Pathogenomic Analysis of the Common Bovine *Staphylococcus aureus* Clone (ET3): Emergence of a Virulent Subtype with Potential Risk to Public Health

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A common clone (ET3) of *Staphylococcus aureus* is responsible for a large proportion of cases of bovine mastitis and occasionally causes zoonotic infections of humans. In the present study, we report the identification of a virulent clonal subtype (ST151) of ET3, which resulted in increased tissue damage and mortality in a mouse model of mastitis. ST151 has undergone extensive diversification in virulence and regulatory-gene content, including the acquisition of genetic elements encoding toxins not made by other ET3 strains. Furthermore, ST151 had elevated levels of RNAIII and cytolytic toxin–gene expression, consistent with the enhanced virulence observed during experimental infection. Previously, the ST151 clone was shown to be hypersusceptible to the acquisition of vancomycin-resistance genes from Enterococcus spp. Taken together, these data indicate the emergence of a virulent subtype of the common ET3 clone, which could present an enhanced risk to public health.

Staphylococcus aureus is an important human pathogen that is responsible for life-threatening diseases such as toxic-shock syndrome, septicemia, and endocarditis. It is also a major cause of bovine mastitis, which has resulted in large financial losses to the dairy industry worldwide [1, 2]. The recent and ongoing epidemic of community-acquired methicillin-resistant *S. aureus* associated with life-threatening necrotizing pneumonia, severe sepsis, and necrotizing fasciitis is extremely worrisome [3]. In particular, the identification of an increasing number of antibiotic-resistant clones—which are associated with severe community-acquired infections—encoding the Panton-Valentine leukocidin (PVL) [4, 5] highlights the need for surveillance, to identify new clones with enhanced pathogenic potential. Emerging human pathogens can often originate from animal reservoirs, and recent reports have indicated the transfer of *S. aureus* strains between animal and human hosts [6–8].

A large proportion of cases of bovine mastitis are caused by a single clone (ET3), which is identified by multilocus enzyme electrophoresis (MLEE) [9, 10]. Although the ET3 clone is primarily found in bovines, it is occasionally associated with human infections [10], which demonstrates its zoonotic potential. The bovine ET3 lineage contains strains that differ in clinical-disease association [9], suggesting that strain-dependent and/or host factors may influence pathogenic potential. The virulence of *S. aureus* strains relies on the coordinated expression of an array of virulence factors under the control of an integrated regulatory network [11, 12].

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Table 1. Multilocus sequence typing analysis of bovine ET3 *S. aureus* strains.

Strain	Country	Electrophoretic type	Sequence type	Reference
RF103	Ireland	ET3	71	[9]
RF104	Ireland	ET3	ST71	[9]
RF123	Ireland	ET3	ST71	[9]
MSA1369	United States	ET3	ST71	[9]
RF26	Ireland	ND	ST97	[17]
RF28	Ireland	ND	ST97	[17]
RF31a	Ireland	ND	ST97	[17]
RF111	Ireland	ET3	ST97	[9]
RF115	Ireland	ET3	ST97	[9]
RF116	Ireland	ET3	ST97	[9]
RF108	Ireland	ET3	ST151	[9]
RF113	Ireland	ET3	ST151	[9]
RF120	Ireland	ET3	ST151	[9]
RF121	Ireland	ET3	ST151	[9]
RF122	Ireland	ET3	ST151	[9]

NOTE. ND, not determined.

Many virulence factors of *S. aureus* are encoded by mobile genetic elements, which means that new variants with increased virulence can evolve rapidly [13–16].

We investigated the variation in pathogenic potential within the major clone of bovine *S. aureus*. The ET3 lineage has undergone considerable genomic diversification that has resulted in at least 3 clonal subtypes, including one (ST151) with increased virulence in a mouse model of mastitis. The ST151 strains encode toxins that are not made by the other ET3 strains, and they have elevated RNAIII and cytotoxin-gene expression levels that are consistent with their increased pathogenic potential. The virulent ST151 subtype was recently found to be very susceptible to the acquisition of vancomycin-resistance genes [16] and, therefore, could represent an enhanced risk to public health.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. aureus* strains were taken from cases of bovine mastitis in Ireland and the United States (table 1). *S. aureus* strains were grown on tryptic soy agar overnight at 37°C or in brain-heart infusion broth or tryptic soy broth at 37°C, with aeration.

Multilocus sequence typing (MLST) of* S. aureus *strains. Genomic DNA was isolated from stationary-phase *S. aureus* cells by use of the Edge Biosystems DNA extraction kit, as recommended by the manufacturers, with the inclusion of a lysostaphin (100 μg/mL) incubation step for 10 min at 37°C. MLST was performed as described elsewhere [18]. PCR products were sequenced by use of an ABI 3730 sequencer (Applied Biosystems) and the Big Dye Terminator (version 3.1) ready-reaction cycle-sequencing kit (Applied Biosystems). Allele numbers and sequence types (STs) were assigned according to the *S. aureus* MLST database (http://saureus.mlst.net).

Quantitative real-time reverse-transcriptase polymerase chain reaction (PCR). Total bacterial RNA was isolated (for 16 h) from stationary-phase cells grown in brain-heart infusion broth. Cells were harvested by centrifugation, and RNA was isolated by the use of the RNeasy mini kit (QIAGEN), after incubation with lysostaphin (100 μg/mL) (Ambi) for 30 min at 37°C. The mRNA from at least 3 separate total-RNA extractions per strain was reverse-transcribed to cDNA by the use of Superscript III reverse transcriptase (Invitrogen), and quantitative real-time PCR was performed by use of SYBR Green Supermix-UDG (Invitrogen) and the Mx3000P quantitative-PCR instrument (Stratagene). Relative cDNA levels were determined by the use of gene-specific primers for *hla*, *hlb*, *lukf-PV*, *sltD*, and *RNAIII* (table 2). The 16S rRNA gene was used as an internal control. Primers for *hla*, *RNAIII*, and *16S rRNA* have been described elsewhere [19]. Statistical analysis was performed by use of the program Minitab (version 14); 2-way *t* tests and confidence intervals were determined for *hla*, *hlb*, and *RNAIII* expression.

Western immunoblot analysis. For α-toxin detection, *S. aureus* stationary-phase culture supernatants (15 mL) grown in brain-heart infusion broth were harvested by centrifugation and were concentrated by use of Ultrafree-15 centrifugal filters (Mil-

Table 2. Oligonucleotide primers used in the present study.

Designation	Nucleotide sequence (5'→3')
hlbRTF	tgaagatggtggcgtagcgattgt
hlbRTR	tcatgtccagcaccacaacgagaa
pvlRTF	gctcaacatatcacacctgtcagcga
pvlRTR	aagtgggttgggtatagcctgagt
sltDRTF	ggtgtttcaactggtacagctagtc
sltDRTR	tccttcaatgcatccgtaccacca
pvlprobeF	tcagtcgctacatcaattaca
pvlprobeR	gggcatttgatgtgttgg
sltDprobeF	catgcacaaatttcagaac
sltDprobeR	ctctaacacggtaacacattc
agrCprobeF	atggaaacaataaacaacatag
agrCprobeR	ggttctatttaaagataggtatg
tstprobeF	ttccttaggatctatgcg
tstprobeR	gagttagctgatgacg
paiSprobeF	atgcatgtgccaaagtattttc
paiSprobeR	ttatgaatatggatgaatatttttc
sarTprobeF	atgaatgatttgaaaagc
sarTprobeR	ctacatttattcaagtaaccc
sarUprobeF	aatgaacacacagtattttcac
sarUprobeR	tcttttgagtacataatgttc
Cap5IJprobeF	gatagcagagaatttaagaaatc
Cap5IJprobeR	Cttatgtttcactgaacctttc
Cap8IJKprobeF	Caattgactgggcatctaat
Cap8IJKprobeR	Gtgatgattaatatgacttg

Table 3. Analysis of virulence of ET3 subtypes in a mouse model of mastitis.

Strain (Sequence type)	Mice inoculated, no.	Inoculum/ gland, cfu	Status of mice after 48 h, no.		Mean viable-cell counts/gland, cfu
			Dead	Alive	
RF123 (ST71)	5	10^8	0	5	5.2×10^7
RF111 (ST97)	5	10^8	1[a]	4	4.0×10^8
RF116 (ST97)	5	10^8	0	5	1.6×10^8
RF108 (ST151)	5	10^8	5	0	6.8×10^8
RF120 (ST151)	3	10^8	3	0	4.9×10^8

NOTE. A Kruskall-Wallis nonparametric test was used to compare mouse survival after inoculation with a dose of 10^8 cfu/gland. $P = .0002$ for ST151 vs. ST97; $P = .0005$ for ST151 vs. ST71.

[a] One animal inoculated with strain RF111 was in extremis and had to be euthanized and therefore is scored as dead.

lipore). Western immunoblotting was performed as described elsewhere [20]. Semiquantitative spot densitometry was carried out with the ChemiImager 4000i.V4 program, by use of a MultiImager light cabinet (Alpha Innotech).

DNA microarray hybridization and data analysis. DNA microarray hybridization and data analysis were performed as previously described [21], with some modifications. In brief, genomic DNA was labeled by use of an ENzo BioAraray Terminal Labeling kit with biotin-dUTP (Enzo Diagnostics). Labeled DNA was hybridized to custom Affymetrix GeneChips (RML-Chips) containing 3961 probe sets specific for *S. aureus* strains COL, EMRSA16, MSSA476, RF122, TSS, 8325, Mu50, and N315, as described elsewhere [21]. Affymetrix GeneChip software (GCOS version 1.4) was used to perform preliminary analysis of the custom chips at the probe-set level, and *.Cel files representing individual biological replicates were scaled to a mean of 500. A pivot table containing all *P* values and signal intensities for each gene was created. The pivot file was then imported into GeneSpring GX7.3 (Agilent Technologies), where hierarchical clustering by use of a Pearson correlation was performed. The pivot table was also imported into Partek software (Partek), to produce a principal-component-analysis plot. Lack of hybridization for a specific gene indicates that either the open reading frame (ORF) is not present in the strain or that it contains sufficient nucleotide sequence divergence that hybridization does not occur under the conditions of stringency employed.

Southern blot hybridization. Southern hybridizations were performed as described elsewhere [14], with PCR-amplified probes specific for selected ORFs. Genomic DNA from strain RF122 was used for the generation of probes for the *tst*, *agrC*, *paiS*, *lukf-PV*, *sltD*, and *cap8IJK* loci. Probes specific for the *cap5IJ*, *sarT*, and *sarU* loci were generated by use of genomic DNA from ST97 strain RF26.

Mouse model of mastitis. Experiments were performed under a license issued to P.J.H., in strict accordance with the University of Dublin's regulations for animal experimentation. Stationary-phase *S. aureus* cells were harvested by centrifugation in a Microspin 24 centrifuge (Sorvall) for 10 min, were washed twice in phosphate-buffered saline (PBS), and were resuspended in 2 mL of PBS. Viable-cell counts were determined by dilution in PBS and plating on tryptic soy agar, followed by incubation overnight at 37°C. Cultures were stored in 2-mL microfuge tubes at −70°C and were diluted in PBS, to contain the appropriate challenge dose in 0.1 mL. Female white mice were used to establish intramammary infection, as described elsewhere [22]. After 48 h, the number of surviving mice was used to determine the relative virulence of the strains. A nonparametric Kruskall-Wallis test was used to determine the statistical significance of the virulence differences between the ET3 subgroups.

Histological examination of mammary tissues. Dissected mammary glands were cut in half longitudinally. One of the halves was used for histological examination, and the other half was ground to an even-tissue suspension, by use of a sterile mortar and pestle, before calculation of viable-cell counts per gland. For histological examination, half of each gland was fixed in buffered formalin. After fixation, tissues were dehydrated in increasing concentrations of ethanol and were embedded in paraffin wax before being sectioned and stained with hematoxylin and eosin.

RESULTS

Identification of subtypes of the common bovine* S. aureus *clonal lineage ET3. From isolates from Ireland and the United States, a total of 15 *S. aureus* strains were chosen to represent the genetic diversity identified within the ET3 bovine lineage, including 3 strains that had not been analyzed by MLEE but that had represented a common genotype specific to ET3 strains (table 1). MLST analysis of the 15 strains revealed the existence of 3 previously identified ST groups: ST71 ($n = 4$), ST97 ($n = 6$), and ST151 ($n = 5$) (table 1). ST71 and ST97 differed at a single locus only (*arcC*), indicating relatively recent divergence. Surprisingly, ST151 differed from ST71 and ST97 at all 7 loci, indicating that it has not shared a recent ancestor with ST97 or ST71.

ET3 subtypes differ in intramammary virulence. Increasingly infective doses (10^7–10^9 cfu/gland) of strains representing the ET3 subtypes were administered to groups of 5 mice, and symptoms of infection and mortality were monitored over a 48-h period. High doses, typical of those used in previous studies, were necessary to cause infection [22]. For strains ST71 and ST97, a dose of 10^9 cfu/gland was necessary to cause overt illness (data not shown); however, for strain ST151 (RF108), a dose of 10^8 cfu/gland resulted in the death of all 5 mice, and a dose of 10^7 cfu/gland resulted in the overt illness of 2 of 5 mice. The results obtained by use of an inoculum of 10^8 cfu/gland are represented in table 3. To determine whether the increased virulence was representative of the effect in other members of the ST151 group, an additional ST151 strain (RF120) was examined, and it was found that a dose of 10^8 cfu/gland resulted in the death of 3 of 3 mice. Overall, the virulence of the ST151 strains was greater than that of the ST97 ($P = .0002$) or ST71 ($P = .0005$) strains. There was no virulence difference between the ST71 and ST97 strains. The progression of infection over a 48 h period was monitored by observing the clinical symptoms and by histopathologically examining the mammary tissue of pairs of mice at 2, 8, 24, and 48 h or at death, if earlier (figure 1). Mammary-gland tissue infected with *S. aureus* strain RF111 or RF116 of ST97 showed widespread dilation of the stroma, with migrating neutrophils and extensive stromal edema. Mammary-gland tissue infected with *S. aureus* strain RF123 of ST71 had alveoli containing a mixture of milk products, cellular debris, and some neutrophils (figure 1). Sections of mammary-gland tissue infected with *S. aureus* strains RF108 and RF120 of ST151 showed widespread and extensive areas of necrosis that involved the epithelium of alveoli and ducts (figure 1). Overall, the strains of ST97 and ST71 caused localized or moderate damage to secretory tissue, with marked edema of the stroma, whereas the strains of ST151 caused extensive and very severe damage to the glands.

ET3 subtypes contain extensive variation in genome content. To investigate the genetic basis for the virulence difference between ET3 subtypes, a whole-genome DNA microarray, representative of the genome sequence of 1 ST151 strain of bovine origin (RF122; GenBank access number AJ938182) and 7 *S. aureus* strains of human origin [21], was used to compare the genome content of the strains RF111, RF116, RF123, and RF120 employed in the mouse model of mastitis. In gene content, extensive variation between the 3 ET3 subtypes was discovered. The ST151 strains RF122 and RF120 shared a very similar gene complement, with a difference of only 39 ORFs, including a pathogenicity island (SaPIbov) in RF122, which encodes genes for TSST-1, the SEC bovine variant, and SEL [14]. In gene content, strain RF120 varied from ST97 strains RF111 and RF116 by 388 and 409 ORFs, respectively, and from RF123 (ST71) by 426 ORFs. Strains RF123 (ST71) and RF111 (ST97) varied by 250 ORFs, and strains RF123 and RF116 varied by 215 ORFs. The 2 ST97 strains differed by 81 ORFs. Overall, the large gene-content variation between ST151 and the other subtypes was consistent with the considerable genome diversification that occurred after their separation from a common ancestor.

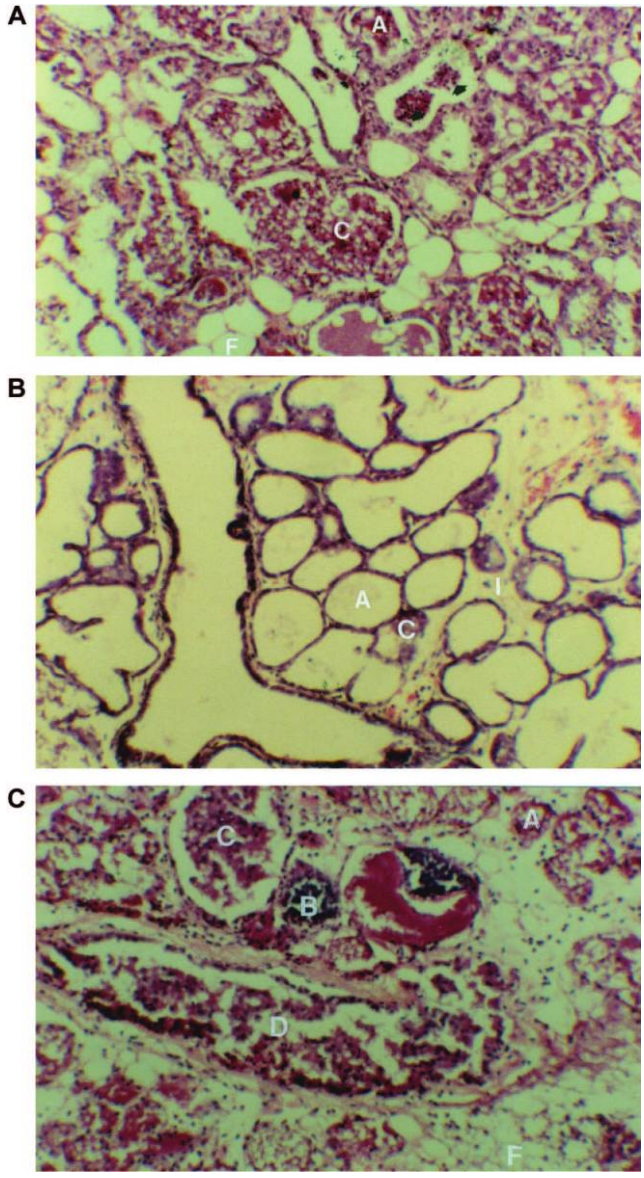

Figure 1. Hemotoxylin-and-eosin–stained sections of mouse mammary gland infected with ST71 *S. aureus* strain RF123 *(A)*, ST97 *S. aureus* strain RF111 *(B)*, and ST151 *S. aureus* strain RF108 *(C)*, 24 h after inoculation with *Staphylococus aureus* strains from bovine intramammary infection. RF123 shows the presence of secretion, cellular debris, and some neutrophils in the lumina of the acini. The small black arrows indicate neutrophils. RF111 shows the presence of dilated acini, some with cellular debris. The stroma is edematous and shows typical signs of inflammation. RF108 shows extensive tissue damage and necrosis involving acini, ducts, and stroma. A, acinus; B, bacterial clumps; C, cellular debris; D, ducts; F, fat cells; I, interlobular stroma. Magnification, ×43.

Importantly, numerous genes that encode factors that may influence pathogenic potential were variably present in strains employed in the virulence studies. The distribution of selected

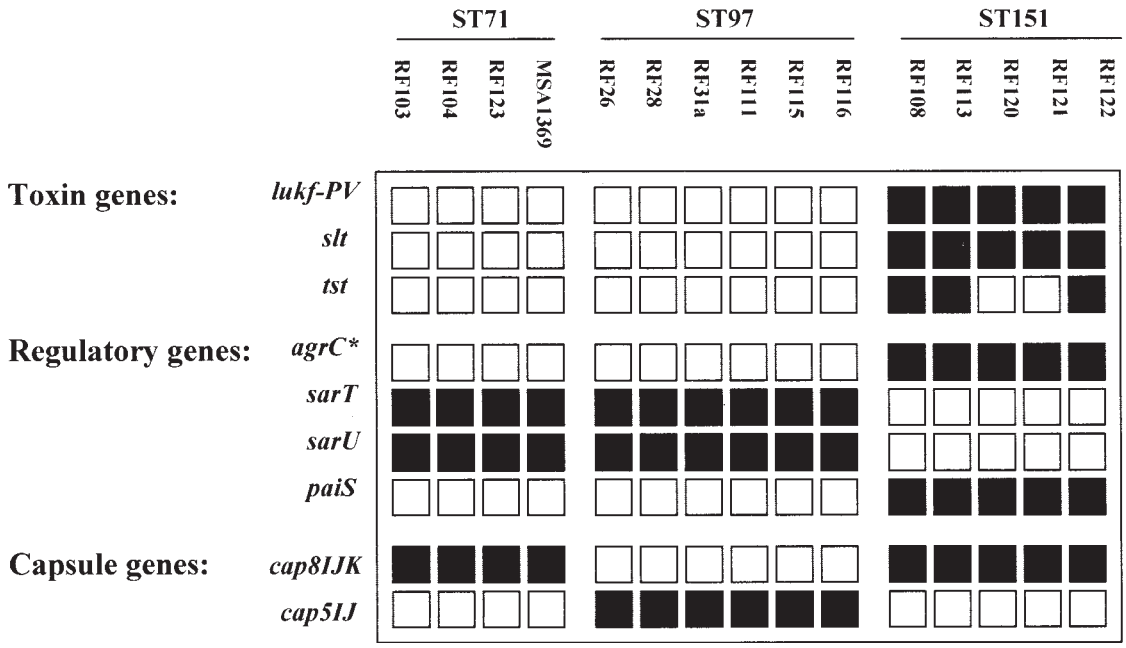

Figure 2. Distribution of selected virulence and regulatory determinants in ET3 strains identified by whole-genome microarray and Southern blot–hybridization analysis. The black and white squares indicate the presence or absence, respectively, of specified genes. The asterisk (*) denotes a probe specific to the 5' unique sequence of the *agrC* gene of strain RF122.

genes likely to directly influence the outcome of infection was examined in all 15 ET3 strains by use of Southern blot hybridization (figure 2). Of note, the virulent ST151 group of strains has acquired genes encoding cytolytic toxins not made by ST71 or ST97. For example, ST151 strains have acquired a phage (ϕPV83) encoding a potent leukocidin (LukF-PV/LukM); ϕPV83 is closely related to ϕPVL, which encodes the Panton-Valentine leukocidin [23–25] associated with severe human infections, and LukF-PV/LukM and PVL share an ~80% amino-acid identity. ST151 strains also encode a novel streptolysin-like toxin (SLT) encoded by a 6-kb genomic island (*sab1372–sab1383*) with ~30% homology to the *sag* operon encoding streptolysin-S (SLS) made by the human pathogen group A streptococcus (GAS) [26].

Variation in the global regulatory-gene complement of ET3 subtypes was identified (figure 2). For example, the results of microarray hybridization indicated that ST151 strains belong to *agr* group II, whereas ST71 and ST97 strains contain *agr* group I alleles. Furthermore, nucleotide-sequence analysis of *agrC* of RF122 revealed a unique 5' sequence not found in the 4 Agr groups identified to date [27]. Southern blot analysis, with a probe specific for the unique sequence of *agrC*, revealed that all ST151 strains contain the novel *agrC* allele (figure 2).

Differences in the complement of genes encoding the Sar family of transcriptional regulators were discovered, with ST151 strains lacking the *sarT* and *sarU* genes found in ST71 and ST97 strains (figure 2). SarT and SarU are reported to influence the interaction between SarA and Agr and to affect virulence-gene expression [28, 29]. In addition, ST151 strains contain a gene (*sab1256c; paiS*) encoding a homolog of PaiB of Bacillus sp., a negative regulator of protease synthesis and sporulation [30]. Interestingly, the *paiS* gene in strain RF122 is located within a 35-kb region previously demonstrated to influence *agr* expression in human strains [31]. The *paiS* locus is not present in ST71 or ST97 strains.

Although ST97 and ST71 subtypes contained many virulence genes in common, some differences were identified that could influence their pathogenic potential. Notably, ST71 and ST151 strains contain genes that would encode for a capsule serotype 8, whereas ST97 strains contain genes that would encode for a capsule serotype 5 (figure 2).

ST151 strains have elevated levels of cytolytic toxin–gene expression. Considering both the intramammary-virulence variation between representatives of the ET3 subtypes and the marked increase in mammary-tissue necrosis caused by ST151 strains, compared with ST71 or ST97 strains, we speculated that these differences may reflect cytolytic toxin–gene expression differences between ET3 subtypes.

Although the levels of α-toxin gene (*hla*) expression within ET3 subtypes varied somewhat, taken together, ST151 strains had significantly higher levels of *hla* expression than did ST97 ($P = .001$) or ST71 ($P = .003$) strains (figure 3). Western immunoblot analysis also indicated elevated expression levels of Hla in ST151 strains (figure 3). Semiquantitative spot densitometry indicated that the relative quantity of α-toxin was elevated in ST151 strains (5.854 ± 0.997), compared with that in ST97

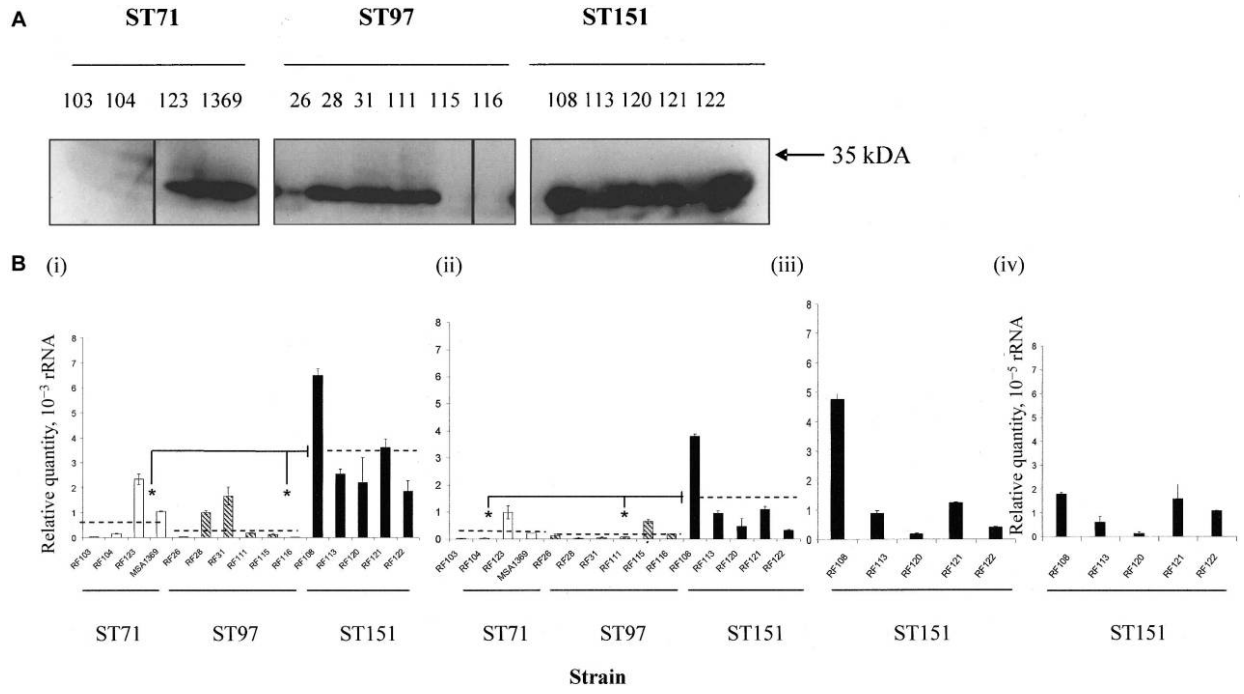

Figure 3. Cytolytic toxin expression in ET3 strains. *A,* Western immunoblot analysis of Hla expression in all strains. *B,* Quantitative real-time polymerase chain reaction gene-expression analysis of *hla (i), hlb (ii), lukf-pv (iii),* and *sltD (iv).* Transcription-level results are expressed as the relative quantity of target cDNA, compared with that of the internal control (i.e., 16S rRNA). The broken line represents the mean value, for each group/sequence type (ST), of *hla* expression (ST71, 0.895; ST97, 0.506; ST151, 3.34) and of *hlb* expression (ST71, 0.316; ST97, 0.187; ST151, 1.32). Statistically significant gene-expression differences between ST groups are indicated by an asterisk (*). The white, hatched, and black bars represent ST71, ST97, and ST151 strains, respectively.

(2.65 ± 1.21; $P = .001$) and ST71 (2.54 ± 2.01; $P = .039$) strains. Furthermore, β-toxin gene (*hlb*) expression was greater in ST151 strains than in ST71 ($P = .048$) and ST97 ($P = .027$) strains (figure 3). In *hla* and *hlb* expression levels, there was no significant difference between ST71 and ST97 subtypes.

Microarray analysis had revealed that genes specific for a potent leukocidin (*lukf-PV/lukM*) and streptolysin S-like toxin (*slt*) were present in ST151 strains but absent from ST97 and ST71 strains. Quantitative real-time PCR analysis of the *lukf-PV* gene revealed that it was expressed in all ST151 strains, at levels comparable to those in *hla* and *hlb* (figure 3). Low-level expression of *slt* was detected in all ST151 strains examined (figure 3). These data indicate that ST151 strains have significantly higher levels of cytolytic toxin gene expression than do strains of the other ET3 subtypes.

ST151 strains have elevated levels of RNAIII expression. Quantitative real-time PCR analysis revealed that ST151 strains produce much higher levels of RNAIII (>30-fold difference; figure 4) than do strains of the other ET3 subtypes ($P = .000$; 100% confidence). In addition, ST97 strains had significantly higher levels of RNAIII than did ST71 strains ($P = .007$). ST71 strains RF103 and RF104 failed to produce any detectable RNAIII under the conditions tested, suggesting that they may represent *agr*-negative mutants. These data demonstrate that, in comparison with ST97 and ST71 strains, ST151 strains have very elevated levels of RNAIII expression, consistent with the high levels of *hla*- and *hlb*-gene expression.

DISCUSSION

Within natural populations of *S. aureus*, variation in genome content is very large. However, our understanding of how this variation contributes to differences in pathogenic potential is limited. The recent emergence of several new virulent clones associated with severe community-acquired human infections has highlighted the importance of investigations into the evolution of strains with increased threat to public health.

In the present study, we report that the common bovine clone of *S. aureus* consists of at least 3 subtypes identified by MLST, indicating that clonal variation is greater than previously thought. We found that the ST151 subtype resulted in very elevated levels of tissue necrosis and mortality in a mouse model of mastitis indicating increased pathogenic potential compared with strains of the other ET3 subtypes (figure 1 and table 3). Comparative genomic hybridizations revealed that ST151 strains differed in the content of >400 ORFs, compared with ST97 and ST71 strains, demonstrating that extensive genome diversification, including many episodes of horizontal gene transfer, has occurred within the ET3 lineage. Very recently, bovine ST151 strains were shown to have acquired mutations in

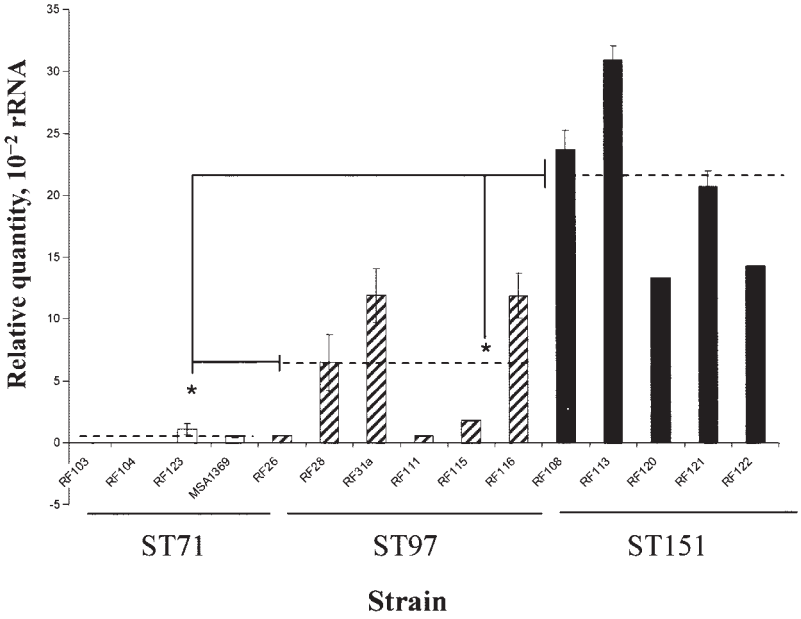

Figure 4. Quantitative real-time polymerase chain reaction analysis of RNAIII expression levels. Results are expressed as the relative quantity of target cDNA, compared with that of the internal control, 16S rRNA. The broken line represents the mean value, for each group/sequence type (ST), of RNAIII expression (ST71, 0.427; ST97, 5.55; ST151, 20.63). Statistically significant differences in RNAIII levels between ST groups are indicated by an asterisk. White, hatched, and black bars represent ST71, ST97, and ST151 strains respectively.

restriction-modification genes, leading to hypersusceptibility to acquisition of foreign genes [16]. This may help to explain, in part, the variation in genome content of the ST151 clone, compared with other ET3 strains. However, the data suggest that ST151 has not shared a very recent ancestor with the other subtypes.

The severe necrosis and mammary tissue damage induced by ST151 strains suggests either high levels of bacterial cytolytic toxin activity or indirect immunopathology. Comparative genomic analysis of ET3 subtypes identified several genes that could influence cytolytic potential. For example, all of the ST151 strains examined were found to encode LukF-PV/LukM, which previously had been reported to be the most cytotoxic of the leukocidins made by bovine strains [32] and which had been shown to induce severe inflammation when administered to the bovine udder [33]. It is possible that LukF-PV/LukM contributes to the increased virulence of ST151 strains, a hypothesis that we are currently investigating. ST151 strains were found to encode a protein (i.e., SLT) with homology to the SLS toxin made by GAS. In GAS, SLS is a potent hemolytic toxin that contributes to the symptoms of severe necrotizing fasciitis in a murine model [34], but the role that SLT plays in the pathogenesis of *S. aureus* infections remains to be investigated. In addition to cytolytic toxin genes, most ST151 strains examined contained the pathogenicity island SaPIbov encoding the superantigen TSST-1, which is responsible for the symptoms of human menstrual toxic-shock syndrome [35, 36]. Moreover, ST151 strains encode up to 9 enterotoxins that are capable of causing human staphylococcus-related food poisoning (GenBank access number AJ938182).

A previous study [22] of a single-bovine *S. aureus* strain (M60) reported that both α-toxin expression and β-toxin expression contributed to the severity of disease in a mouse model of mastitis. We discovered that the virulent ST151 strains had significantly higher expression levels of both *hla* and *hlb* than did subtypes ST97 and ST71—and that *lukf-pv*, found only in ST151 strains, was expressed at a similarly high level (figure 3). The high cytolytic toxin–gene expression in ST151 strains in vitro correlates with the severe necrosis caused during experimental infection with ST151. We suggest that the increased virulence of the ST151 clone may be due to increased cytoloytic toxin production and not due to an increased growth yield. This is indicated by the lack of difference between viable-cell counts in the glands after infection with different subtypes (table 3). Expression of *hla*, *hlb*, and *pvl* has previously been shown to be under the control of *agr* [37, 38]. Consistent with the central role that ST151 strains play in the control of cytolytic toxin expression, we found that ST151 strains have very elevated levels of RNAIII, compared with the other ET3 subtypes. In general, strains of subtypes ST97 and ST71 produced lower levels of RNAIII, and 2 strains of ST71 produced no detectable RNAIII under the conditions examined, which indicates that they may represent naturally occurring *agr* mutants. Human clinical *S. aureus* strains with *agr* mutations have been well documented [39, 40], and the diminished virulence of *agr*-null clinical strains could translate into prolonged survival in the host, a characteristic of chronic bovine mastitis.

The genetic basis for elevated RNAIII levels in ST151 strains is unclear, but it could be a reflection of the differences between ET3 subtypes in regulatory gene content. In particular, in ST151 strains, the presence of a novel *agrC* allele, the absence of *sarU* and *sarT* genes, and the presence of a gene encoding a putative novel transcriptional regulator (i.e., PaiS), within a gene cluster previously shown to influence *agr* expression [31], may contribute to increased RNAIII levels.

The recent observation that the ST151 clone is hypersusceptible to the acquisition of vancomycin-resistance genes from Enterococcus spp. [16] is worrisome, and colocalization of vancomycin-resistant Enterococci and ST151 strains in the agricultural setting suggests that gene-transfer opportunities could arise. ST151 could hypothetically act as an intermediate host for *vanA* genes before they are transfered to human-adapted clones. However, the previously reported zoonotic transfer of ET3 strains [10] suggests that episodes of human infection caused by virulent ST151 strains cannot be ruled out.

Taken together, these data highlight the variation, in both genome content and pathogenic potential, that exists within natural populations of *S. aureus*, and they provide new insights into the evolution of virulent clones.

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