RESEARCH ARTICLE

Trading Capsule for Increased Cytotoxin Production: Contribution to Virulence of a Newly Emerged Clade of *emm89 Streptococcus pyogenes*

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ABSTRACT **Strains of *emm89 Streptococcus pyogenes* have become one of the major causes of invasive infections worldwide in the last 10 years. We recently sequenced the genome of 1,125 *emm89* strains and identified three major phylogenetic groups, designated clade 1, clade 2, and the epidemic clade 3. Epidemic clade 3 strains, which now cause the great majority of infections, have two distinct genetic features compared to clade 1 and clade 2 strains. First, all clade 3 organisms have a variant 3 *nga* promoter region pattern, which is associated with increased production of secreted cytolytic toxins SPN (*S. pyogenes* NADase) and SLO (streptolysin O). Second, all clade 3 strains lack the *hasABC* locus mediating hyaluronic acid capsule synthesis, whereas this locus is intact in clade 1 and clade 2 strains. We constructed isogenic mutant strains that produce different levels of SPN and SLO toxins and capsule (none, low, or high). Here we report that *emm89* strains with elevated toxin production are significantly more virulent than low-toxin producers. Importantly, we also show that capsule production is dispensable for virulence in strains that already produce high levels of SPN and SLO. Our results provide new understanding about the molecular mechanisms contributing to the rapid emergence and molecular pathogenesis of epidemic clade 3 *emm89 S. pyogenes*.**

IMPORTANCE ***S. pyogenes* (group A streptococcus [GAS]) causes pharyngitis ("strep throat"), necrotizing fasciitis, and other human infections. Serious infections caused by *emm89 S. pyogenes* strains have recently increased in frequency in many countries. Based on whole-genome sequence analysis of 1,125 strains recovered from patients on two continents, we discovered that a new *emm89* clone, termed clade 3, has two distinct genetic features compared to its predecessors: (i) absence of the genes encoding antiphagocytic hyaluronic acid capsule virulence factor and (ii) increased production of the secreted cytolytic toxins SPN and SLO. *emm89 S. pyogenes* strains with the clade 3 phenotype (absence of capsule and high expression of SPN and SLO) are highly virulent in mice. These findings provide new understanding of how new virulent clones emerge and cause severe infections worldwide. This newfound knowledge of *S. pyogenes* virulence can be used to help understand future epidemics and conduct new translational research.**

Received 10 August 2015 **Accepted** 10 September 2015 **Published** 6 October 2015

Citation Zhu L, Olsen RJ, Nasser W, de la Riva Morales I, Musser JM. 2015. Trading capsule for increased cytotoxin production: contribution to virulence of a newly emerged clade of *emm89 Streptococcus pyogenes*. mBio 6(5):e01378-15. doi:10.1128/mBio.01378-15.

Editor Eric J. Rubin, Harvard School of Public Health

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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Understanding the evolutionary genetic changes underpinning emergence of new pathogenic strains is of practical importance because it provides useful information for development of effective strategies to control infectious diseases in humans, domesticated animals, and crops. The combination of population genomics, epidemiology, evolutionary biology, molecular genetics, and microbial pathogenesis makes it possible to precisely delineate these genetic changes at the nucleotide level.

Streptococcus pyogenes (group A streptococcus [GAS]) is a human pathogen that causes many diseases ranging in severity from minor skin and throat infections to fatal invasive episodes (1). Based on variation in the *emm* gene encoding the antiphagocytic M protein, *S. pyogenes* can be classified into ~200 *emm* types (2). For reasons that are not well understood, in the last 10 years *emm89* strains have rapidly become one of the major *emm* types causing severe invasive *S. pyogenes* infections in several geographic regions (3–18). We recently sequenced the genomes of 1,125 *emm89* strains isolated on two continents from 1995 to 2013 and discovered the existence of three major phylogenetic groups of *emm89* strains, designated clade 1, clade 2, and epidemic clade 3 (19). We discovered that clade 3 strains emerged, disseminated extensively, and rapidly displaced clade 1 and clade 2 strains in an epidemic wave of *emm89* disease.

Population genomic analysis revealed two major features of epidemic clade 3 *emm89* strains. First, all clade 3 *emm89* strains have the variant 3 *nga* promoter region sequence, which is identical to the *nga* promoter region present in pandemic *emm1* strains. The variant 3 promoter region is associated with elevated production of SPN (*S. pyogenes* NADase) and SLO (streptolysin O), two potent secreted cytolytic toxins that contribute to virulence (19). Second, all clade 3 *emm89* strains studied lack the *hasABC* gene region containing hyaluronic acid (HA) capsule synthesis genes.

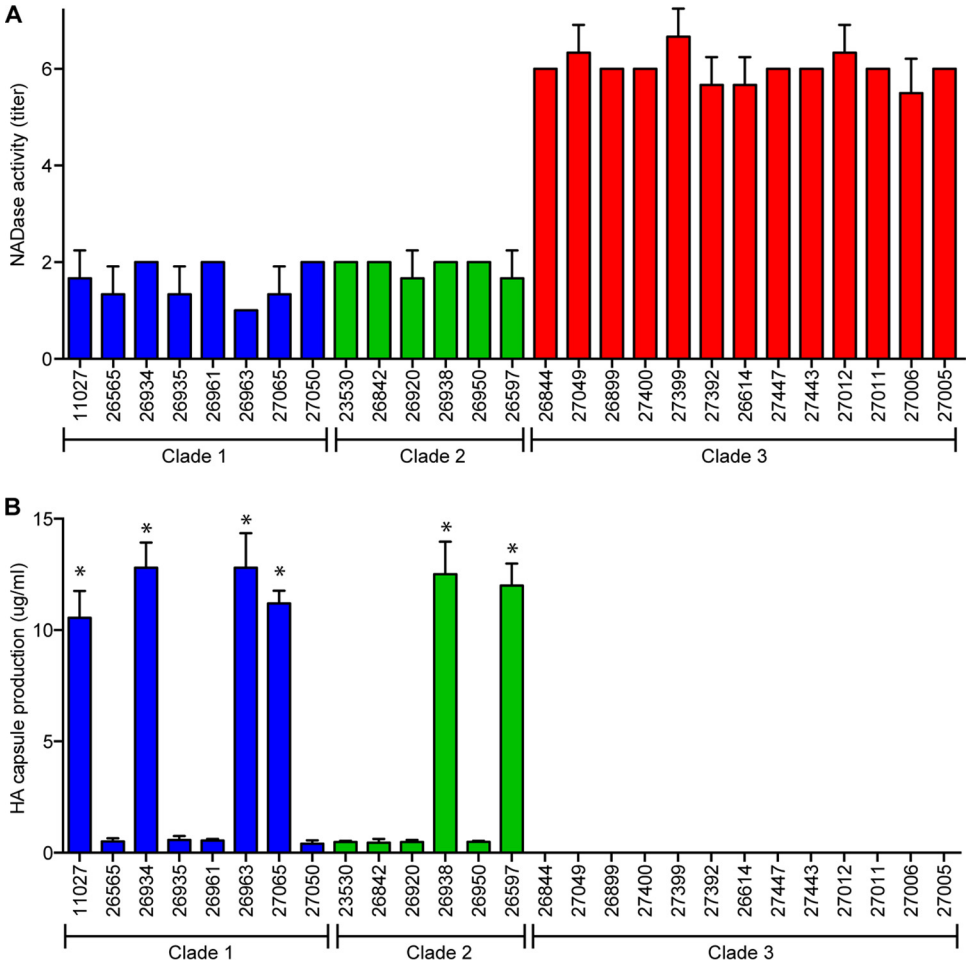

FIG 1 *In vitro* characteristics of 27 *emm89* isolates. (A) NADase activities of *emm89* isolates in the culture supernatant. (B) Production of hyaluronic acid capsule by *emm89* isolates. Asterisks indicate that strains 11027, 26934, 26963, 27065, 26938, and 26957, which produce higher levels of HA capsule, also have a 38-bp deletion in the *hasA* upstream region (Fig. 3). NADase assays and capsule assays were performed in triplicate on three separate occasions. Replicate data are expressed as the mean ± SD.

We hypothesized that the genetic changes in the *nga* promoter region and capsule synthesis genes have altered the virulence phenotype of *emm89* strains. To test this hypothesis, we constructed a panel of isogenic mutant strains that vary in level of production of secreted SPN and SLO cytolytic toxins and HA capsule. These mutant strains recapitulate the level of SPN and SLO toxin and capsule production made by genomically representative members of each of the three *emm89* clades. The isogenic mutant strains were tested for virulence in a mouse model of necrotizing fasciitis.

RESULTS

Variant 3 *nga* promoter sequence is associated with increased production of secreted SPN and SLO toxins. Based on our analysis of 1,125 genome sequences, *emm89* strains cluster into three major phylogenetic groups referred to as clades. Strains in each clade have a distinct *nga* promoter sequence (2). That is, clade 1 strains all have the variant 1 pattern, clade 2 strains have the variant 2 pattern, and epidemic clade 3 strains have the variant 3 pattern. Three reference strains, MGAS11027 (clade 1), MGAS23530 (clade 2), and MGAS26844 (clade 3), whose genomes were sequenced to closure at high fold coverage, were chosen for subsequent analyses. The *nga*, *slo*, and intervening *ifs* genes are organized as an operon and expressed as a single transcript (20–22) (see Fig. 2A). *nga* and *slo* encode SPN and SLO, respectively (22). In principle, variation in the promoter region sequence could alter the level of *nga* and *slo* transcripts and thereby SPN and SLO production. To test this idea, we first compared the secreted SPN (NADase) activities of 27 *emm89* isolates belonging to clade 1, clade 2, and clade 3 (All 27 strains are wild type in transcriptional regulators known to control SPN, SLO production, and capsule production, i.e., *covR/S* and *rocA* [23–26]). Compared to clade 1 and clade 2 isolates, clade 3 isolates produced increased NADase activity (Fig. 1). We next examined the transcript levels of *nga* and *slo* and SPN and SLO production in the three reference strains representing the three clades. Consistent with the hypothesis, strain MGAS26844 (variant 3 pattern) had a significantly higher *nga* and *slo* transcript levels and production of SPN and SLO compared to strain MGAS23530 (variant 2 pattern) and strain MGAS11027 (variant 1 pattern) (Fig. 2B).

To further test the hypothesis that variation in the promoter region alters gene transcript levels, we generated isogenic mutant strains by replacing the *nga* promoter of the variant-3 parental

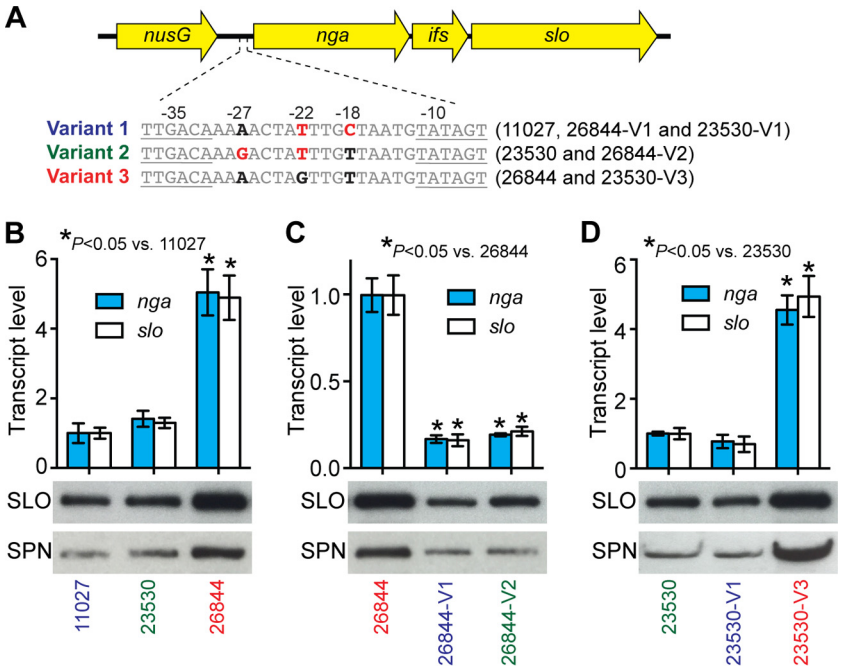

FIG 2 *In vitro* characteristics of *emm89* reference strains and isogenic mutant strains with different *nga* promoter region sequences. (A) Schematic showing the *nga* promoter region sequences of the strains assayed. (B to D) qRT-PCR analysis of *nga* and *slo* transcript levels and Western immunoblot analysis of secreted SLO and SPN in the culture supernatant. Transcript analysis was done in triplicate on three separate occasions.

strain 26844. The results (Fig. 2C) show that converting the *nga* promoter region of strain MGAS26844 to either variant 1 sequence or variant 2 sequence significantly reduced both *nga* and *slo* transcript levels and production of SPN and SLO cytotoxins. We next generated isogenic mutant strains by converting the *nga* promoter region of parental strain MGAS23530 (variant 2) to variant 1 sequence and variant 3 sequence. Consistent with the hypothesis, the isogenic mutant strain with the variant 3 sequence expressed significantly more *nga* and *slo* transcript and SPN and SLO proteins (Fig. 2D). In contrast, conversion to the variant 1 *nga* promoter sequence did not significantly alter the level of transcript or SLO and SPN production. Collectively, these results show that sequence variation in the *nga* promoter region alters transcription of *nga* and *slo* and production of SPN and SLO. Epidemic clade 3 *emm89* strains with the variant 3 *nga* promoter pattern have the highest *nga-slo* transcript level and produce the largest amount of secreted SPN and SLO.

A 38-bp deletion in the upstream region of *hasA* is associated with increased production of HA capsule. Epidemic clade 3 strains lack the *hasABC* genes for capsule synthesis, whereas clade 1 and clade 2 strains have these genes. However, the abundance of capsule production varies from strain to strain among clade 1 and clade 2 strains (Fig. 1B). For example, representative strain MGAS23530 (clade 2) produces a moderate amount of capsule (~0.3 μg/ml), and HA capsule production by representative strain MGAS11027 (clade 1) is ~40 times higher (~12 μg/ml) (Fig. 3C). Analysis of the genome sequence data found two distinct *hasA* promoter region patterns present in clade 1 and clade 2 strains. We designate these two variants as pattern A and pattern B. Patterns A and B are present in both clade 1 and clade 2 strains. Compared to pattern A, pattern B has a 38-bp deletion that may remove a transcriptional terminator (Fig. 3A). Consistent with this idea, we found that regardless of clade assignment, strains with the 38-bp deletion produce a higher level of capsule (Fig. 1B and 3A). Falaleeva et al. showed that deletion of this transcriptional terminator region resulted in increased capsule production (27). To directly study if the 38-bp deletion results in increased production of capsule, we generated an isogenic mutant derivative of parental reference strain MGAS23530 (23530-CapH) by replacing the *hasA* promoter with the promoter sequence present in pattern B strains. Deletion of the 38-bp region resulted in an ~15-fold increase in *hasA* expression and ~40-fold increase in HA capsule production, levels similar to those obtained for the naturally occurring pattern B strain MGAS11027 (Fig. 3B and C). These results demonstrate that removal of the 38-bp region upstream of the *hasA* promoter region results in increased *hasA* transcript and HA capsule synthesis.

An isogenic acapsular *emm89* strain with the variant 3 *nga* promoter region is highly virulent in a mouse model of necrotizing fasciitis. Two key genetic features of the epidemic *emm89* clone are absence of HA capsule synthesis genes and presence of the variant 3 *nga* promoter region that results in increased production of the secreted SPN and SLO toxin virulence factors. We hypothesized that the absence of capsule together with increased SPN/SLO production (phenotypes that occur naturally in clade 3 strains) significantly increases strain virulence of *emm89 S. pyogenes*. We have previously shown that reduction of SPN/SLO production in the genetically representative clade 3 strain MGAS26844 significantly reduced its virulence in a mouse infection model (19). These data confirmed our hypothesis that increased toxin production is associated with increased virulence. However, strain MGAS26844 is acapsular, as are the isogenic mutant derivatives, which means that the effect of capsule loss on virulence in these strains was not determined.

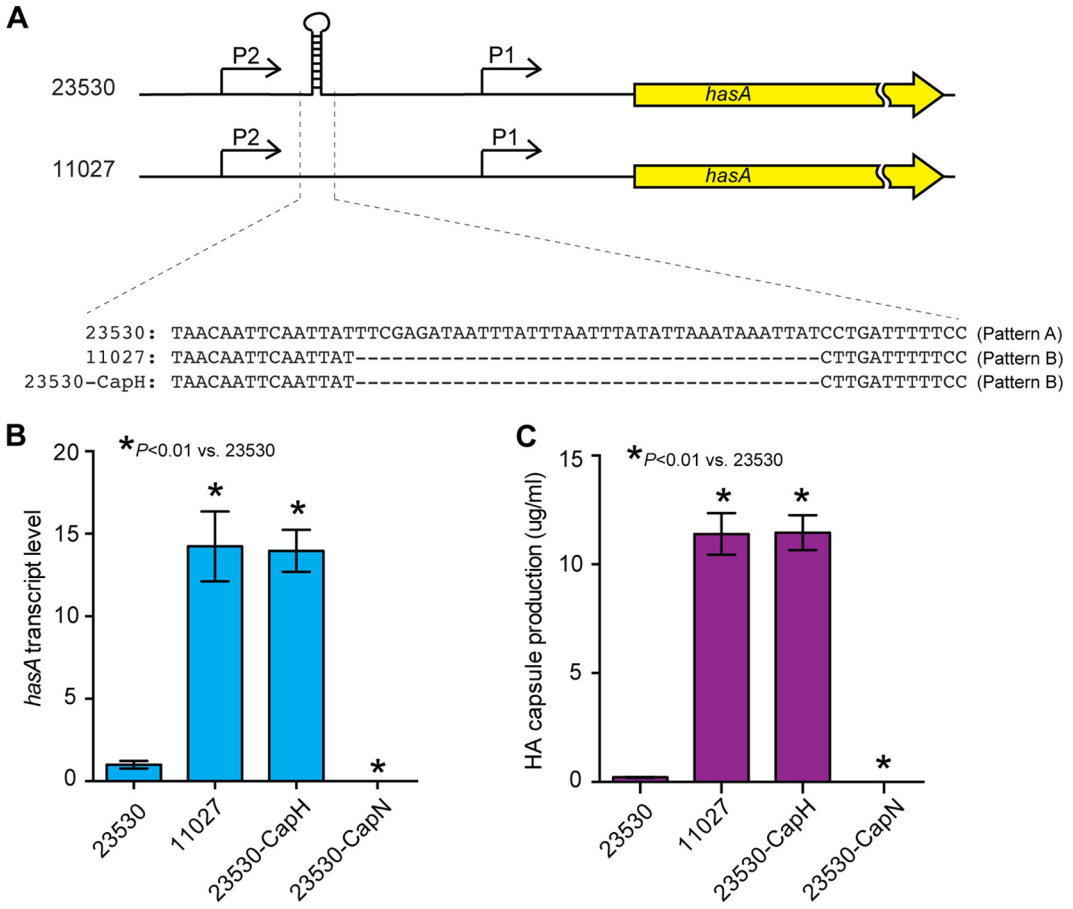

FIG 3 *In vitro* characteristics of *emm89* reference strains and isogenic mutant strains with different *hasA* upstream promoter region sequences. (A) Schematic showing the *hasA* upstream promoter region sequences of the assayed strains. The relative position of the "region of deletion" relative to P1 and P2 was graphed according to Falaleeva et al. (27). (B and C) *hasA* transcript level and hyaluronic acid capsule production of assayed strains. Transcript analysis and capsule assays were done in triplicate on three separate occasions.

We next generated a panel of isogenic mutant strains of clade 2 strain MGAS23530 (weakly encapsulated, low SPN/SLO production) by altering the *nga* promoter region sequence and deleting the *hasABC* locus (Fig. 4; Table 1). The virulence of these strains was compared using a mouse model of necrotizing fasciitis (Fig. 5). Consistent with our hypothesis, the acapsular variant 3 strain (23530-V3-CapN) caused significantly greater near mortality than the acapsular or weakly encapsulated strains that express comparatively smaller amounts of SPN and SLO toxin (Fig. 5A, 23530-V1-CapN, 23530-V1, 23530-V2-CapN, and 23530-V2). Similarly, gross and microscopic examination of infected limbs found that the acapsular variant 3 isogenic mutant strain (23530-V3-CapN) caused significantly larger lesions with more tissue destruction (Fig. 5E to J). Interestingly, loss of HA capsule did not result in decreased virulence (Fig. 5). To the contrary, loss of capsule caused a substantial increase of virulence in an *emm89* strain with the variant 3 *nga* promoter (Fig. 5D and 6). This result suggests production of a low level of capsule is not sufficient to protect *emm89* organisms from host innate immunity. Taken together, these data strongly support our hypothesis that the epidemic clade 3 genotype (absence of capsule combined with strong *nga* promoter expression) confers an increased virulence phenotype to the epidemic *emm89* clade 3 organisms.

HA capsule production is dispensable for full virulence in *emm89* strains that produce high levels of SPN and SLO toxins. The data show that transition from low HA capsule production to complete absence of capsule did not result in decreased virulence (Fig. 5). To further study if HA capsule production plays a role in the virulence in *emm89* strains, we compared the virulence levels of heavily encapsulated *emm89* strains (generated by deleting a 38-bp region upstream of *hasA* [Fig. 4; Table 1]) and acapsular organisms. Our virulence study results show that for *emm89* strains that produce low levels of toxins (strains with a variant 1 or variant 2 *nga* promoter region), high capsule production rendered the organisms more virulent (Fig. 6A and B). However, for *emm89* strains that produce high levels of SLO and SPN cytolytic toxins (strains with a variant 3 *nga* promoter region), the acapsular isogenic mutant strain is equally virulent to the heavily encapsulated strain (Fig. 6C and D). In other words, production of a high level of HA capsule did not increase virulence in strains producing large amounts of SLO and SPN. Again, intriguingly, the poorly encapsulated strain was less virulent (23530-V3) (Fig. 6C and D). Taken together, capsule production (regardless of whether high or low) is dispensable for virulence in *emm89* strains that produce high levels of the secreted cytolytic toxins SLO and SPN.

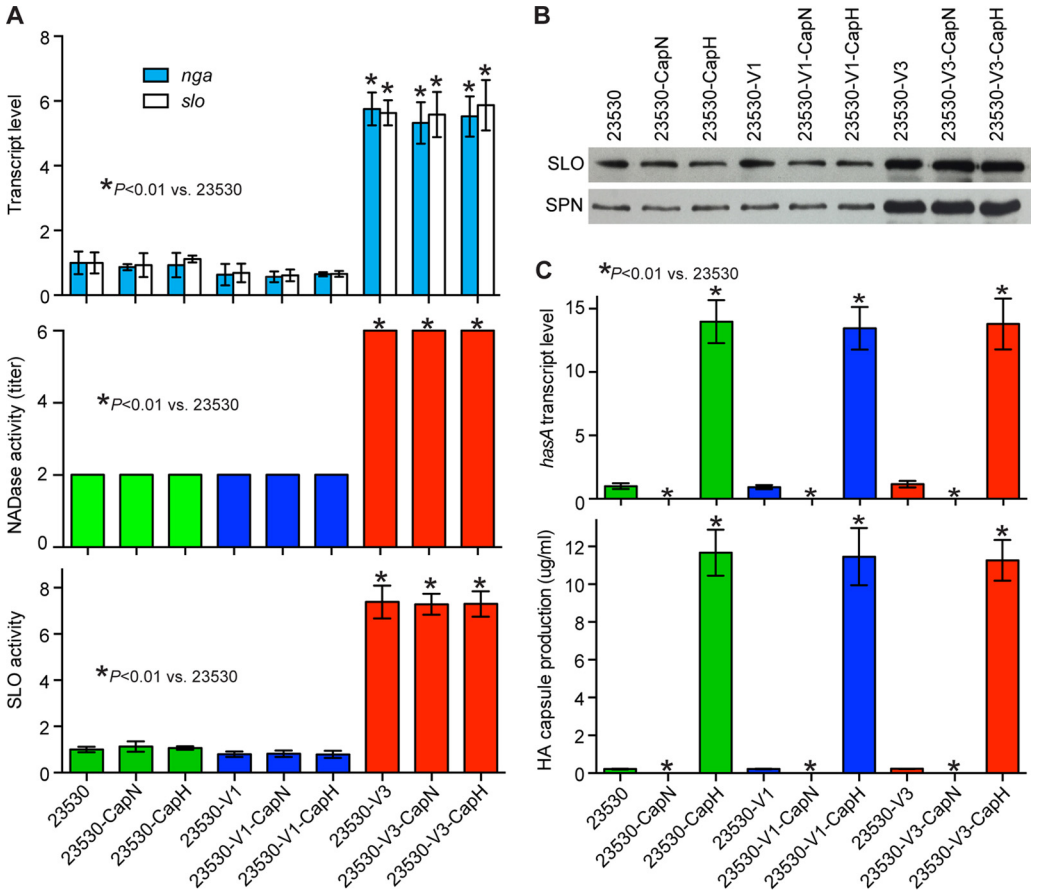

FIG 4 *In vitro* characteristics of nine *emm89* strains with three *nga* promoter patterns that produce different levels of hyaluronic acid capsule. (A) Relative transcript levels of *nga* and *slo* and enzymatic activities of SPN (NADase) and SLO in the culture supernatant. (B) Western immunoblot analysis of SLO and SPN in the culture supernatant. (C) Transcript level of *hasA* and hyaluronic acid capsule production of strains analyzed.

DISCUSSION

One distinctive feature of the epidemic *emm89* population is the lack of the *hasABC* locus responsible for production of HA capsule. Capsule has been studied for more than 100 years and has long been considered an important virulence factor for *S. pyogenes* strains, in part because of its role in resisting phagocytosis and killing by human polymorphonuclear leukocytes (PMNs) (28–30). Many lines of evidence, including virulence studies using isogenic mutant strains, have strongly supported the concept that HA capsule production is a crucial factor in the complex interaction between pathogen and host (28–30). These extensive studies led to the generally accepted idea that HA capsule is required for invasive infections and colonization. However, Flores et al. (31) recently reported that human disease isolates of *emm4* and *emm22* *S. pyogenes* lack the *hasABC* genes, a discovery effectively ruling out the idea that production of HA capsule is obligatory for virulence. In addition, the investigators showed that *emm4* and *emm22* strains proliferated *ex vivo* in human blood. Our work here adds *emm89* strains to the list of *S. pyogenes* organisms that do not require HA capsule to cause human infections or, unexpectedly, epidemic disease.

A second distinctive feature of epidemic clade 3 *emm89* strains is that they all have the variant 3 *nga* promoter region pattern. Compared to the variant 1 and variant 2 *nga* promoter region sequences present in clade 1 and clade 2 strains, respectively, the variant 3 promoter region sequence is associated with increased expression of *nga* and *slo* and increased production of SPN and SLO cytotoxins. SPN and SLO have been reported to protect *S. pyogenes* from phagocytic killing and increase intracellular survival (32, 33). In the absence of HA capsule, a high level of SPN

TABLE 1 Characteristics of the strains used in this study

Strain (MGAS no.)[a]	*nga* promoter	Production of:		Clade
		SPN or SLO	Capsule	
11027	Variant 1	Low	High	1
26844	Variant 3	High	None	3
26844-V1	Variant 1	Low	None	
26844-V2	Variant 2	Low	None	
23530	Variant 2	Low	Low	2
23530-V1	Variant 1	Low	Low	
23530-V3	Variant 3	High	Low	
23530-CapH	Variant 2	Low	High	
23530-V1-CapH	Variant 1	Low	High	
23530-V3-CapH	Variant 3	High	High	
23530-CapN	Variant 2	Low	None	
23530-V1-CapN	Variant 1	Low	None	
23530-V3-CapN	Variant 3	High	None	

[a] MGAS no., Musser group A *Streptococcus* number.

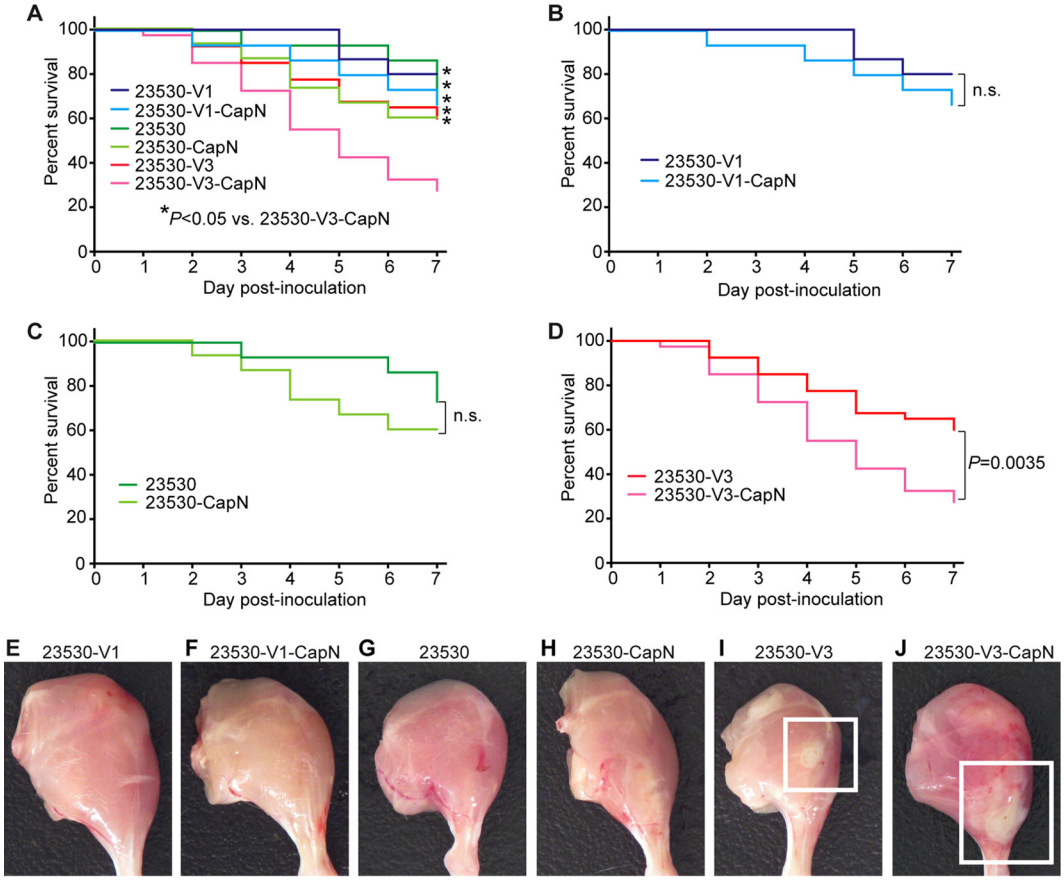

FIG 5 Acapsular *emm89* strain with a variant 3 *nga* promoter is highly virulent in a mouse model of necrotizing fasciitis. (A) Kaplan-Meier near-survival curves of mice infected with strain MGAS23530 (low toxin, low capsule), 23530-V1 (low toxin, low capsule), 23530-V3 (high toxin, low capsule), 23530-CapN (low toxin, no capsule), 23530-V1-CapN (low toxin, no capsule), and 23530-V3-CapN (high toxin, no capsule). (B to D) Subsections of panel A that show comparisons between capsule-positive strains and capsule-negative strains. (E to J) Gross pathology analysis of strain virulence in a mouse model of necrotizing fasciitis. The boxed region in panels I and J marks the comparatively larger lesion caused by variant 3 strains expressing large amounts of SPN and SLO toxins.

and SLO production could provide a critical defense against host immunity. In this regard, we note that for *emm89* strains that are high producers of SPN and SLO, HA capsule production is dispensable for virulence in a mouse infection model. The acapsular strain 23530-V3-CapN is as virulent as heavily encapsulated strain 23530-V3-CapH, as assessed by ability to cause near mortality and tissue destruction. In contrast, for strains that produce relatively smaller amounts of SPN and SLO, a high level of HA capsule production is important for virulence.

Population genomic analysis showed that epidemic clade 3 *emm89* evolved from a clade 2 strain (19). The precise nature and order of the molecular events that generated epidemic clade 3 is not known. In particular, we do not know if loss of the *hasABC* capsule synthesis genes preceded acquisition of the variant 3 *nga-slo* region or vice versa. Our inability to differentiate between these two possibilities is due in part to a lack of strains that have one of these two genotypic characteristics in the absence of the other. That is, our analysis did not identify strains with the *hasABC* capsule genes together with the variant 3 *nga-slo* region or which have the variant 2 *nga-slo* region but lack the HA capsule synthesis genes. The mouse infection data suggest that increased production of SPN and SLO is a more important contributor to virulence than HA capsule production. This leads us to speculate that the key event immediately preceding the successful emergence of epidemic clade 3 organisms was gain of the variant 3 *nga-slo* region by horizontal gene transfer and homologous recombination in an *emm89* strain that lacked the *hasABC* genes. It is possible that analysis of additional *emm89* strains may identify strains that will permit us to differentiate between the possibilities. We note that it is a formal possibility that the evolutionary genetic events occurred simultaneously, although this seems unlikely.

One intriguing finding of our study is that low HA capsule production is not sufficient to provide adequate protection against host defenses for *emm89 S. pyogenes*. For strains that produce low levels of SPN and SLO, poorly encapsulated strains are as attenuated as acapsular strains (Fig. 5 and 6). Interestingly, for high-toxin producers, poorly encapsulated strain 23530-V3 is significantly less virulent than acapsular strain 23530-V3-CapN (Fig. 5 and 6). One potential explanation for this unusual phenomenon is that acapsular *emm89* organisms that produce high levels of SPN and SLO are more cytotoxic for host cells. Under this idea, compared to capsule-positive strains (even poorly encapsulated strains), acapsular organisms attach to the host cell better and are more likely to be internalized and

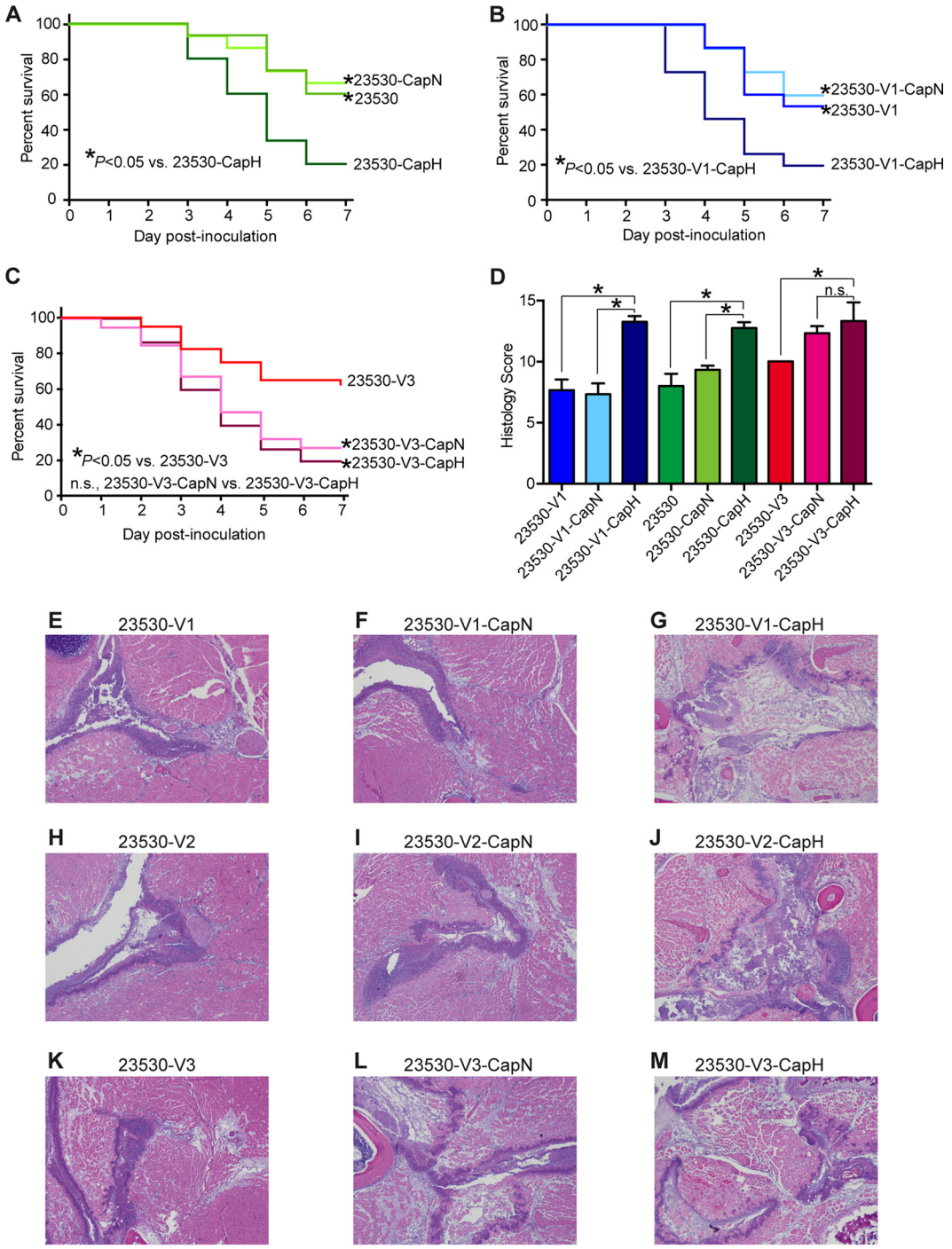

FIG 6 Capsule production is dispensable for *emm89* virulence among strains that produce high levels of SPN and SLO. (A to C) Kaplan-Meier near-survival curves of mice infected with strains that produce different levels of capsule and toxins. (D) Histopathology scores of mouse limb tissue infected with each strain. Data are expressed as means ± SEM. (E to M) Histopathologic analysis of the virulence of isogenic mutant strains in a mouse model of necrotizing fasciitis. Original magnification, 4×.

intoxicate the host cell. For high-toxin producers, more internalization may result in more abundant cytotoxicity, tissue destruction, and host pathology.

In summary, by analyzing isogenic mutant strains that recapitulate key genetic aspects of *emm89* strains from different phylogenetic clades, we discovered that an acapsular *emm89* strain producing high level of SPN and SLO (the two main features of epidemic *emm89* strains) is highly virulent in a mouse model of necrotizing fasciitis. In addition, our data show that HA capsule production is dispensable for virulence in *emm89* strains that produce high levels of SPN and SLO cytotoxins. The sum of the data provide additional evidence to support the idea we recently put

TABLE 2 Primers used for construction of isoallelic mutant strains

Primer	Sequence	Sequence underlined
ngapro-1	CGCGTGGATCCCCGTGCTATCTTGTTGTCTATGGGT	BamHI
ngapro-V1A	TAAGTAAACTATACATTAGCAAATAGTTTTTGTCAATGCGA	SNPs to introduce
ngapro-V1B	TCGCATTGACAAAAACTATTTGCTAATGTATAGTTTACTTA	SNPs to introduce
ngapro-4	CGCGTGGATCCTTTATCAATCTCAATGTGATGCGGT	BamHI site
ngapro-V3A	TAAGTAAACTATACATTAACAACTAGTTTTTGTCAATGCGA	SNPs to introduce
ngapro-V3B	TCGCATTGACAAAAACTAGTTGTTAATGTATAGTTTACTTA	SNPs to introduce
ngapro-seq	CGTGTTTACAAACCAATGGATGACT	
hasABCdel-1	CGCGTGGATCCCTTTAGGTTGATACTGGGCGATGGA	BamHI site
hasABCdel-2	TGGTCCTCGATAGAGCGTTTAGCTTTATTTTAGCAAACTTTTCAAATAGT	
hasABCdel-3	ACTATTTGAAAAGTTTGCTAAAATAAAGCTAAACGCTCTATCGAGGACCA	
hasABCdel-4	CGCGTGGATCCAATCCAAATTTATCCCCAACATCGT	BamHI site
11027has-1	CGCGTGGATCCCTTTAGGTTGATACTGGGCGATGGA	BamHI site
11027has-2	CGCGTGGATCCTCTACGGTTAAAAAAACGTCAGCGT	BamHI site
hasA−1	CCGCGTGGATCCAATAAAGGAAAACGCCATGCTCAAG	BamHI site
hasA−2	CCGCGTGGATCCATCATCCCCAATGCTAACAGGTAAA	BamHI site

forward that upregulation of SPN and SLO production is a key trigger for epidemic disease caused by *S. pyogenes*.

MATERIALS AND METHODS

Construction of isogenic mutant strains. Strain MGAS23530 was used as the parental organism for construction of isogenic mutant derivatives. Isogenic mutant strain 23530-V1 was generated by converting the *nga* promoter to the variant 1 pattern. Briefly, using MGAS23530 genomic DNA as the template, overlap extension PCR was performed with primer sets ngapro-1/V1A and ngapro-V1B/4 to generate a 1,097-bp fragment flanking the *nga* promoter region. SNPs G-27A and T-22G were introduced into the amplicon with primers ngapro-V1A and ngapro-V1B. The double-SNP-containing amplicon was digested with BamHI and ligated into the BamHI site of plasmid pBBL740 (34). The ligation product was column purified and transformed into strain MGAS23530. Allelic exchange was performed as previously described (34). The resulting derivative strain, 23530-V1, was sequenced (sequencing primer, ngapro-seq) (Table 2) to ensure that the native MGAS23530 sequence GACTAT was replaced with the derived two-single-nucleotide polymorphism (SNP) sequence AACTAG located in the −10 and −35 spacer region of the *nga* promoter. (The changed residues are underlined.) Analogous to the procedure used to generate 23530-V1, strain 23530-V3 was generated by converting the *nga* promoter to the variant 3 pattern. SNPs G-27A and T-18C were introduced into the *nga* promoter region with primers ngapro-V3A and ngapro-V3B. Capsule-negative derivative strain 23530-V3-CapN was generated by deleting the *has* operon (*hasA*, *hasB*, and *hasC*) genes. Briefly, primer sets hasABCdel-1/2 and hasABCdel-3/4 (Table 2) were used for overlap extension PCR to amplify and join the two DNA fragments upstream and downstream of the *hasABC* locus. PCR product was cloned into the BamHI site of suicide plasmid pBBL740 and then transformed into the designated *S. pyogenes* strains. Allelic exchange was performed as previously described. PCR (primer set hasABCdel-1/4) (Table 2) was used to screen for *hasABC*-negative strains. Capsule-negative derivative strains 23530-CapN and 23530-V1-CapN were made acapsular by disrupting the *hasA* gene with suicide plasmid pBBL740. Briefly, primers hasA-1 and hasA-2 (Table 2) were used to amplify an ~350-bp internal region of *hasA*. The PCR product was cloned into the BamHI site of pBBL740. Recombinant plasmid was used to transform *S. pyogenes* strains and disrupt *hasA*.

Isogenic mutant strain 23530-V3-CapH was constructed by converting the *hasA* promoter region of strain 23530-V3 to the sequence present in strain MGAS11027. Briefly, using genomic DNA of strain MGAS11027 as the template, primers 11027has-1 and 11027has-2 were used to amplify a 1.4-kb fragment that spans the *hasA* promoter region. The amplicon was ligated into the BamHI site of plasmid pBBL740 and transformed into strain MGAS23530. Allelic exchange was performed as previously described (34). Isogenic mutant strains 23530-V1-CapH and 23530-CapH were generated by the same method.

The genome of all mutant strains was sequenced, and no spurious mutations were identified.

qRT-PCR analysis of *nga*, *slo*, and *hasA* transcripts in *emm89 S. pyogenes* strains grown in THY broth. RNA prepared from *S. pyogenes* cultures grown to an optical density at 600 nm (OD_{600}) of 0.5 was extracted with an RNeasy minikit (Qiagen) and converted into cDNA using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was performed with TaqMan Fast Universal PCR master mix (Applied Biosystems) and an ABI 7500 Fast System (Life Technologies) instrument. The sequences of the TaqMan primers and probes used are listed in Table 3. Each experiment was performed in triplicate with the mean values (± standard deviation [SD]) shown. Statistical differences between strains were determined using Student's *t* test.

Western immunoblot analysis of SLO and SPN in the culture supernatant. Western immunoblot analysis was done as described previously (19). Briefly, cell-free supernatants of *S. pyogenes* cultures grown to OD_{600} of 0.5 were collected by centrifugation at 4,000 rpm for 10 min and filtered through a 0.2-μm filter. Proteins in the supernatant were concentrated with 2-ml centrifugal filters (Amicon) and assayed for presence of SLO and SPN with anti-SLO antibody (American Research Product) and anti-NADase antibody (Abcam), respectively.

Measurement of SLO and NADase activities in the culture supernatant. SLO activity and NADase activity in the culture supernatant was assayed as described previously (35).

Capsule assay. Hyaluronic acid capsule production was assayed as described previously (31).

Mouse virulence experiments. Six-week-old female CD1 mice (Harlan Laboratories) were used for the necrotizing fasciitis studies as described previously (36). This study was approved by the Institutional Animal Care and Use Committee of the Houston Methodist Research Institute (AUP-0615-0041). Mice were randomly assigned to treatment groups and inoculated in the right hindlimb to a uniform depth with 2.5×10^8 CFU of *emm89* strains in 100 μl phosphate-buffered saline (PBS). Stocks of each strain were prepared at known CFU and stored at −80°C. Inocula were prepared immediately before infection by diluting frozen stocks in PBS to the desired number of CFU. For survival experiments ($n = 15$ mice/strain for variant 1 and 2 strains and $n = 40$ mice/strain for variant 3 strains), near mortality was determined by observation using predefined internationally recognized criteria for sacrificing mice, including loss of >10% of body weight, failure to eat or drink for 24 h, reduction of body condition score by <2, becoming unable to ambulate, rupture of the abscess at the inoculation site, or rupture of an abscess at a

TABLE 3 Primers used for TaqMan qRT-PCR analysis

Primer name	Sequence[a]
rpsl-forward	CGTGTTGGAACAATGACACCTAA
rpsl-reverse	CTTCGATAAGGTTGCTCAAACGT
rpsl-probe	6FAM-CCTAACTCAGCCCTTCGTAAATTCGCTCGT-TAMRA
M89hasA-forward	ACCGTTCCCTTGTCAATAAAGG
M89hasA-reverse	CGTCAGCGTCAGATCTTTCAAA
M89hasA-probe	6FAM-CGCCATGCTCAAGCGTGGGC-TAMRA
M89nga-forward	GAATTAGGCGACACCTACACTAA
M89nga-reverse	GTGACCTCTGACAAGGCTAAA
M89nga-probe	6FAM-TGAGGTAACAGAGGTCCATCAGGGA-TAMRA
M89slo-forward	TGGGACAACAACTGGTATAGTAAG
M89slo-reverse	GTGCACTCTCTAGCCATGATAC
M89slo-probe	6FAM-AGCACAGTTATCCCACTAGGAGCT-TAMRA

[a] 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

site of dissemination (37). Data are shown as a Kaplan-Meier survival curve, with statistical differences between strain groups determined with the log-rank test. The larger number of mice used for the variant 3 strains was determined using a power calculation after conducting a pilot experiment. For histology evaluation ($n = 3$ mice/strain), lesions were excised, visually inspected, and photographed. The tissue was fixed in 10% phosphate-buffered formalin, decalcified, and embedded in paraffin using standard automated instruments. Histology was scored by a pathologist blinded to the strain treatment groups as described previously (38). Data are shown as means ± standard errors of the means (SEM), with statistical differences between strain groups determined using the Wilcoxon rank sum test.

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