprofiling, and they exhibit the greatest signal in a genome-wide screen for genes influencing *S. aureus* survival in human cells. This effect is likely to be mediated in part via *SSR*42, a long-noncoding RNA. We show that *rsp* controls *SSR*42 expression, is induced by hydrogen peroxide, and is required for normal cytotoxicity and hemolytic activity. Rsp inactivation in laboratory- and bacteremia-derived mutants attenuates toxin production, but up-regulates other immune subversion proteins and reduces lethality during experimental infection. Crucially, inactivation of *rsp* preserves bacterial dissemination, because it affects neither formation of deep abscesses in mice nor survival in human blood. Thus, we have identified a spontaneously evolving, attenuated-cytotoxicity, nonhemolytic *S. aureus* phenotype, controlled by a pleiotropic transcriptional regulator/noncoding RNA virulence regulatory system, capable of causing *S. aureus* bloodstream infections. Such a phenotype could promote deep infection with limited early clinical manifestations, raising concerns that bacterial evolution within the human body may contribute to severe infection.**

Staphylococcus aureus | bloodstream infection | *rsp* | SSR42 | toxicity regulator

The bacterium *Staphylococcus aureus* constitutes a major pathogen causing an array of diseases including deep abscesses, endocarditis, sepsis, and necrotizing pneumonia (1). The toll of severe disease and mortality inflicted by *S. aureus*, the ongoing rise in multiple antibiotic-resistant strains, and the prolonged hospital stays it causes make it one of the most important human pathogens (2, 3).

Despite much effort, the determinants of *S. aureus* virulence remain incompletely understood. It is known that *S. aureus* can secrete a wide range of proteins, including adhesins (4), nucleases (5, 6), complement control proteins (7–9), and multiple toxins, which interfere with host immune function. Toxins elicit cytotoxicity toward a variety of cells ranging from epithelial cells to leukocytes (1, 4, 10), and their secretion is associated with lethality in some disease models (11–14). Additionally, some bacterial lineages, such as USA300, display high levels of toxicity, which may be linked to their evolutionary success (13, 15).

S. aureus asymptomatically colonizes the anterior nares of one-third of the human population, and this bacterial reservoir represents a source for invasive infection (1, 16). However, bacterial isolates from blood differ phenotypically from those from the nares, exhibiting decreased cytotoxicity (17) and reduced hemolysis (18). This finding is surprising because carried isolates represent the source for most human disease, and invasive and carried isolates are closely related genetically (19). One possible explanation for the low-hemolysis phenotype of the bloodstream isolates involves their carrying mutations in transcription factors.

Significance

Staphylococcus aureus **is a major cause of life-threatening bacterial infection. A significant risk factor for infection is nasal carriage. Previously, we reported spontaneous mutations during carriage associated with infection, including loss-of-function of the gene *repressor of surface proteins* (*rsp*). Here we use genomic screens, experimental assays, and molecular examination of *rsp* mutants from patients to understand how *rsp* is involved in infection; we find it has far-reaching effects on gene regulation. Paradoxically, *rsp* mutants exhibited attenuated toxicity and reduced disease severity early in experimental infection, without sacrificing the ability to cause abscesses and bloodstream infection. This work reveals a complex relationship between correlates of disease in the laboratory and in patients, demonstrating that life-threatening disease can be associated with reduced severity early in infection.**

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For example, a major regulator of *S. aureus* cytotoxicity and hemolysis, accessory gene regulator (*agr*), is known to be mutated in a proportion of bacteria recovered from within human host cells (20–22). Such mutants have also been noted among hospital-derived isolates of virulent clones of *S. aureus* (23). They exhibit prolonged intracellular residence due to attenuated cytotoxicity and consequent delays in initiation of host cell death (24–26).

However, other genetic mechanisms might also control the induction of an attenuated cytotoxic state. One candidate for such a role was suggested by a study of a patient with long-term nasal *S. aureus* carriage. Within this population, isolates with reduced cytotoxicity evolved through a loss-of-function mutation in the gene *repressor of surface proteins* (*rsp*), a gene encoding an AraC-family transcriptional regulator. The occurrence of this mutation accompanied the progression to a fatal bacteremia (27) and caused a reduction in the cytotoxicity of the nasal *S. aureus* population (17).

Here, we used an unbiased genome-wide screen for staphylococcal genes involved in prolonged intracellular survival. We show that *rsp* and the long noncoding RNA (ncRNA) *SSR*42 were by far the most significantly recovered genes from the screen. We demonstrate that *rsp* controls *SSR*42 expression, is required for normal cytotoxicity and hemolytic activity, is required for lethality in experimental infection, and is induced by hydrogen peroxide. Crucially, inactivation of *rsp* preserves bacterial dissemination, because it neither affects formation of deep abscesses in mice nor survival in human blood. Thus, we have identified a pleiotropic transcriptional regulator/ncRNA virulence regulatory system that controls hemolysis and cytotoxicity and a low-cytotoxic phenotype that plays a central role in invasive *S. aureus* infection. This study provides an important demonstration of how within-host bacterial evolution can radically alter bacterial phenotypes pertinent to disease severity and outcome.

Results

Rsp and the ncRNA *SSR*42 Are Required for Intracellular Cytotoxicity and Hemolysis of *S. aureus*. To identify genes mediating prolonged intracellular survival (perhaps due to attenuated cytotoxicity) in *S. aureus*, we used an unbiased genome-wide approach: We generated a transposon mutant library pool comprising ~25,000 independent mutants within the highly cytotoxic isolate *S. aureus* 6850 (28). We then screened it for transposon mutants that were recovered from epithelial (HeLa) cells after internalization as described in *Materials and Methods*. Changes of frequencies of transposon insertion sites (TIS) in the recovered bacterial pools were compared with those of the inoculum by TIS deep sequencing, hereafter referred to as TnSeq (Fig. 1*A* and *SI Appendix*, Fig. S1*A*).

We found that mutants in the *rsp* locus and the ncRNA *SSR*42 located directly upstream of *rsp* were significantly enriched in the intracellular fraction (Fig. 1 *B* and *C*, Table 1, and *SI Appendix*, Fig. S1 and Dataset S1) (adjusted *P* values 3.6×10^{-4} and 2.4×10^{-9}). Replication of *rsp* mutants in vitro and within HeLa cells did not differ significantly compared with wild-type bacteria (*SI Appendix*, Fig. S1 *B* and *C*). We therefore excluded differences in intracellular growth as a reason for the frequent recovery of *rsp* mutants. We also excluded differential gentamicin susceptibility as an explanation for the enhanced survival of *rsp* mutants observed in the screen (*SI Appendix*, Fig. S1*D*).

We therefore generated targeted mutants of *rsp* to study its contribution to virulence. In *S. aureus* 6850, we deleted the complete ORF, leaving the adjacent ncRNA as well as downstream ORFs intact (*SI Appendix*, *SI Materials and Methods*). Furthermore, we transduced the insertional mutation within *rsp*, NE1304, into a clean genomic background of *S. aureus* USA300 (*SI Appendix*, Table S5). Hemolysis and cytotoxicity are hallmarks of *S. aureus* virulence, and both are regulated by the *agr* quorum sensing system. However, we found that hemolysis on sheep blood agar plates was also strongly *rsp*-dependent (Fig. 2*A*). We also noted that cytotoxicity toward epithelial cells was *rsp*-dependent (Fig. 2*B*). We observed enhanced cytotoxicity and hemolysis in *rsp* complementants relative to wild-type, likely

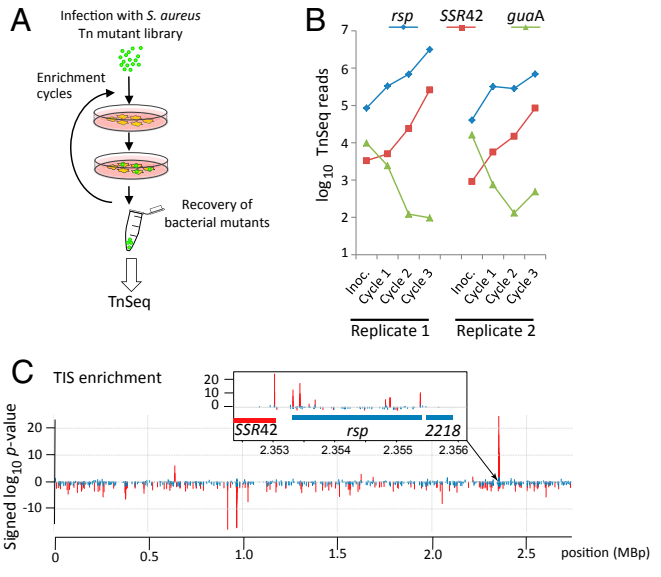

Fig. 1. A genome-wide screen for noncytotoxic *S. aureus* identifies *rsp* and *SSR*42. (*A*) HeLa cells were infected with a mariner transposon mutant library of *S. aureus* 6850. Viable bacteria were recovered from host cells 8 h after infection and were used to reinfect epithelial cells in three consecutive enrichment cycles. Pools of recovered bacteria and the respective inoculum were analyzed by TnSeq. (*B*) Sequence reads from transposons within the genes encoding *rsp* (blue) and *SSR*42 (red) were strongly enriched in noncytotoxic mutants ($P < 0.001$). By contrast, transposon insertions in genes such as the drug target *gua*A (75) (green) were significantly depleted. (*C*) Genome-wide significance (signed log10 *P* values) of changes in TIS frequencies demonstrate that the locus encoding *rsp* and *SSR*42 is most significantly enriched (*Inset*). Positive and negative values on *y* axis, respectively, indicate enrichment and depletion in TIS reads compared with the inoculum. Significant changes (adjusted $P < 0.05$) are highlighted in red.

because of enhanced *rsp* expression in complementants [relative expression level was 11.99 ± 5.16 (mean ± SD), 95% CI 6.57–17.41)] relative to wild-type, as determined by quantitative RT-PCR (qRT-PCR).

To analyze the kinetics of cytotoxicity, we infected HeLa with wild-type, isogenic *rsp* mutants, as well as complemented mutants, and compared with strains deficient in either *agr* or *sae*, both global regulators of *S. aureus* virulence. We determined intracellular cytotoxicity at 1.5, 4, 8, and 24 h after infection. *agr* and *sae* mutants were strongly attenuated over the course of infection (Fig. 2*C*). The *rsp* mutant was attenuated at 4 and 8 h after infection compared with the wild-type ($P = 0.015$ and 0.029, respectively), but appeared to display similar cytotoxicity after 24 h ($P = 0.192$) (Fig. 2*C*). However, *rsp* mutation neither influences internalization of *S. aureus* by HeLa cells nor phagosomal escape (*SI Appendix*, Fig. S2), both of which have been shown to be associated with cytotoxicity (29–31). Thus, our data suggest that *rsp*-defective *S. aureus* remain within the host cell longer and delay pathogen-induced cell death.

***rsp* Is Required for Lethality in Murine Infection, but Not for Abscess Formation.** Because in vitro toxicity has been linked to severe outcome in acute mouse infection models (32), we investigated whether *rsp* altered progression of experimental *S. aureus* infection. Despite recovering *rsp* mutants from human bloodstream infection, we observed reduced lethality in a lung-challenge model when comparing survival of mice infected with either *rsp* mutant, their respective wild-type, or complemented strains. Remarkably, all mice infected with *rsp* mutants survived for 3 d (Fig. 3*A*), whereas mortality was 100% at day 2 in the USA300 background and reached 40% in the 6850 background (*SI Appendix*, Fig. S3*A*) ($P < 0.0001$ and $P = 0.01$ for USA300 and *S. aureus* 6850, respectively).

Table 1. TnSeq screening results of *S. aureus* Himar1 transposon mutant library in epithelial cells

Mutant*	*P* value†	Product
Inactivated genes enriched in intracellular *S. aureus*‡		
*ssr*42	$<10^{-6}$	Small stable RNA (SSR) 42
rsp	0.0004	AraC-type transcriptional regulator
geh	0.037	Glycerol ester hydrolase
*ruv*A	0.045	Holliday junction DNA helicase RuvA
*hem*L	0.050	Glutamate-1-semialdehyde aminotransferase
Inactivated genes depleted in intracellular *S. aureus*		
*gua*A	0.0003	Bifunctional GMP synthase
0220	0.0004	Transmembrane efflux pump protein, putative
*pbu*X	0.0004	Xanthine permease, putative
*pur*M	0.003	Phosphoribosylformyl glycinamidine cyclo-ligase PurM
1920	0.008	ATP-dependent RNA helicase, DEAD box family, putative
2160	0.008	Phosphosugar-binding transcriptional regulator, putative

*Gene or locus IDs according to NCBI GenBank accession no. CP006706.1 (i.e., 0181 represent RSAU_000181).
†The *P* values were corrected for multiple testing and genes/loci showing $P < 0.05$ were reported as significantly increased or decreased. For further details, see *SI Appendix*, Table S1.
‡Trend followed by the mutant in the given genes/loci throughout the intracellular passages of screening.

A model in which abscesses form after intravenous administration of *S. aureus* (33) supported this observation (Fig. 3*B*). Starting from day 2 after infection, clinical severity scores increased in the mice infected with wild-type bacteria compared with the group infected with the *rsp* mutant (*SI Appendix*, Fig. S3 *B* and *C*); severity scores on day 2 differed ($P = 0.04$) and on day 3 ($P = 0.0002$). Mice challenged with *rsp* wild-type bacteria also lost more weight (*SI Appendix*, Fig. S3) (day 2 difference, $P = 0.01$) and, as in the pulmonary model, survival was significantly reduced compared with the *rsp* mutant ($P = 0.05$) (Fig. 3*B*). These results show that *rsp* influences bacterially induced lethality in vivo and that this observed mortality occurred in the first days after experimental infection.

However, 3 h after injection of the USA300 strain, or its *rsp* insertion mutant, viable bacteria were detectable in multiple tissues, with high concentrations in liver and spleen, but with low renal concentrations of both strains (Fig. 3*C*). By 48 h, both wild-type and *rsp* mutant bacteria showed increased bacterial load (Fig. 3*C*) and had clear histological evidence of abscess formation (*SI Appendix*, Fig. S4). Compatible with the similar bacterial loads, the numbers of abscesses identified histologically were similar. Their architecture also appeared similar (*SI Appendix*, Fig. S4). This finding indicates that *rsp* inactivation does not inhibit bacterial dissemination from the blood, survival, or proliferation in tissues in mice.

***rsp* Mutants Are Isolated from the Human Bloodstream and Survive in Human Blood.** In a previous longitudinal study of asymptomatic *S. aureus* carriage, one patient, designated patient P, was recruited and was admitted to hospital with a *S. aureus* bloodstream infection 15 mo after joining the study. Bloodstream isolates recovered from this patient differed by a small number of mutations from the ancestor (Fig. 4*A*), one of which caused a stop codon in *rsp*, as described (27). Subsequently, we identified a second patient, patient S, who was treated for a *S. aureus* bloodstream infection at a hospital in Oxfordshire, United Kingdom, with a nasal

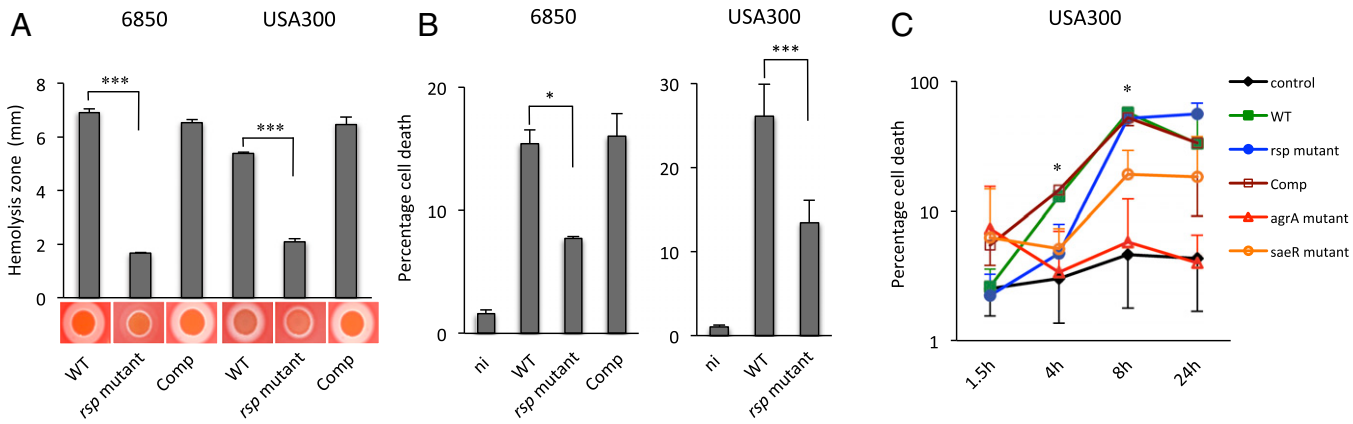

Fig. 2. *S. aureus rsp* mutants are less hemolytic and show altered kinetics of cytotoxicity. (*A*) Hemolysis by *S. aureus* is drastically reduced in an *rsp* mutant but is readily restored to wild-type (WT) levels by expressing *rsp in trans* (Comp) in both *S. aureus* backgrounds, 6850 and USA300. Statistical analysis was performed by one-way ANOVA and Tukey's post hoc analysis. ***$P < 0.001$. (*B*) Host cell cytotoxicity assayed at 4 h after infection is significantly reduced in *rsp* mutants compared with wild-type (WT) and complemented mutants (Comp) in infected HeLa epithelial cells for both *S. aureus* strains, 6850 and USA300. ni, uninfected control. Statistical significance was determined by one-way ANOVA. *$P < 0.05$; ***$P < 0.001$. (*C*) Mutation within *S. aureus rsp* delays pathogen-induced cytotoxicity. HeLa cells were left uninfected (control) or infected with *S. aureus* wild-type (USA300 WT), an isogenic *rsp* mutant, and a complemented mutant (USA300 Comp) along with mutants within the global regulators *agr*A and *sae*R (*SI Appendix*, Table S1). Kinetics of cytotoxicity were monitored over time by propidium iodide staining and flow cytometry, here depicted on the *y* axis using a log scale. Statistical analysis at each time point was performed by one-way ANOVA and Tukey's post hoc analysis. *$P < 0.05$ (*rsp* mutant compared with wild-type).

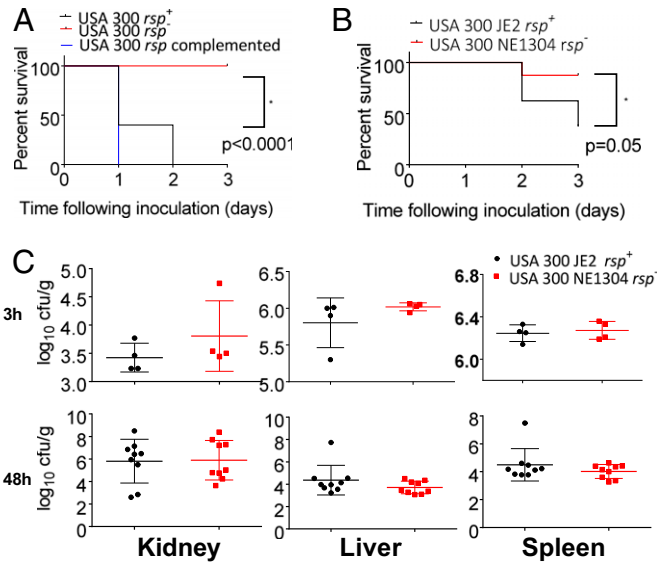

Fig. 3. *rsp* mutants exhibit reduced lethality in mouse models but are capable of forming deep abscesses. (*A*) In a murine pneumonia model, infected mice survived when challenged with lethal doses of *rsp* mutants of strain USA300 LAC*, whereas wild-type and complemented strains were virulent ($n = 10$). The comparison shown is by log-rank test between wild-type and *rsp* mutant organisms. (*B*) In intravenous infections, mice were challenged with *S. aureus* USA300 JE2 or its *rsp* mutant. Significantly enhanced lethality was seen in the wild-type relative to the mutant. (*C*) Bacterial counts in kidney, liver, and spleen were comparable 3 and 48 h after intravenous infection. Shown is the number of colony-forming units (cfu) per gram of tissue.

swab subsequently taken as part of routine surveillance. The bloodstream isolate differed from the nasal isolate by only one mutation (Fig. 4*A*), located in the DNA binding domain of *rsp* (Fig. 4*B*), which is predicted to abrogate DNA binding (*SI Appendix*, *SI Materials and Methods*). The nasal isolate carried the common (wild-type) allele, so we considered it to be the ancestor.

Compatible with murine deep abscess formation after intravenous challenge, we observed that bacterial survival in human blood was similarly *rsp*-independent. We inoculated wild-type or *rsp* mutant bacteria into whole blood drawn from healthy human donors and quantified viable bacterial counts over time (Fig. 4*C*). The studied isolates included the highly cytotoxic *S. aureus* background of strain JE2 (34, 35), a member of the epidemic, highly pathogenic methicillin-resistant *S. aureus* (MRSA) USA300 lineage (ST-8), as well as the common ST-15 (patient P) and ST-59 (patient S) lineages (*SI Appendix*, Table S1). Rsp-associated differences in bacterial survival in human blood were not observed (Fig. 4*C*). Thus, the enhanced early cytotoxicity observed in *rsp* wild-type organisms appears dispensable for bloodstream survival and dissemination after intravenous challenge.

***rsp* Is a Global Regulator of *S. aureus* Immune Modulators and Toxins.** The spontaneous evolution of *rsp* loss-of-function mutations found in human bloodstream infections, and the *rsp* mutants' capability to survive ex vivo in human blood, demonstrate that they are not avirulent in humans. However, this observation raises questions as to whether, in the absence of *rsp*, *S. aureus* might elaborate an alternative set of virulence proteins other than toxins. Rsp is a transcription regulator; hence, we tested this hypothesis by analysis of differential transcription and protein expression between wild-type strains and *rsp* mutants in three genetic backgrounds of *S. aureus* isolates.

Initially, we studied bacteria from the stationary phase of growth to minimize growth-phase specific differences between strains. In USA300 and patient P and patient S strain backgrounds, we found transcription to differ between loss-of-function mutants (rsp^-) and wild-type (rsp^+) isolates in ∼30% of the 2,368 genes present in all three strains, using a statistical model designed to detect consistent effects of *rsp* mutations across strains (*SI Appendix*, *SI Materials and Methods*). Transcription was similar across genetic backgrounds (ρ between 0.67 and 0.79), indicating broadly consistent effects of the *rsp* defects studied (Fig. 5*A* and *SI Appendix*, Fig. S5). However, interactions between *rsp* genotype and genetic background were also evident (Dataset S2).

Among the genes up-regulated in *rsp* mutants (highly regulated genes in Table 2 and all results in *SI Appendix*, Fig. S2), we found a strong enrichment for involvement in pathogenesis ($P = 10^{-5.0}$), such as *map* ($2^{1.38}$-fold), a reported immunomodulatory molecule (36); *nuc* ($2^{0.8}$-fold), a nuclease capable of lysing neutrophil extracellular traps (6); the Ig-binding protein *sbi* (37) ($2^{1.22}$-fold); and capsule biosynthesis genes ($\geq 2^{1.0}$-fold), whose product impedes phagocytosis (38). Genes influenced by *rsp* also include reported complement inhibitors such as extracellular proteases *sspABC* (39) ($\geq 2^{0.8}$-fold), the extracellular fibrinogen binding protein *efb* (40) ($2^{2.08}$-fold), complement regulator bind ing protein *sdrE* (41)($\geq 2^{0.9}$-fold), and the protease aureolysin *aur* (42) ($2^{1.2}$-fold). Genes associated with adhesion to squamous

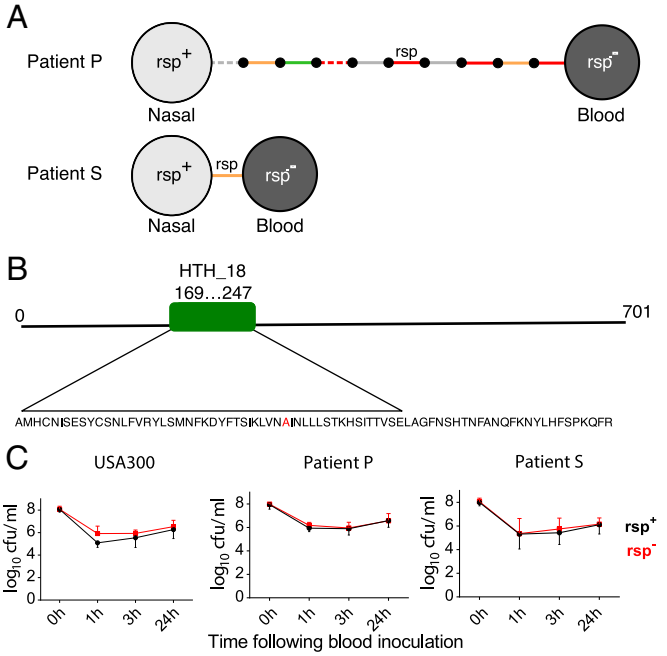

Fig. 4. Low hemolytic *rsp* mutants are recovered from patients and occur naturally. (*A*) *S. aureus* from bloodstream infections carry mutations in *rsp*. Two bloodstream isolates were obtained from patients P and S and compared with their respective carried strains. Isolates are represented by light (rsp^+) and dark (rsp^-) gray circles. Intergenic (gray), synonymous (green), nonsynonymous (orange), and nonsense (red) SNPs and indels are represented by solid and dashed lines, respectively. Small black circles represent hypothetical intermediate genotypes. The ordering of mutations along the branch in patient P is arbitrary. Remarkably, only a single mutation (A204P) separated the bacteremic from a carriage isolate in patient S. (*B*) Rsp is highly conserved in *S. aureus* and contains a helix-turn-helix domain (amino acids 169–247). The observed substitution in the patient S rsp^- isolate (indicated in red) occurs in the center of this domain, substituting an alanine with a proline and thereby predicted to disrupt the 3D structure of the DNA binding region. (*C*) Bacterial survival with the same strains used in Fig. 3, as well as with pairs of clinical isolates (*A*), was assessed after inoculation into human blood from three healthy donors. Bacterial survival was measured at three different time points (1, 3, and 24 h). There was no significant difference in blood survival observed between the *rsp* mutant (black lines) and wild-type bacteria (red lines). Statistical significance was determined by general linear modeling, modeling counts at each time point as a function of *rsp* genotype, and genetic background of the organism.

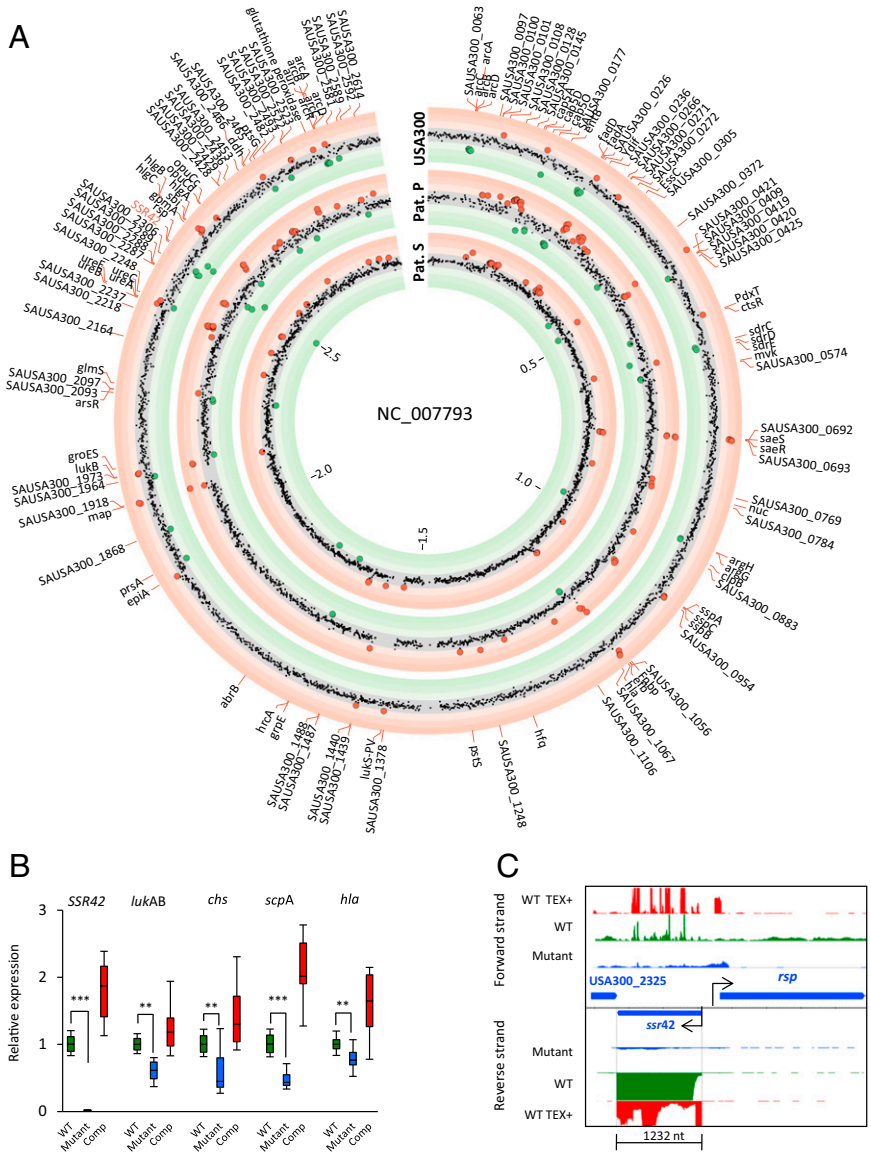

Fig. 5. *rsp* is a global regulator of *S. aureus* virulence. *S. aureus* gene expression was measured in stationary growth phase, showing the transcripts of the *rsp* mutant relative to the wild-type. (*A*) Effects of *rsp* mutation on gene transcription were determined by DESeq2 fitting of three separate models, one for each of three *S. aureus* backgrounds, estimating effects per gene from RNA-seq data. Concentric red rings indicate $\log_2$ fold induction, and green rings repression by the mutant relative to the wild-type. Differentially regulated genes are shown as large dots (red, up-regulated in mutant relative to wild-type; green, down-regulated in mutant relative to wild-type) within each strain background (USA300 and patients P and S); see also *SI Appendix*, Fig. S5. (*B*) Transcriptomic profiling revealed several *rsp*-dependently transcribed genes, including the ncRNA *SSR42*, as well as the virulence factor genes *luk*AB, *chs*, *scp*A, and *hla*. We verified the relative expression of these factors during exponential growth phase by comparing *S. aureus* USA300 wild-type (WT; green), its isogenic *rsp* mutant (blue), and a complemented mutant (Comp; red), by qRT-PCR. Similar results were obtained for stationary phase cultures as well as for *S. aureus* 6850 (*SI Appendix*, Fig. S6). Box plots show the median and quantiles, with whiskers indicating the range of the data. Statistical analysis was performed by one-way ANOVA and Tukey's post hoc analysis. $^{**}P < 0.01$; $^{***}P < 0.001$. (*C*) The transcriptome landscape at the *rsp* locus demonstrates *rsp*-dependent transcription of the ncRNA *SSR42*. RNA-seq of wild-type *S. aureus* USA300 (WT; green histograms) and its isogenic *rsp* mutant (blue histograms) demonstrated a loss of *SSR42* transcription in the absence of *rsp*. TSS of the wild-type *S. aureus* USA300 were enriched by treatment with Terminator-5′ phosphate-dependent EXonuclease (WT TEX+; red histograms; *SI Appendix, SI Materials and Methods*), because this enzyme degrades RNA lacking a physiological 5′ terminus. Subsequent RNA-seq revealed that *SSR42*, which is situated immediately upstream of *rsp*, is transcribed in antiparallel direction and is 1,232 nt long.

cells and colonization were found to be down-regulated (*sdrCD*) (43) ($2^{-0.87}$ and $2^{-0.23}$-fold, respectively) (*SI Appendix*, Fig. S2).

Additionally, we studied transcription in the USA300 background using RNA sequencing (RNA-seq) and qPCR in both exponential and stationary growth phases (Fig. 5*B*, *SI Appendix*, Fig. S6, and Datasets S2 and S3). Comparing these results, we noted that some important Rsp targets such as α-hemolysin (*hla*) demonstrated decreased transcription in *rsp* mutants during exponential growth, but increased transcript levels during stationary phase (Table 3). This finding indicates that some *rsp* effects may be modified by quorum-sensing mechanisms.

Rsp Influences Abundance of Secreted Proteins. Noting that Rsp affected gene transcription of many secreted proteins (Table 2 and *SI Appendix*, Fig. S6), we tested whether the effect of Rsp on transcripts was detectable at a protein level in the supernatants of *S. aureus* strains ST-8 (USA300), ST-15 (patient P), and ST-59 (patient S). We compared protein abundances (Dataset S4) with intracellular RNA levels in stationary phase (Fig. 6). For these analyses, cells were grown in α-MEM, because this medium afforded enhanced sensitivity of detection over growth in tryptone soy broth (*SI Appendix*, Fig. S7). A high proportion (113 of 636; 18%) of the proteins detected in the supernatant of any of the strains analyzed were affected by *rsp* mutation. As predicted from functional assays, toxins [α-hemolysin Hla, γ hemolysin components HlgA-C, the Panton-Valentine Leukocidin LukS, and LukAB (also known as LukGH (44)] had decreased abundances in *rsp* mutant organisms, as did the neutrophil chemotaxis inhibitor CHIPS (*chs* gene product) (Fig. 6*A*). However, for a subset of genes, including the toxins *hla*, *luk*B, and *hlg*B, we noted stationary-phase RNA abundances of these genes to be significantly increased in *rsp* mutant organisms (Fig. 6*B*). This discordance between transcript and protein levels, which has been previously observed in the context of *hla* expression and translation, suggests the existence of posttranscriptional control(s) (35) on toxin secretion.

Thus, Rsp has pleiotropic effects on the bacterial cell, inducing some virulence factors (such as toxins) and significantly reducing the concentrations of others known to be involved in pathogenesis and complement evasion, including the ESAT-6 homolog EsxA, nuclease (36), the complement control protein Efb (40), and lipase (Fig. 6).

Rsp Controls, and Is Adjacent to, the Highly Transcribed RNA *SSR42*. Having demonstrated that Rsp controls an extensive set of genes enriched in virulence factors, we sought to explore mechanisms by which Rsp might exert its effects. We precisely mapped transcription start sites (TSS) in wild-type and *rsp* mutants in a

Table 2. The 20 most up- and down-regulated genes in *rsp* mutant compared with wild-type

Gene*	Fold-change	Product
Up-regulated in *rsp* mutant		
0693	6.00	Putative lipoprotein
0692	5.82	Conserved hypothetical protein
0409	5.10	Conserved hypothetical protein
1056	4.53	Conserved hypothetical protein
efb	4.23	Fibrinogen-binding protein
*sae*R	4.06	DNA-binding response regulator SaeR
*sae*S	4.00	Sensor histidine kinase SaeS
1918	3.36	Truncated β-hemolysin
*hlg*A	3.34	Gamma-hemolysin component A
*ure*C	3.23	Urease, α-subunit
0108	3.20	Antigen, 67 kDa
1052	3.182	Fibrinogen-binding protein
*ure*B	3.160	Urease, β-subunit
0274	3.117	Conserved hypothetical protein
0278	3.095	Conserved hypothetical protein
0273	3.031	Putative membrane protein
*hlg*C	3.010	Gamma-hemolysin component C
2524	3.010	Conserved hypothetical protein
0272	2.868	Conserved hypothetical protein
0238	2.848	Transcriptional antiterminator, BglG family
*luk*A (G)	2.828	Leukocidin LukA/G
Down-regulated in *rsp* mutant		
2493	0.56	Conserved hypothetical protein
2311	0.56	Conserved hypothetical protein
*ent*B	0.55	Isochorismatase
*sdr*C	0.55	SdrC protein
*grp*E	0.54	Cochaperone GrpE
*hrc*A	0.53	Heat-inducible transcription repressor
2310	0.53	Conserved hypothetical protein
*ars*R	0.51	Arsenical resistance operon repressor
2245	0.49	Staphylococcal accessory regulator R
0372	0.48	Putative lipoprotein
0225	0.48	Putative acyl-CoA acetyltransferase FadA
*fad*D	0.470	Acyl-CoA dehydrogenase FadD
0226	0.463	3-hydroxyacyl-CoA dehydrogenase
*arc*A	0.460	Arginine deiminase
*fad*E	0.454	Acyl-CoA synthetase FadE
0229	0.444	Putative acyl-CoA transferase FadX
2453	0.435	ABC transporter, ATP-binding protein
2306	0.435	ABC transporter, ATP-binding protein
2307	0.297	ABC transporter, permease protein
0179	0.255	Putative D-isomer specific 2-hydroxyacid dehydrogenase
*SSR*42	0.004	Small stable RNA 42

*Gene symbol or last four digits of locus tag (i.e., 0238 represents SAUSA300_0238).

USA300 background. This finding showed that *SSR*42 is situated directly upstream of *rsp* and is transcribed in an antiparallel orientation in a highly *rsp*-dependent manner (Fig. 5*C*). *SSR*42 expression was almost completely lost in the absence of *rsp* in exponential growth (Fig. 5 *B* and *C*). RNA-seq in the stationary growth phase showed that, in wild-type bacteria of USA300, P, and S background, *SSR*42 comprised 6.4 ± 1.9% (mean ± SD) of RNA mapping to the genome compared with 0.02 ± 0.01% in *rsp* mutants. We showed *SSR*42 to be longer than previously noted (45) at 1,232 nt (Fig. 5*C*), and as such to be the longest non-ribosomal ncRNA identified in *S. aureus*.

***rsp* and Its Targets Are Induced by Hydrogen Peroxide.** Given the widespread effects of *rsp*, we decided to investigate mechanisms of induction of the *rsp* regulon. Using the *S. aureus* Transcriptome Meta-Database (46), we observed extensive overlap between *rsp*-regulated genes and genes differentially regulated in response to challenge with hydrogen peroxide or azurophilic granules, both

Table 3. Exemplar genes which show expression discordance between stationary and exponential growth phase

Gene	Fold-change		Product
	Exponential	Stationary	
hla	0.48	2.57	α-Hemolysin
coa	0.05	1.16	Coagulase
chs	0.06	1.37	Chemotaxis-inhibiting protein CHIPS
sbi	0.38	2.33	IgG-binding protein SBI
*luk*A(G)	0.26	2.828	Leukocidin A/G
*luk*B(H)	0.19	2.713	Leukocidin B/H

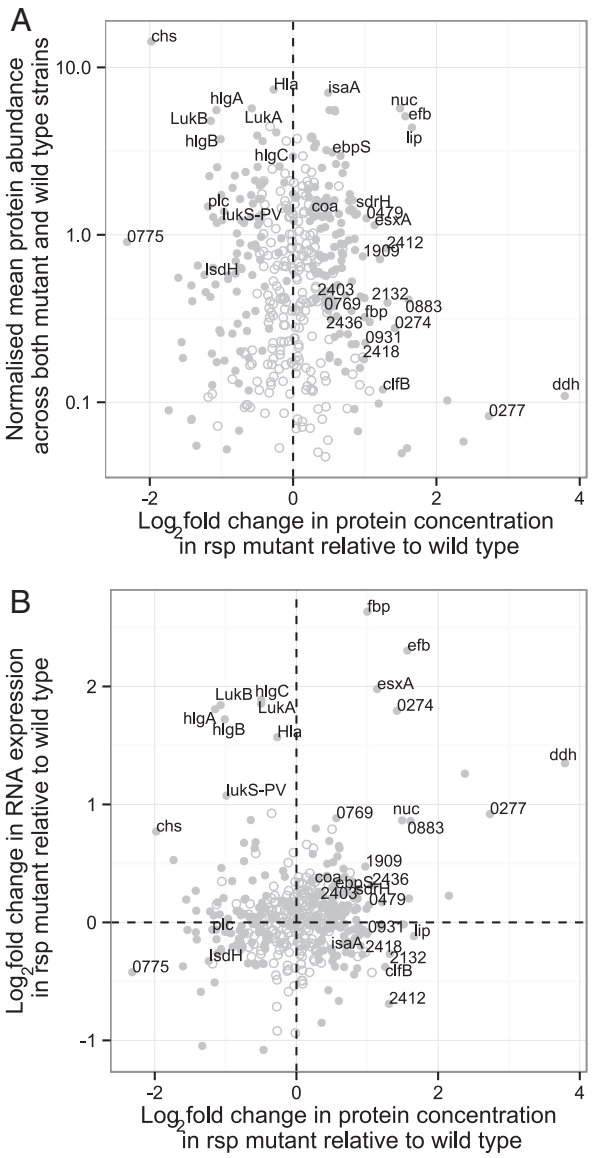

Fig. 6. Complex *rsp*-dependent control mechanisms influence toxin production. MS was performed on the supernatant of stationary phase culture of USA300 *S. aureus* wild-type and an *rsp* mutant. Fold-change in the protein concentration in *rsp* mutant relative to wild-type organisms (*x* axis) was compared with protein abundance in both wild-type and mutant organisms (*A*), and fold-change of RNA expression in *rsp* mutant relative to wild-type organisms (*B*), as derived from RNA-seq performed at the same time point. Each dot represents a gene, and numbers denote locus identifiers (e.g., 0274 refers to SAUSA300_0274). Open circles indicate that the effect of *rsp* is not significant ($P > 0.05$) for this gene, whereas filled symbols indicate that the effect of *rsp* is significant on that gene. The dotted lines indicate the condition where *rsp* mutation has no effect. In *B*, the upper right quadrant identifies genes that are increased in RNA as well as protein level. The upper left quadrant shows genes with increased RNA expression, but lower levels of protein in stationary phase. A number of important toxins, including Hla, HlgA, HlgC, and LukB, fall within this category.

secretions from neutrophils (47). For example, 73 of 113 significantly peroxide-regulated genes were also *rsp*-regulated. Because neutrophils are one of the first cells recruited to invading bacteria and produce bactericidal reactive oxygen species (ROS) (48), we tested the hypothesis that *rsp* might be activated when bacteria encountered the antimicrobial ROS, hydrogen peroxide. Supporting this idea, we observed that *rsp* was rapidly up-regulated in *S. aureus* after hydrogen peroxide treatment ($2^{2.24}$-fold) (Fig. 7*A*). A subset of *rsp* target genes was similarly induced by ROS in a clearly *rsp*-dependent manner, which included SSR42 ($2^{2.14}$-fold), *isd*A ($2^{1.99}$-fold), *luk*AB ($2^{2.03}$-fold), *scp*A ($2^{1.43}$-fold), *hlg*A ($2^{1.48}$-fold), *hlg*C ($2^{1.31}$-fold), and *luk*S-PVL ($2^{1.50}$-fold). Induction of the general stress protein 20U (*dps*), which is known to react to oxidative stress (47), is also partially *rsp*-dependent (*SI Appendix*, Fig. S8*A*). By contrast, the genes *hla* ($2^{1.20}$-fold) and *chs* ($2^{1.06}$-fold) were not significantly altered in the given time (Fig. 7*A*). Similar trends were seen in a different strain background (*SI Appendix*, Fig. S8*B*). This finding indicates that *rsp*-dependent regulatory pathways for early gene induction respond to peroxide challenge.

Because a fraction of *rsp*-regulated gene products are related to immune evasion and neutrophil killing (49), we examined the cytotoxic potential of both bacteria and bacterial supernatants toward neutrophils. *rsp*-dependent neutrophil killing was evident in both investigated strain backgrounds (Fig. 7*B*). Furthermore, neutrophil cell death increased in an *rsp*-dependent manner, when intoxicated with bacterial supernatants from cultures that were challenged with hydrogen peroxide (Fig. 7*C*). Thus, hydrogen peroxide, which is also produced by neutrophils, induces an *rsp*-dependent response that is directed against leukocytes.

To determine whether the ability of *S. aureus* to infect and reside within neutrophils was *rsp*-dependent, we incubated two strains of *S. aureus* with primary human neutrophils and quantified intracellular *S. aureus* by plating. After 2 h of incubation, *rsp* mutants of both genetic backgrounds were present and viable in the neutrophils at similar amounts, indicating similar capability to infect and reside in a viable state. Both strains grew within neutrophils over the next 2 h, but counts were significantly higher for wild-type than for *rsp* mutants ($P = 0.02$), compatible with rapid bacterial replication within neutrophils, as well as neutrophil lysis (Fig. 7 *B* and *D*), being facilitated by *rsp* (Fig. 7*D*).

Thus, *rsp*-dependent mechanisms detect oxidative stress, increase bacterial replication in neutrophils, shorten intracellular bacterial residence, and produce mediators that kill neutrophils and epithelial cells. They also increase mortality in experimental infection, but are dispensable for bloodstream infection and abscess formation.

Discussion

We have shown that naturally occurring loss-of-function mutants in *S. aureus* enact global regulatory changes in gene expression that reduce bacterial toxicity and prolong bacterial residence inside mammalian cells, while maintaining the ability to survive, proliferate, and cause disseminated infection within the human body.

Specifically, we have characterized the pleiotropic transcription factor *rsp* (50), a member of the AraC family of transcriptional regulators (AFTR). We found that Rsp regulates the duration of *S. aureus* residence inside cells, cytotoxicity toward epithelial cells and neutrophils, and lethality in animal models of acute *S. aureus* infection (Table 4). Genome-wide screening suggests that loss-of-function mutants in *rsp* and the ncRNA *SSR42* have the strongest influence among *S. aureus* genes on prolonging intracellular residence of *S. aureus*. The prolonged intracellular residence in the host cell cytoplasm is associated with a delayed cytotoxicity that differs in kinetics from that seen in mutants of the key virulence regulators, *agr*A and *sae*R mutants (Fig. 2*B*). *rsp* expression is not required for dissemination of *S. aureus* from the blood or for deep abscess formation, and we have identified and characterized *rsp* loss-of-function mutants found in human bacteremia. Thus, our data suggest that *S. aureus* can adopt an attenuated cytotoxic phenotype, with prolonged intracellular residence (51–53), which permits effective dissemination of the organism with few initial symptoms, followed by deep abscess establishment. The attenuated toxicity phenotype was not noted in isolates from skin and soft tissue infection (17), suggesting that such attenuated toxicity and intracellular survival may be particularly important in bloodstream infection, as opposed to other forms of infection, such as skin and soft tissue infection. Our findings that *rsp* contributed to lethality in pulmonary disease is also supported by a recent study of cutaneous *S. aureus* disease (54). In both models, toxins have been shown to be key mediators of disease (13, 55).

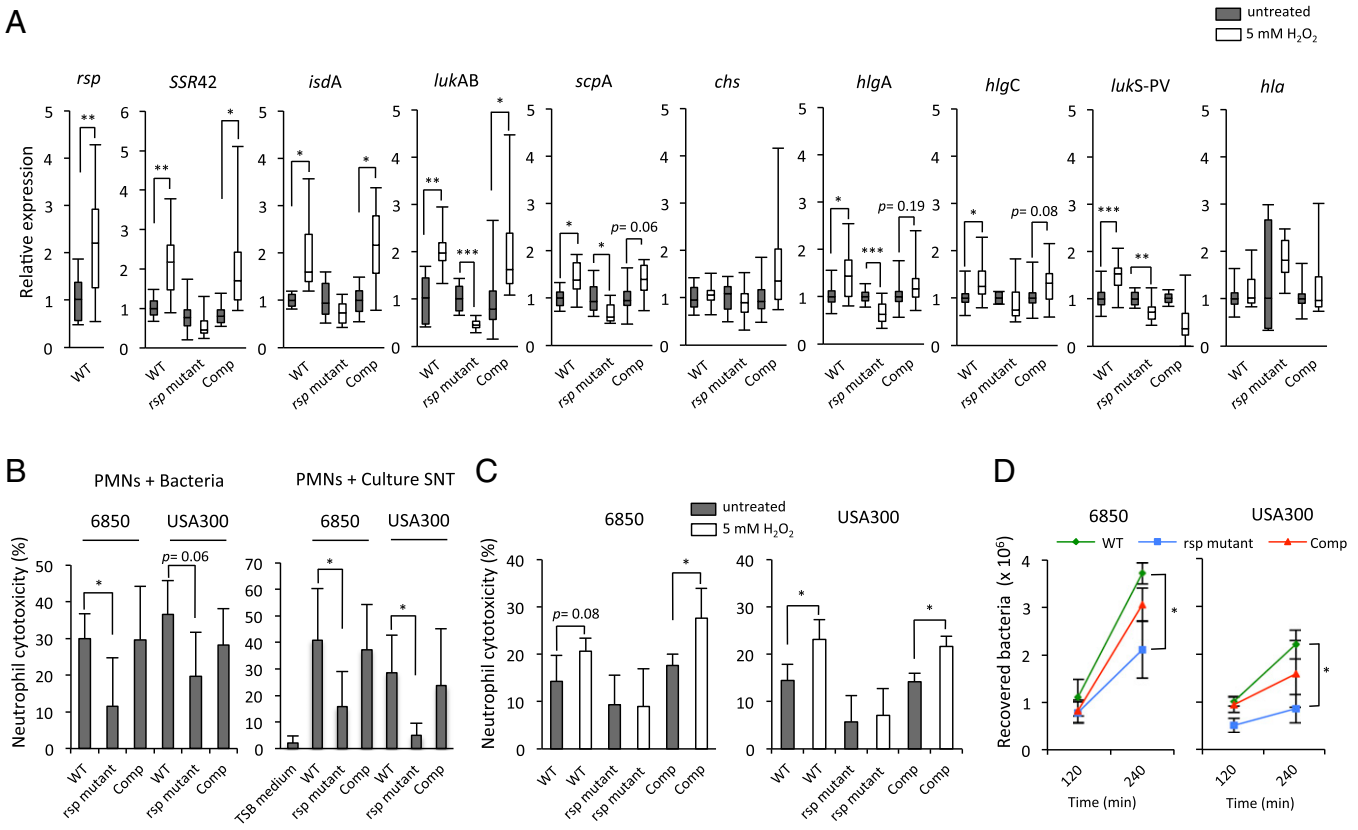

Fig. 7. *rsp* effects are induced by hydrogen peroxide and regulate cytotoxicity toward PMNs. (*A*) Upon 10-min exposure to 5 mM hydrogen peroxide, transcription of *rsp* and its targets is up-regulated in exponentially growing wild-type *S. aureus* LAC* (WT). This response to hydrogen peroxide was *rsp*-dependent and rescued in complemented mutants (Comp). Values indicate expression levels of peroxide-challenged bacteria (open bars) relative to untreated controls (filled bars). Statistical analysis was performed by pairwise *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (*B*) Bacterial infection of PMN with wild-type, *rsp* mutant, and complementants, as well as treatment with bacterial culture supernatant for 4 h, demonstrated that host cell death levels were significantly decreased and complementable in *rsp* mutants. Statistical significance was determined by one-way ANOVA and Tukey's post hoc analysis. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (*C*) *S. aureus* culture supernatants collected from wild type, *rsp* mutant, and complementants after peroxide challenge exhibit increased cytotoxic potential in an Rsp-dependent manner. Human PMNs were intoxicated with the supernatants, and cytotoxicity was determined by LDH release. The vertical axis indicates the percentage of neutrophil cell death compared with complete cell lysis (positive control). Statistical analysis was performed by pairwise *t* test. $*P < 0.05$. (*D*) *S. aureus* (strains 6850 and USA300) *rsp* wild-type (WT), *rsp* mutant, and complementants (Comp) were used to infect neutrophils. Intracellular bacteria were recovered 2 and 4 h after infection, and number of viable bacteria was determined. Statistical analysis at each time point was performed by one-way ANOVA and Tukey's post hoc analysis. $*P < 0.05$ (*rsp* mutant compared with wild type).

We demonstrated that Rsp and *SSR*42 represent a regulatory system consisting of a protein and ncRNA in *S. aureus*. Structurally, it consists of the two adjacent genes, in antiparallel localization and with two distinct TSS (Fig. 5*C*). *SSR*42 is an Rsp target, as evidenced by the absence of transcription after *rsp* inactivation by transposon insertion, ORF deletion, point mutation in DNA binding domains, or translational termination, even though *SSR*42 comprises ∼5% of nonribosomal RNA in wild-type cells. Some of the Rsp effects are mediated by *SSR*42, because *SSR*42-dependent production of the Rsp targets, α-toxin, has been demonstrated (45). Synergy of *SSR42*-mediated effects with direct effects of Rsp itself, such the recently demonstrated binding of Rsp to the *agr* promoter, may also occur (54).

We found that *rsp* transcription was induced by hydrogen peroxide, which is produced by neutrophils in vivo upon stimulation. This finding suggests a model in which *rsp* fulfills an environment-sensing role: On encountering phagocytes, it initiates a specific response that consists of virulence factors that target phagocyte functions, including α and γ hemolysins, *luk*AB (*luk*GH), and the Panton–Valentine leukocidin. This model is compatible with the function of other AFTR members, which, in other bacterial genera, regulate carbon metabolism, stress responses, and virulence in response to changing environmental conditions such as antibiotic use and stress (56, 57). In *S. aureus*, the AFTRs *rbf, rsr*, and *ary*K promote biofilm formation (58), modulate *sar*R and *agr* in a skin infection model (59), and potentiate toxin expression and virulence (60), respectively.

Curiously, we have observed that a subset of genes (Fig. 7) attenuated by *rsp* cannot be readily complemented by supplying *rsp in trans*. Explanations for this phenomenon could be due to: (*i*) the highly complex virulence regulatory cross-talk in *S. aureus*; (*ii*) the involvement of posttranscriptional mechanisms, as we detected by comparing transcriptomic and proteomic data (Fig. 6); or (*iii*) the requirement for an *SSR*42-dependent *cis*-interaction, and thus will require further study.

In summary, our results provide new evidence that a *S. aureus* regulatory system involving the *rsp* transcription factor is subject to spontaneously occurring loss-of-function mutations during evolution within the human body. These knockout mutants display attenuated lethality in the initial stages of experimental infection, but still invade deep tissues, causing severe disease. Although *rsp* loss-of-function stands alone as an interesting mechanism, it has wider significance as an example of how within-host bacterial evolution affects key regulatory pathways, thus influencing disease progression and clinical outcome.

Materials and Methods

Transposon Mutant Library Generation and TnSeq. *S. aureus* strain 6850 was transformed with plasmid pBTn, and mutagenesis was performed as described (61). TnSeq DNA libraries were generated, and Illumina-specific adaptors were

Table 4. Effect of *rsp* mutation on *S. aureus*

Pathway/effect/ phenomenon	*rsp* wild-type	*rsp* mutants
Intracellularity	Rapid host cell lysis after endocytosis	Prolonged intracellular residence after endocytosis
Cytotoxicity	Cytotoxicity (epithelial cells, neutrophils)	Reduced cytotoxicity
Hemolysis	Normal α-toxin hemolysis	Strongly reduced α-toxin hemolysis
SSR42 expression	SSR42 expression (6% of mRNA)	Absent SSR42 expression
Virulence	Lethality (murine sepsis and pneumonia)	Reduced lethality
Peroxide response	Virulence response to peroxide	Reduced peroxide response
Source	Identified in nasal carriage isolates	Identified in bloodstream isolates

ligated to the fragments, and these were enriched for TIS by PCR. After sequencing on the Illumina Hi-Seq 2500 platform, sequences were mapped (62) to the *S. aureus* 6850 genome (63), and differences in frequencies between the samples were detected with DEseq2 (64). For further details, see *SI Appendix, SI Materials and Methods*.

Infection Screens with *S. aureus* Transposon Mutant Libraries. For in vitro cell death screens, HeLa cell monolayers were infected for 1 h with pooled mutant libraries of *S. aureus* 6850 at a multiplicity of infection (MOI) of 1. Extracellular bacteria were removed by using 20 μg/mL Lysostaphin (AMBI) and 100 μg/mL gentamicin (GIBCO) for 30 min. The infected cells were further incubated for 8 h in RPMI medium containing 100 μg/mL gentamicin to inhibit extracellular growth of bacteria that were released by host cell disruption. The bacteria were recovered (output) by hypoosmotic rupture of the HeLa cells using sterile water and plated onto tryptone soy agar plates; including the inoculum (input), this process completed one cycle of infection. Thereby, the screening process selected for intracellular noncytotoxic bacteria, because if the bacteria killed the epithelial cells or escaped extracellularly, they were killed by gentamicin and thus were not recovered on the agar plates. A three-cycle infection method was adopted to enrich the subsequent effects. The output from one cycle was used as input for the next cycle. All three outputs and the input were subjected to TnSeq (see above).

Clinical Samples. *S. aureus* strains were isolated from two patients with concomitant nasal carriage and bloodstream infection. Patient P was recruited to a previously reported longitudinal study of asymptomatic carriage among adults attending general practices in Oxfordshire, U.K., developing a *S. aureus* bloodstream infection 15 mo after joining the study (27). Patient S was treated for a *S. aureus* bloodstream infection at a hospital in Oxfordshire, with a nasal swab subsequently taken as part of routine surveillance. Microbiological processing was performed as described (27). DNA was extracted by using a commercial kit (FastDNA; MP Biomedicals).

Genome Sequencing, Assembly, and Variant Calling. We used the Illumina HiSeq 2000 platform with 96-fold multiplexing, read lengths of 100 or 150 bp, insert sizes of 200 bp, and mean depth of 125 reads. As described (27), we used Velvet (65) to assemble reads into contigs de novo for each genome. We used Stampy (66) to map the reads of each isolate against MRSA252 (67) and a host-specific draft genome assembled by Velvet. We used xBASE (68) to annotate the draft genome assemblies. SAMtools (69) and Picard (broadinstitute.github.io/picard/) were used to call single-nucleotide polymorphisms (SNPs) from mapping, which we filtered by using published criteria (27). We additionally used Cortex (70) to detect SNPs and indels.

RNA Extraction, RNA-Seq, and Real-Time RT-PCR. Bacterial mRNA was extracted by using TRIzol (71) or RNeasy (QIAGEN), and reverse transcription was performed according to manufacturer's guidelines (QIAGEN/Superscript II; Invitrogen). For determination of TSS, processed transcripts were depleted by using Terminator 5′-phosphate-dependent exonuclease (TEX) kit (Epicentre) as described (72). The cDNA was sequenced on the Illumina HiSeq 2000/2500 platforms, and reads were adapter-removed, trimmed, and mapped to the respective bacterial genomes. DESeq2 (64) was used to analyze differential gene expression.

Proteomics. Proteins were precipitated from bacterial culture supernatant, resolubilized, and digested with trypsin. Desalted peptides were separated on a Dionex Ultimate 3000 UPLC system (Thermo Scientific) and introduced to a TripleTOF 5600 mass spectrometer (AB Sciex) by electrospray ionization. Collision-induced dissociation-fragment data were converted to MASCOT format, and MS/MS spectra were interpreted with PEAKS (73). The reference protein database used for identification is provided in Dataset S5. Statistics was performed with DESeq2 (64).

Infection Experiments. HeLa cells were seeded in tissue culture microwell plates (Corning) or in μ-Plate ibiTreat (ibidi) and infected with *S. aureus* at a MOI of 10. The extracellular bacteria were removed by treatment with Lysostaphin and gentamicin for 30 min and further incubated with gentamicin. Cell death was measured by staining with Annexin-V/PI/7-AAD. Human neutrophils were infected with *S. aureus* (74) at a MOI of 10. Cell death was measured by lactate dehydrogenase (LDH) assay, and bacterial titers were enumerated to see intraphagosomal survival.

Female BALB/c mice 8 wk of age were administered *S. aureus* either intravenously through the lateral tail vein (sepsis model) or intranasally (pneumonia model), with previously titrated bacterial suspensions. Weight and clinical score were determined. Animals were killed, and bacterial titers were enumerated from organs by plating.

Ethical Framework. Animal studies were either approved by the local government of Franconia, Germany (approval nos. 2531.01-06/12 and 2532-2-155) and performed in strict accordance with the guidelines for animal care and experimentation of German Animal Protection Law or were approved under the Animal (Scientific Procedures) Act 1986 (Project license 30/2825) and were approved by the University of Oxford Animal Care and Ethical Review Committee. Both sites conformed to Directive 2010/63/EU of the European Union. Work with human neutrophils was approved by the Ethics Commission of the University of Wuerzburg (Code 2015091401). Patient P isolates were obtained during participation in a study of *S. aureus* carriage in Oxfordshire. This study was approved by Oxfordshire Research Ethics Committee B (approval reference 08/H0605/102 granted September 2, 2008) and obtained individual written consent from all participants. Patient S isolates were collected from routine clinical samples. Ethical approval for sequencing *S. aureus* isolates from routine clinical samples and linkage to patient data without individual patient consent in Oxford and Brighton in the U.K. was obtained from Berkshire Ethics Committee (10/H0505/83) and the U.K. National Information Governance Board [8-05(e)/2010].

For additional experimental details, please see *SI Appendix, SI Materials and Methods*.

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