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A point mutation in the *agr* locus rather than expression of the Panton-Valentine leukocidin caused previously reported phenotypes in *Staphylococcus aureus* pneumonia and gene regulation

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Abstract

The role of Panton-Valentine leukocidin (PVL) in *Staphylococcus aureus* pathogenesis is controversial. Here, we show that an unintended point mutation in the *agr* P2 promoter of *S. aureus* caused the phenotypes in gene regulation and murine pneumonia attributed to PVL by Labandeira-Rey *et al.* (*Science* 315:1130-3, 2007). In agreement with previous studies that failed to detect similar effects of PVL using community-associated methicillin-resistant *S. aureus* strains, we found no significant impact of PVL on gene expression or pathogenesis after we repaired the mutation. These findings further contribute to the idea that PVL does not have a major impact on *S. aureus* pathogenesis. Moreover, our results demonstrate that a single nucleotide polymorphism in an intergenic region can dramatically impact bacterial physiology and virulence. Finally, our work emphasizes the need to frequently evaluate the integrity of the *S. aureus agr* locus.

Keywords

Staphylococcus aureus; MRSA; Community-associated MRSA; Panton-Valentine Leukocidin; *agr*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major problem worldwide and kills more people annually in the United States than AIDS [1]. This is in part due to the evolving epidemic of community-associated MRSA (CA-MRSA), which are also the leading cause of skin and soft tissue infections reporting to the emergency department in the United States [2].

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However, the molecular determinants responsible for the ability of CA-MRSA strains to spread and infect otherwise healthy people outside of the hospital setting have remained poorly defined [3]. Cytolytic peptides, the α-type phenol-soluble modulins (PSMs), and α-toxin have a crucial impact on the development of CA-MRSA disease in mice [4,5]. However, these two virulence factors are not exclusively produced in CA-MRSA. Possibly, differences in expression of these genes can partially explain the enhanced virulence of CA-MRSA strains [4]. In contrast, many CA-MRSA strains have the *lukSF-PV* genes encoding the Panton-Valentine leukocidin (PVL), while *lukSF-PV* are present less frequently in other strains [3]. Therefore, PVL has been suspected to represent a major factor contributing to CA-MRSA virulence, and a correlation between presence of the *lukSF-PV* genes and specific types of skin and soft tissue infections has been noted previously [6].

Several recent studies have investigated the role of PVL in *S. aureus* pathogenesis using animal infection models. Using isogenic *lukSF-PV* deletion mutants in the most prominent CA-MRSA strains, Voyich *et al.* and Bubeck Wardenburg *et al.* found no difference in murine skin and soft tissue, bacteremia, and pneumonia models compared with the corresponding parental strains. Based upon these results, the authors concluded that PVL is not a major virulence factor in CA-MRSA disease [5,7]. Diep *et al.* found only a transient positive effect of PVL in a CA-MRSA bacteremia model in rabbits [8]. This effect was limited to colonization of the kidneys and may be explained by PVL initially priming the host innate immune system.

In contrast, Labandeira-Rey *et al.* reported that PVL is a significant factor contributing to murine pneumonia [9]. Of note, by using laboratory strains of *S. aureus* with the *lukSF-PV* genes introduced on the PVL-encoding phage ϕSLT, Labandeira-Rey *et al.* [9] did not directly address the function of PVL in CA-MRSA, but tested its role solely as an *S. aureus* virulence factor in general. Subsequently, Bubeck Wardenburg *et al.* ruled out the possibility that the differential results with regard to the role of PVL in murine pneumonia were due to the use of different strains of mice [10].

In addition to the murine pneumonia results, Labandeira-Rey *et al.* reported a global gene regulatory effect of PVL reminiscent of that caused by the accessory gene regulator (*agr*) in *S. aureus* [9,11]. In contrast, Diep *et al.* failed to find a gene regulatory effect of PVL using isogenic *lukSF-PV* deletion mutants in CA-MRSA strains [8]. Thus, key results obtained by Labandeira-Rey *et al.* are at variance with those achieved by other groups, which prompted us to re-evaluate the strains used by these authors as a potential source for the different experimental outcomes.

Methods

Bacterial strains and growth conditions

RN6390 is a laboratory *S. aureus* strain and the parental strain of all other strains used herein (table 1). Strains LUG855, LUG776, and LUG862 were kindly provided by M. Gabriela Bowden (Texas A&M University System Health Science Center) [9]. LUG855 contains the ϕSLT phage carrying among other genes the *lukSF* genes encoding PVL [9]; LUG776 contains ϕSLT in which the *lukSF* genes were deleted [9]; LUG862 is LUG776 carrying a plasmid with the *lukSF* genes (pLUG534) [9] and was grown with addition of 10 μg/ml chloramphenicol. All strains were kept in glycerol stocks after being received and were not passaged. Tryptic soy broth (TSB) was used for all experiments.

Oligonucleotides and quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed in triplicate using *gyrB* RNA as a control as described [4]. All oligonucleotides were synthesized by Sigma (table 2).

Immunoblotting

Immunoblots were performed using 10% Tris/glycine SDS-PAGE gels and blotting on nitrocellulose. Horse radish peroxidase conjugates were used as second antibodies and immune reactions were made visible using enhanced chemiluminescence.

DNA sequencing and manipulation

DNA encompassing the entire *agr* locus was sequenced in all strains at the Genomics Unit, Research Technologies Branch, NIAID. The mutation identified in LUG855 was repaired using the allelic replacement method with plasmid pKOR1 as described [12]. To that end, a ~ 1.6 kb fragment containing the site of the mutation was amplified from RN6390 genomic DNA using primers GBatt1 and GBatt2 introducing att1 and att2 sites, respectively, and cloned in pKOR1. The resulting plasmid was used for allelic replacement. The entire *agr* system of the resulting, repaired clone was sequenced and the sequence was found to be exactly the same as in RN6390.

Purification of AgrA

Purification of AgrA was performed as described previously [13]. Obtained AgrA was analyzed by SDS-PAGE, N-terminal sequencing, and analytical RP-HPLC/ESIMS, and found to be correct and pure.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described [13]. Final DNA concentrations were 25 pM. Binding reactions were performed in 20 μl, incubated at room temperature for 30 min and run on an 8% non-denaturing 0.5 × TBE gel.

Measurement of δ-toxin concentration

Determination of δ-toxin production in *S. aureus* culture filtrates was performed in triplicate using RP-HPLC/ESI-MS as described [4].

Murine pneumonia model

For murine lung infections examining weight loss, lung CFU recovery and histopathology, *S. aureus* strains were prepared as described by Labandeira-Rey *et al*. [9]. Briefly, overnight cultures grown in TSB were refreshed 1:100 in media and grown with shaking to an OD_{600} of 1.0. Bacteria were sedimented by centrifugation, washed in PBS, and suspended at a concentration of $4\text{-}6\times10^7$ CFUs per 20-μl volume of PBS for intranasal inoculation. 7 week old mice (BALB/cAnNHsd) were anesthetized prior to inoculation of the *S. aureus* suspension into the left nare as previously described [14]. Microbiologic and pathologic correlates of disease were assessed 48 hours post-infection, also as previously described [14]. Animal experiments were reviewed, approved and supervised by the IACUC at the University of Chicago.

RNA isolation, transcriptional profiling, and quantitative RT-PCR

RNA isolation and cDNA preparation from cultures grown to early stationary growth phase (7 h) were performed as previously described [15]. Biotinylated *S. aureus* cDNA was hybridized to custom Affymetrix GeneChips (RMLChip 7) with 100% coverage of chromosomal genes from USA300 and scanned according to standard GeneChip protocols (Affymetrix). Each experiment was replicated 3 times. Affymetrix GeneChip Operating Software (GCOS v1.4, http://www.affymetrix.com) was used to perform the preliminary analysis of the custom GeneChips at the probe-set level. Subsequent data analysis was performed as described [15]. Briefly, each comparison gene list was filtered first using the 0.05 significance level, false discovery rate corrected p-values resulting from a two-way ANOVA. Second, a filter was

placed upon fold change (2X), then call consistency and finally signal above background to produce the final gene list for each comparison. The complete set of microarray data was deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE14394.

Results

The hemolysis-negative phenotype of LUG855 cannot be complemented by lukSF-PV

Strains LUG855 and LUG776 (table 1), represent the only isogenic strain pair compared in the Labandeira-Rey *et al.* study [9]. Therefore, we focused our evaluation on those strains, the respective complementation strain LUG862, and the background strain RN6390. First, we determined β-hemolysis, which is dependent on simultaneous expression of δ-toxin and other *agr*-regulated toxins, and thus a common and simple readout for *agr* functionality. While LUG855 was non-β-hemolytic, both LUG776 and LUG862 strains were β-hemolytic, even though the *lukSF* genes were re-introduced into LUG862 (figure 1*B*). Restoration of a β-hemolysis-negative phenotype in LUG862 would be expected if PVL expression were responsible for the β-hemolysis-negative phenotype of LUG855. Notably, all tested cultures showed a consistent β-hemolysis phenotype, indicating that there were no contaminations or mixed cultures (shown for LUG776 and LUG855 in figure 1*C*). Furthermore, we determined δ-toxin production by HPLC/MS (figure 1*D*). Production of this toxin is a direct readout of *agr* functionality, as the δ-toxin encoding gene *hld* is embedded within the region encoding the intracellular effector molecule of *agr*, RNAIII [16]. The results confirmed those achieved by measuring β-hemolysis, inasmuch as all strains were clearly δ-toxin-positive, whereas LUG855 was δ-toxin-negative. These findings indicated that the β-hemolysis- and δ-toxin-negative phenotype observed in LUG855 could not be restored by genetic complementation, lending support to the hypothesis that the phenotype of LUG855 reported by Labandeira-Rey *et al.* [9] was not due to PVL.

LUG855 contains a point mutation in the agr P2 promoter that strongly impairs binding of AgrA

To test whether the observed phenotypes were rather caused by a spontaneous mutation in the *agr* locus, we sequenced the entire *agr* locus in all strains (from position 2092803 to position 2097710 in the genome sequence of *S. aureus* strain NCTC 8325). The *agr* sequences of RN6390, LUG776, and LUG862 exactly matched that published for the RN6390 progenitor strain, NCTC 8325 (NC_007795). However, strain LUG855 had a one-base pair mutation (G to A) in the intergenic region between the RNAIII and AgrB encoding regions. The mutation mapped to one of the consensus sequences in the binding site of the AgrA response regulator protein in the P2 promoter [17], which drives transcription of the *agrBDCA* operon [18] (figure 1*A*). This auto-regulatory interaction is crucial for *agr* function. To confirm that this one-base pair mutation affects binding of AgrA to the P2 promoter, we performed EMSAs with purified AgrA protein. We found that AgrA binding to the mutated P2 region of LUG855 was dramatically decreased compared to the corresponding region in LUG776 (figure 1*E*).

Gene and protein expression analysis of an agr-repaired LUG855 indicates that the LUG855 phenotype is due to the agr mutation and not PVL

To determine whether the identified mutation in the *agr* locus of LUG855 is responsible for the *agr*-negative phenotype and differences in gene expression reported by Labandeira-Rey *et al.* [9], we repaired the mutation in strain LUG855 using the allelic replacement strategy according to Bae and Schneewind [12]. The entire *agr* system of the resulting clone LUG855r was sequenced and found to be correct, exactly matching that of RN6390 and the other *S. aureus* strains.

We first determined global gene expression using microarray analysis. Table 3 shows the total number of differentially regulated genes and figure 2 shows selected differentially regulated virulence factors (see GEO, http://www.ncbi.nlm.nih.gov/geo/, GSE14394 for detailed results). Comparing strains LUG855 and LUG776, we confirmed the global changes in gene expression (277 genes differentially-expressed) that are characteristic of genes controlled by *agr* [11] and were in general described by Labandeira-Rey *et al.* [9] (figure 2). However, there were only 10 genes up- or down-regulated in the repaired LUG855 compared with LUG776. Several of these 10 differentially-expressed genes were either only slightly above the 2-fold cutoff or represented homologues of *lukS-PV* or *lukF-PV*, which may be explained by cross-hybridization. In addition, only expression of *spa* (encoding protein A) and *clpB* were considerably different between the strains, an effect that is not easily explained, but may be caused by differential integration of the phage. Notably, the observed change in *spa* expression level was opposite that described by Labandeira-Rey *et al.* [9]. In addition, there was very pronounced similarity in genes differentially expressed between LUG855/LUG776 and LUG855/LUG855r comparisons (figure 2), further indicating that the explanation for differential gene expression in LUG776/LUG855 is identical to that in LUG855/LUG855r – namely, the mutation in the *agr* locus. The results of the LUG855/LUG776 and LUG855/LUG855r comparisons are consistent with the observed decrease in AgrA–P2 promoter interaction (figure 1*E*); i.e., a pronounced decrease, yet not complete absence of *agrA*, *agrB*, *agrC*, and *agrD* transcripts in LUG855. Also, these results are in good agreement with the values for *agr* genes obtained by Labandeira-Rey et al. in their LUG855/LUG776 comparison [9]. The overall results of the gene expression analyses using the complementation strain LUG862 were consistent, as there was a much greater number of differentially expressed genes when comparing this strain to the *agr*-mutated LUG855 than to LUG855r or LUG776, confirming that the extensive changes in gene expression are due to a difference in *agr* rather than *lukSF-PV* (Table 3). Furthermore, this analysis revealed that the comparison of LUG862 with LUG776 or LUG855 by Labandeira-Rey *et al.* was not appropriate to investigate the impact of PVL on virulence, as our results demonstrate a significant influence of the plasmid background (> 100 differentially expressed genes were identified by comparing LUG862 and LUG855r) and Labandeira-Rey *et al.* did not compare to a strain with a control plasmid. In summary, our results demonstrate that the global gene regulatory effect described in strain LUG855 by Labandeira-Rey *et al.* was not caused by PVL. In contrast, our results strongly suggest that they were caused by an unintended mutation in the *agr* locus of LUG855.

To further confirm this idea, we determined gene expression of major virulence determinants by qRT-PCR (figure 3*A*). Notably, we analyzed *spa* encoding protein A that was suggested by Labandeira-Rey *et al.* to underlie the ability of PVL to promote *S. aureus* necrotizing pneumonia [9]. Furthermore, we tested transcription of the genes encoding the surface proteins ClfB and SdrD, which were also suggested to contribute to pathogenesis via the reported gene regulatory effect of PVL [9], and α-toxin and α-type PSMs, the only factors determined so far to impact pathogenesis of CA-MRSA [4, 5]. Since the β-hemolysis data suggested that *agr* was defective in the original unrepaired LUG855 strain, we also tested expression of RNAIII transcripts (figure 1*D*) as a direct readout for *agr* activity. Compared to LUG776, there were no significant differences in the levels of these transcripts in the repaired LUG855r strain (figure 3*A*). Protein expression analyses of α-toxin and protein A using immunoblots confirmed these results on a translational level (figure 3*B*). These results contrast those of the original, mutated LUG855 strain, which showed a phenotype dramatically different than those of all other strains (figure 3*A*,*B*). In agreement with the microarray results, these data further indicate that the *agr* mutation but not PVL was the underlying cause of the differences reported between LUG855 and LUG776 [9].

Additionally, we found that expression of PVL in LUG855 was very low (figure 3*A*,*B*), most likely owing to strong *agr* control of *lukSF-PV* [13], which was defective in LUG855. These

observations provide a possible explanation for our inability to detect a difference in *lukSF-PV* expression in the microarray experiment that compared LUG776 with LUG855, whereas there was a significant difference in *lukSF-PV* transcript levels when we compared LUG855r with LUG776, as expected from a correct, repaired LUG855r strain expressing large amounts of PVL. Therefore, the experiments of Labandeira-Rey *et al.* using LUG855 were based on an *S. aureus* strain that produced little PVL, further supporting the idea that the phenotypic differences observed in those experiments were not due to PVL.

Use of the repaired LUG855 demonstrates that PVL does not impact murine pneumonia

Our finding demonstrating that the LUG855 strain contained an unintended mutation in *agr*, which dramatically changed gene expression, suggested that the virulence phenotype described by Labandeira-Rey *et al.* using the same strain was influenced by the *agr* mutation and not PVL. To evaluate this hypothesis, we repeated the murine pneumonia model of Labandeira-Rey *et al.* [9] using the same mouse strain, experimental conditions, and experimental readouts with the repaired LUG855r and corresponding isogenic *lukSF-PV*-negative LUG776. There was no statistically significant difference in weight loss (figure 3*C*). Furthermore, the histopathology in all lung sections of mice infected with the 2 strains was similar, inasmuch as the severity of pneumonia was similar in all tissue sections and was interpreted as moderate to severe (data not shown). Moreover, survival rates of mice infected with LUG855r and LUG776 were the same, as all mice survived the experiment. Notably, Labandeira-Rey *et al.* also failed to detect differences in mouse survival in this model when comparing LUG855 and LUG776 (100% survival for both strains) [9]. They only detected significant differences in survival rates when PVL was over-expressed from a plasmid and using a non-isogenic comparison. In addition, we measured the concentration of CFUs in lung tissue, which were not significantly different between the 2 groups of mice (figure 3*D*). Together, these findings demonstrate that PVL does not have an effect on *S. aureus* pathogenesis in this murine model of *S. aureus* pneumonia.

Introduction of the PVL phage itself impacts gene expression

Labandeira-Rey *et al.* also compared PVL-positive and -negative strains produced in the *S. aureus* SH1000 background, which like RN6390 is a derivative of the 8325-4 laboratory strain with a repaired *rsbU* gene [19]. However, this strain pair was not isogenic, as the control strain did not contain the ϕSLT phage with deleted *lukSF-PV* genes, such as LUG855. To investigate whether the phage itself, rather than *lukSF-PV*, has an effect on gene expression, we compared the LUG776 (containing the ϕSLT phage without *lukSF-PV*) to the RN6390 background strain. We found that one group of genes with unknown function (homologues of the USA300 1377, 1378, 1379, and 1380 genes), located adjacent to the PVL phage, showed drastically increased expression in RN6390 (table 4). Furthermore, we found that *clpB* and *spa* genes were differentially regulated, lending support to our hypothesis that the differential expression of *spa* and *clpB* observed in the LUG855r versus LUG776 comparison may have been caused by differential phage integration. Notably, this analysis showed that the ϕSLT phage background without *lukSF-PV* has a significant impact on gene expression, emphasizing that comparisons of ϕSLT phage-containing with non-ϕSLT phage-containing strains are not valid to investigate regulatory or phenotypic effects caused by *lukSF-PV*.

Discussion

The *S. aureus* global gene regulator *agr* controls expression of multiple virulence factors and metabolic genes and is reportedly prone to spontaneous mutation [20]. As (i) the alteration in global gene expression attributed to PVL by Labandeira-Rey *et al.* [9] was reminiscent of that caused by mutations in *agr*, and (ii) control experiments in that study were not sufficient to rule out the possibility that the observed effects might have originated from a spontaneous

mutation in another gene locus, we hypothesized that an unintended mutation in *agr* may have caused the PVL-associated phenotype reported by Labandeira-Rey *et al.* Failure to rule out spontaneous mutation in *agr* by appropriate genetic controls previously led to erroneous reports describing regulatory functions for the *xprA* [21], *svrA* [22], and *traP* [23-25] genes.

In the present study we demonstrated that the gene expression differences and the virulence phenotype in experimental murine pneumonia described by Labandeira-Rey *et al.* as attributed to PVL [9] were caused by a mutation in *agr*. This conclusion is based on two main observations. First, we detected an unintended *agr* mutation in the LUG855 strain used by Labandeira-Rey *et al.* that caused decreased binding of AgrA to the *agr* P2 promoter and dramatically decreased *agr* activity. Second, we could not reproduce the aforementioned results of Labandeira-Rey *et al.* by using a corrected LUG855 strain in which the *agr* mutation was repaired.

We are aware of the fact that the conclusions by Labandeira-Rey *et al.* were also based on other strain comparisons that revealed differences in virulence. However, these comparisons were not made between isogenic strains and may thus be influenced by factors other than PVL. In support of this, we showed that there are gene expression differences comparing LUG862 with the repaired LUG855r, and RN6390 with LUG776, demonstrating the influence of the plasmid or phage, respectively, independently of *lukSF-PV*.

Several reports indicate that PVL has no or only a minor role for *S. aureus* pathogenesis when PVL is expressed under natural conditions [5,7,8,10]. Only one recent report from the same group that authored the Labandeira-Rey *et al.* study is at variance with these results [26]. Furthermore, there is an increasing frequency of CA-MRSA infections by strains lacking *lukSF* genes [3,27,28]. Thus, although the role of PVL for CA-MRSA disease will certainly need to be investigated further, experimental and epidemiological evidence indicates that it has been greatly overestimated.

Mutations in *agr* occur frequently in vitro [20] and can often be isolated from infections, particularly chronic and biofilm-associated infections [29,30]. Most of these spontaneous mutations are found in the *agrC* gene. To our knowledge, this report is the first to identify a mutation in the *agr* promoter region causing dramatic changes in *agr* activity, thereby identifying a nucleotide position with extreme significance for binding of the regulatory AgrA DNA-binding protein. Our findings demonstrate that a single nucleotide change even outside an open reading frame can have a dramatic and global impact on bacterial gene expression. Furthermore, our finding underscores the importance of appropriate genetic validation and highlights the need to evaluate and re-evaluate *S. aureus* strains in vitro, owing to the frequent spontaneous mutations that occur in the *agr* locus.

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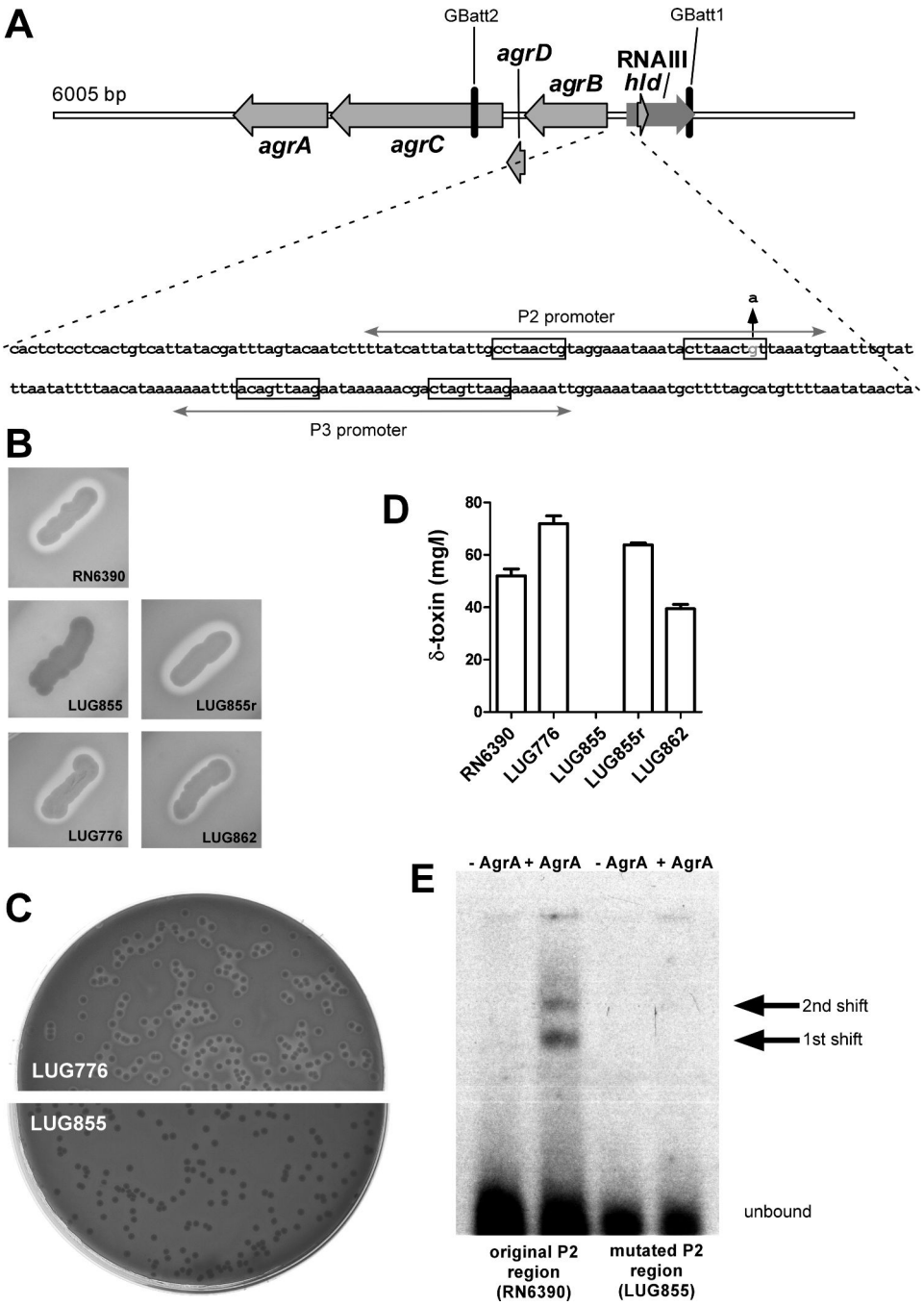

Figure 1.

Mutation in the accessory gene regulator (*agr*) system of *S. aureus* in strain LUG855. (*A*), The *agr* locus of LUG855. Locations of primers used for allelic replacement with pKOR1 (GBatt1, GBatt2) are shown. The DNA sequence of the intergenic region between the *agrBDCA* operon and RNAIII is at the bottom, with locations of the P2 and P3 promoters, the corresponding AgrA consensus binding sequences (in boxes), and the site and nature of the mutation observed in LUG855 (up arrow). (*B*), hemolysis on sheep blood agar plates of streaked cultures. (*C*), hemolysis of LUG776 and LUG855 in stock cultures showing a homogenous phenotype. (*D*), δ-toxin expression by RP-HPLC/ESI-MS. Samples were taken from cultures inoculated from pre-cultures and grown for 7 h. (*E*) EMSA analysis showing dramatic decrease of AgrA

binding to the mutated P2 promoter present in LUG855 compared to the original promoter of RN6390 (same sequence as in LUG776 and the other tested strains except LUG855). AgrA concentration was 10 nMol/L. First and second shifts, as previously observed for AgrA binding by Koenig *et al.* [17], are marked.

gene number (USA300)	gene product function	855 vs 776	855 vs 855r	855r vs 776
SAUSA300_0113	Immunoglobulin G binding protein A precursor	2.84	29.13	10.27
SAUSA300_0114	Staphylococcal accessory regulator A	18.28	20.23	n.c.
SAUSA300_0120	Siderophore biosynthesis LucC protein (EC 6.-.-.-)	6.62	5.73	n.c.
SAUSA300_0121	Multidrug resistance efflux pump	2.51	2.25	n.c.
SAUSA300_0123	Siderophore synthase (EC 6.-.-.-)	3.03	2.56	n.c.
SAUSA300_0180	Multidrug resistance protein ImrP	2.70	2.82	n.c.
SAUSA300_0200	Oligopeptide transport ATP-binding protein OppD	5.69	4.51	n.c.
SAUSA300_0201	Oligopeptide transport system permease protein OppB	8.56	8.61	n.c.
SAUSA300_0202	Oligopeptide transport system permease protein OppC	10.89	7.92	n.c.
SAUSA300_0203	Oligopeptide-binding protein OppA	11.51	9.60	n.c.
SAUSA300_0204	Gamma-glutamyltranspeptidase (EC 2.3.2.2)	8.31	8.60	n.c.
SAUSA300_0256	Murein hydrolase exporter	13.93	6.63	n.c.
SAUSA300_0320	Lipase (EC 3.1.1.3)	3.60	3.92	n.c.
SAUSA300_0547	Fibronectin-binding protein	9.13	14.61	n.c.
SAUSA300_0630	Multidrug resistance ABC transporter ATP-binding and permease protein	2.38	2.85	n.c.
SAUSA300_0887	Oligopeptide transport system permease protein OppB	2.43	2.14	n.c.
SAUSA300_0888	Oligopeptide transport system permease protein OppC	2.58	3.51	n.c.
SAUSA300_0889	Oligopeptide transport ATP-binding protein OppD	3.29	4.18	n.c.
SAUSA300_0890	Oligopeptide transport ATP-binding protein OppF	3.10	3.36	n.c.
SAUSA300_0891	Oligopeptide-binding protein OppA	3.95	3.90	n.c.
SAUSA300_0950	Staphopain (EC 3.4.22.-)	9.31	10.83	n.c.
SAUSA300_0951	Glutamyl endopeptidase precursor (EC 3.4.21.19)	17.37	19.55	n.c.
SAUSA300_0954	Transcriptional regulator, MarR family	7.59	5.44	n.c.
SAUSA300_1058	Alpha-hemolysin	5.64	6.46	n.c.
SAUSA300_1060	Exotoxin	4.20	n.c.	2.2
SAUSA300_1061	Exotoxin	4.83	n.c.	2.55
SAUSA300_1067	Antibacterial protein 3	12.33	14.79	n.c
SAUSA300_1068	PSM beta 1	10.17	11.69	n.c.
SAUSA300_1381	Leukocidin F subunit (PVL)	n.c.	28.04	3650.47
SAUSA300_1382	Leukocidin S subunit (PVL)	n.c.	25.32	1299.69
SAUSA300_1739	Endonuclease (EC 3.1.-.-)	4.14	2.29	n.c.
SAUSA300_1753	Serine protease (EC 3.4.21.-)	270.46	296.52	n.c.
SAUSA300_1754	Serine protease (EC 3.4.21.-)	259.10	278.09	n.c.
SAUSA300_1755	Serine protease (EC 3.4.21.-)	247.67	266.78	n.c.
SAUSA300_1756	Serine protease (EC 3.4.21.-)	266.84	277.70	n.c.
SAUSA300_1757	Serine protease (EC 3.4.21.-)	119.01	116.77	n.c.
SAUSA300_1758	Serine protease (EC 3.4.21.-)	300.24	283.57	n.c.
SAUSA300_1769	Leukocidin S subunit	11.08	n.c.	n.c.
SAUSA300_1890	Staphopain (EC 3.4.22.-)	57.45	49.22	n.c.
SAUSA300_1918	Sphingomyelin phosphodiesterase (EC 3.1.4.12)	21.83	15.34	n.c.
SAUSA300_1922	Staphylokinase precursor	4.95	4.61	n.c.
SAUSA300_1973	Sphingomyelin phosphodiesterase (EC 3.1.4.12)	33.87	20.59	n.c.
SAUSA300_1974	Leukocidin F subunit	6.80	2.53	n.c.
SAUSA300_1975	Leukocidin S subunit	5.87	n.c.	n.c.
SAUSA300_1989	Accessory gene regulator protein B AgrB	4.51	5.43	n.c.
SAUSA300_1990	Autoinducing peptide precursor AgrD	5.46	4.84	n.c.
SAUSA300_1991	Sensory transduction histidine kinase AgrC (EC 2.7.3.-)	4.95	6.38	n.c.
SAUSA300_1992	Accessory gene regulator protein A AgrA	8.92	11.67	n.c.
SAUSA300_2126	Multidrug resistance protein B	2.43	2.19	n.c.
SAUSA300_2161	Hyaluronate lyase precursor (EC 4.2.2.1)	7.56	6.62	n.c.
SAUSA300_2238	Urease gamma subunit (EC 3.5.1.5)	12.70	6.79	n.c.
SAUSA300_2239	Urease beta subunit (EC 3.5.1.5)	16.30	7.53	n.c.
SAUSA300_2240	Urease alpha subunit (EC 3.5.1.5)	11.94	6.53	n.c.
SAUSA300_2241	Urease accessory protein UreE	10.35	5.81	n.c.
SAUSA300_2242	Urease accessory protein UreF	8.78	5.57	n.c.
SAUSA300_2243	Urease accessory protein UreG	7.51	4.74	n.c.
SAUSA300_2244	Urease accessory protein UreD	6.67	4.10	n.c.
SAUSA300_2366	Leukocidin S subunit	30.22	9.48	n.c.
SAUSA300_2453	Lantibiotic transport ATP-binding protein	6.97	10.98	n.c.
SAUSA300_2603	Lipase (EC 3.1.1.3)	11.06	12.72	n.c.
SAUSA300_2616	Cobalt transport protein CbiQ	3.16	3.47	n.c.

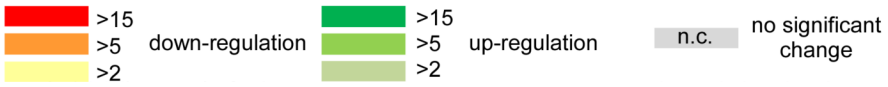

Figure 2.

Microarray analysis of differential gene expression in LUG776, LUG855, and the *agr*-repaired LUG855r. Samples for microarray analysis were harvested from cultures grown to early stationary phase (7 h) and prepared as described in Methods. Results for selected genes involved in virulence are shown. The microarray used was based on the genome for USA300 to include the PVL phage in the analysis.

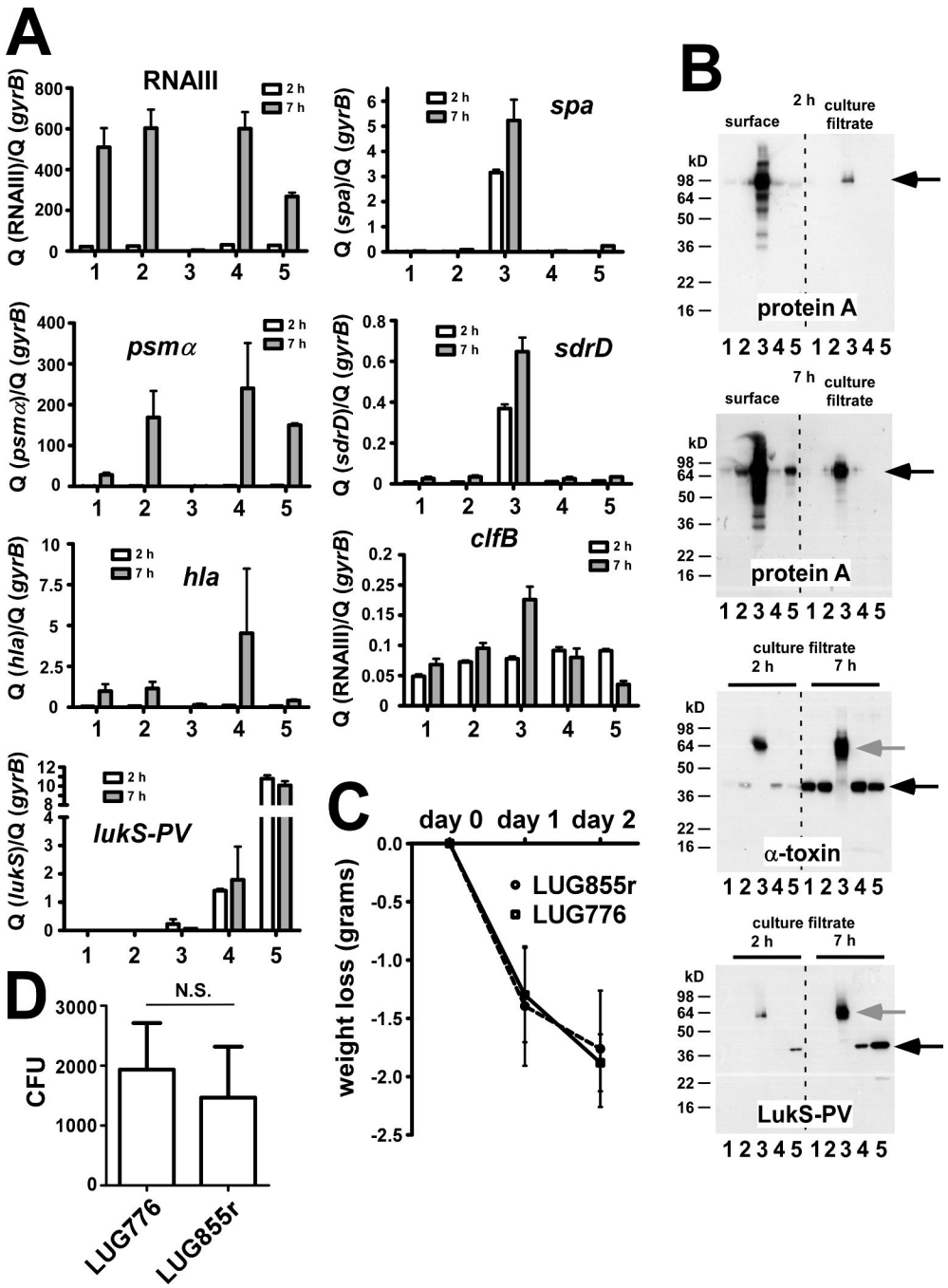

Figure 3.

Gene and protein expression of various key virulence factors and mouse pneumonia model. (*A*), Transcription levels by qRT-PCR. *lukS-PV*: *lukS* component of PVL. (*B*), Immunoblots of protein A, α-toxin, and the LukS component of PVL, using specific antisera (protein A, α-toxin: commercially available from Sigma; LukS-PV: produced by GenScript). Culture filtrate samples were directly used for SDS-PAGE; surface protein samples were obtained by lysostaphin digestion. Black arrow, detected protein, grey arrow, protein A reaction with IgG Fc part. (*A,B*) 1, RN6390; 2, LUG776; 3, LUG855 (original); 4, LUG855 (repaired); 5, LUG862. (*C,D*) Animal model of murine pneumonia with strains LUG776 and the isogenic, repaired LUG855r. The model was performed as described by Labandeira-Rey et al. [9], using

the same mouse strain, inocula, and experimental conditions. Fifteen mice were used for each strain. Data for weight loss (*C*) and CFU/g in lung tissue samples (*D*) are shown. Statistical analysis was by unpaired Student's t-test. Error bars depict SEM.

Table 1

Strains used in this study[1].

Strain	Description	*agr*	*lukSF* (encoding PVL)	Reference
RN6390	*S. aureus* laboratory strain	+	−	[16]
LUG855	RN6390 ϕPVL^{+}	− (point mutation)	+	[9]
LUG855r	RN6390*ϕPVL^{+}	+ (point mutation repaired)	+	This study
LUG776	RN6390 ϕPVL^{-}	+	− (inactivated on phage)	[9]
LUG862	RN6390 ϕPVL^{-} pPVL^{+}	+	+ (plasmid)	[9]

[1] *, point mutation in *agr*; ϕPVL^{+}, lysogenic phage SLT carrying *lukSF* genes; ϕPVL^{-}, lysogenic phage SLT carrying inactivated *lukSF* genes; pPVL^{+}, plasmid carrying *lukSF* genes.

Table 2

Oligonucleotides used in this study.

Name	Sequence
For allelic replacement:	
GBattl	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCGCGAGCTTGGGAGGGGCTCACGACC
GBatt2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAATATGATTAAGGACGCGCTATCAAAC
For EMSAs:	
P2	TACATTTAACAGTTAAGTATTTATTTCCTACAGTTAGGCAATATAATG
P2 Comp	CATTATATTGCCTAACTGTAGGAAATAAATACTTAACTGTTAAATGTA
P2 Mut	TACATTTAATAGTTAAGTATTTATTTCCTACAGTTAGGCAATATAATG
P2 MutComp	CATTATATTGCCTAACTGTAGGAAATAAATACTTAACTATTAAATGTA
For qRT-PCR:	
psmα	
Pl	TATCAAAAGCTTAATCGAACAATTC
P2	CCCCTTCAAATAAGATGTTCATATC
Probe	AAAGACCTCCTTTGTTTGTTATGAAATCTTATTTACCAG
RNAIII	
Pl	GTGATGGAAAATAGTTGATGAGTTGTTT
P2	GAATTTGTTCACTGTGTCGATAATCC
Probe	TGCACAAGATATCATTTCAACAATCAGTGACTTAGTAAAA
clfB	
Pl	TTCCAATGCGCAAGGAACTAG
P2	CAGCATTTACTACAGGTTCAGCAACT
Probe	AGACTACGTACAGCTCTCGTTCTAACACTT
sdrD	
Pl	TCAGATGAGCAAGCTTCACCAA
P2	TTGGTTGAGCATTTACCACTGATT
Probe	ATTCTCTTGCAAATCAGGTTGTAACGCTTCTTG
hla	
Pl	AAAAAACTGCTAGTTATTAGAACGAAAGG
P2	GGCCAGGCTAAACCACTTTTG
Probe	CCTTCTTCGCTATAAACTCTATATTGACCAGCAAT
spa	
Pl	CAGCAAACCATGCAGATGCTA
P2	GCTAATGATAATCCACCAAATACAGTTG
Probe	CATTACCAGAAACTGGTGAAGAAAATCCATTCATTG
For *agr* sequencing:	
2803-3859F	GAATCCGCAGATATTTTGACTGTA
2803-3859R	CCTCACTGTCATTATACGATTTAGT
3653-4609F	CACATCTCTGTGATCTAGTTAT
3653-4609R	TCCACCTACTATCACACTCT
4351-5315F	AAAGAGCCATTTGCCCAATT
4351-5315R	GAACTTGCGCATTTCGTTGT
5108-6146F	TTCACAAATAAACTCGGATG
5108-6146R	CTTACGAATTTCACTGCCTA
5924-6927F	TTTCATTTGCGAAGACGATC
5924-6927R	GATTCACGGAGTAGGAAATT
6701-7710F	CAAATGCACTGTATAGCTGGCTT
6701-7710R	GAATGAAGCAAACACTGCGT

Table 3

Number of genes with significantly differential expression in microarray experiments.

Comparison	number of genes passing significance tests
855[1] vs 776	277
855 vs 855r	296
855r vs 776	10
862 vs 855	386
862 vs 776	65
862 vs 855r	103
776 vs 6390	26

[1] 855, LUG855; 855r, LUG855r; 776, LUG776; 6390, RN6390

Table 4

Gene expression changes in RN6390 versus LUG776.

Gene number (USA300)	Gene product function	RN6390 vs LUG776	
		up-regulated	down-regulated
SAUSA300_0085	Hypothetical protein	2.61	
SAUSA300_0113	Immunoglobulin G binding protein A precursor	3.66	
SAUSA300_0118	Cysteine synthase (EC 2.5.1.47)	2.96	
SAUSA300_0125	Diaminopimelate decarboxylase (EC 4.1.1.20)	2.88	
SAUSA300_0409	Hypothetical exported protein	2.26	
SAUSA300_0508	ClpC ATPase	3.24	
SAUSA300_0509	Arginine kinase (EC 2.7.3.3)	3.16	
SAUSA300_0877	ClpB protein	5.29	
SAUSA300_1377	Hypothetical protein		26.38
SAUSA300_1378	Hypothetical cytosolic protein		22.18
SAUSA300_1379	Hypothetical cytosolic protein		142.65
SAUSA300_1380	Hypothetical protein		159.30
SAUSA300_1383	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)*	6.58	
SAUSA300_1390	Hypothetical protein*	16.36	
SAUSA300_1393	Phage protein*	11.25	
SAUSA300_1397	Major tail protein*	163.08	
SAUSA300_1403	Portal protein*	81.55	
SAUSA300_1407	Transcriptional activator RinA*	45.76	
SAUSA300_1437	Hypothetical protein*	742.69	
SAUSA300_1438	DNA integration/recombination/inversion protein*	116.55	
SAUSA300_2366	Leukocidin S subunit	3.23	
SAUSA300_2367	Hypothetical protein	3.25	
SAUSA300_2493	Hypothetical protein	2.45	

* phage ϕSLT region.