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Extracellular Nucleases of *Streptococcus equi* subsp. *zooepidemicus* Degrade Neutrophil Extracellular Traps and Impair Macrophage Activity of the Host

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ABSTRACT The pathogen *Streptococcus equi* subsp. *zooepidemicus* is associated with a wide range of animals, including humans, and outbreaks frequently occur in pigs, equines, and goats. Thus far, few studies have assessed interactions between the host immune system and *S. equi* subsp. *zooepidemicus* and how these interactions explain the wide host spectrum of *S. equi* subsp. *zooepidemicus*. Neutrophils, the first line of innate immunity, possess a defense mechanism called neutrophil extracellular traps (NETs), which primarily consist of DNA and granule proteins that trap bacteria via charge interactions. Extracellular nucleases play important roles in the degradation of the DNA backbone of NETs. Here, two related extracellular nucleases, nuclease and 5′-nucleotidase (named ENuc and 5Nuc, respectively, in this study), were identified as being encoded by the *SESEC_RS04165* gene and the *SESEC_RS05720* gene (named *ENuc* and *5Nuc*, respectively), and three related gene deletion mutant strains, specifically, the single-mutant Δ*ENuc* and Δ*5Nuc* strains and the double-mutant Δ*ENuc* Δ*5Nuc* strain, were constructed. The Δ*ENuc* and Δ*5Nuc* single-mutant strains and the Δ*ENuc* Δ*5Nuc* double-mutant strain demonstrated lower virulence than wild-type *S. equi* subsp. *zooepidemicus* when the mouse survival rate was evaluated postinfection. Furthermore, wild-type *S. equi* subsp. *zooepidemicus* more frequently traversed the bloodstream and transferred to other organs. Wild-type *S. equi* subsp. *zooepidemicus* induced fewer NETs and was able to survive in NETs, whereas only 40% of the Δ*ENuc* Δ*5Nuc* double-mutant cells survived. *S. equi* subsp. *zooepidemicus* degraded the NET DNA backbone and produced deoxyadenosine, primarily through the action of ENuc and/or 5Nuc. However, the double-mutant Δ*ENuc* Δ*5Nuc* strain lost the ability to degrade NETs into deoxyadenosine. Deoxyadenosine decreased RAW 264.7 cell phagocytosis to 40% of that of normal macrophages.

IMPORTANCE *Streptococcus equi* subsp. *zooepidemicus* causes serious bacteremia in its hosts. However, little is known about how *S. equi* subsp. *zooepidemicus* interacts with the host innate immune system, particularly innate cells found in the blood. *S. equi* subsp. *zooepidemicus* is capable of evading NET-mediated killing via the actions of its potent extracellular nucleases, ENuc and 5Nuc, which directly degrade the NET DNA backbone to deoxyadenosine. In previous studies, other pathogens have required the synergism of nuclease and 5′-nucleotidase to engage in this self-protective process; however, ENuc and 5Nuc both possess nuclease activity and 5′-nucleotidase activity, highlighting the novelty of this discovery. Furthermore, deoxyadenosine impairs phagocytosis but not the intracellular bactericidal activity of macrophages. Here we describe a novel mechanism for *S. equi* subsp. *zooepidemicus* extracellular nucleases in NET degradation, which may provide new insights into the pathogen immune evasion mechanism and the prevention and treatment of bacterial disease.

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Streptococcus equi subsp. *zooepidemicus* is a zoonotic pathogen belonging to Lancefield group C, which includes *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *ruminatorum*. *Streptococcus equi* subsp. *equi* is an important horse pathogen causing strangles, a serious and highly contagious disease of the upper respiratory tract (1). *S. equi* subsp. *zooepidemicus* is thought to be the ancestor of *Streptococcus equi* subsp. *equi* and infects a wild range of animal species, including humans, who contract the disease through contact with infected animals and via consumption of contaminated dairy products (2, 3). Recent *S. equi* subsp. *zooepidemicus* outbreaks in Sweden (2009/2010) and Brazil (1997/1998) led to significant suffering and economic loss (4). The widespread nature of *S. equi* subsp. *zooepidemicus* may be related to its strong ability to evade the immune systems of its hosts.

Neutrophils are regarded as the first line of immune defense against bacterial infection (5) and kill microbes by releasing antimicrobial proteins into extracellular space via degranulation or by phagocytosing invading microbes (6, 7). However, neutrophils also kill pathogens by unleashing neutrophil extracellular traps (NETs) (8). NETs respond to bacteria, fungi, viruses, other chemical compounds, and even host factors such as activated platelets and interleukin-8 (IL-8). Upon neutrophil activation, the nuclear envelope disintegrates, and DNA fibers burst into extracellular space (9).

To survive in the host, many bacteria produce extracellular DNases, such as Sda1 of *Streptococcus pyogenes* and EndA of *Streptococcus pneumoniae*, to degrade the NET DNA backbone (10, 11). These enzymes allow bacteria to evade the host immune system. In recent years, the involvement of many extracellular nucleotidases in the interaction between NETs and bacteria has been identified. For example, adenosine synthase A (AdsA) of *Staphylococcus aureus* acts as a nucleotidase to convert NET degradation products into deoxyadenosine, which activates caspase-3-mediated apoptosis in macrophages and monocytes (12). However, few studies have assessed the interaction between NETs and *S. equi* subsp. *zooepidemicus*, and the mechanism underlying *S. equi* subsp. *zooepidemicus*-mediated bacteremia remains unclear.

Therefore, in this study, we aimed to assess the interaction between NETs and *S. equi* subsp. *zooepidemicus* to determine whether the actions of extracellular nucleases contribute to NET evasion and the spread of *S. equi* subsp. *zooepidemicus* in the host. Here, we found two genes, the *SESEC_RS04165* gene and the *SESEC_RS05720* gene (named *ENuc* and *5Nuc*, respectively, in this study), encoding putative extracellular nucleases. *ENuc* is predicted to encode nuclease (ENuc), and *5Nuc* is predicted to encode 5′-nucleotidase (5Nuc). The properties of the two related extracellular nuclease genes are characterized, and their roles in immune evasion are confirmed.

RESULTS

Determination and identification of *ENuc* and *5Nuc*. Seven genes in wild-type (WT) *S. equi* subsp. *zooepidemicus* are predicted to encode related extracellular nucleases according to a literature search in the NCBI database (see Table 2 for a list); however, it is unknown whether these genes are transcribed. Therefore, reverse transcription-quantitative PCR (qRT-PCR) was performed to study the transcriptional levels of these genes. mRNA expression levels at 2 h were set as 1-fold, and all 7 genes were transcribed during growth, particularly by 10 h (early in the stationary phase). However, the expression levels of *ENuc* and *5Nuc* were significantly higher by 10 h ($P < 0.001$) than those of other nuclease genes. At 10 h, *ENuc* and *5Nuc* transcription levels were 165 times and 67 times higher, respectively, than their transcription levels at 2 h (Fig. 1A).

ENuc (2,781-bp open reading frame [ORF]; NCBI gene identification, *SESEC_RS04165*) and *5Nuc* (2,007-bp ORF; NCBI gene identification, *SESEC_RS05720*) are uncharacterized

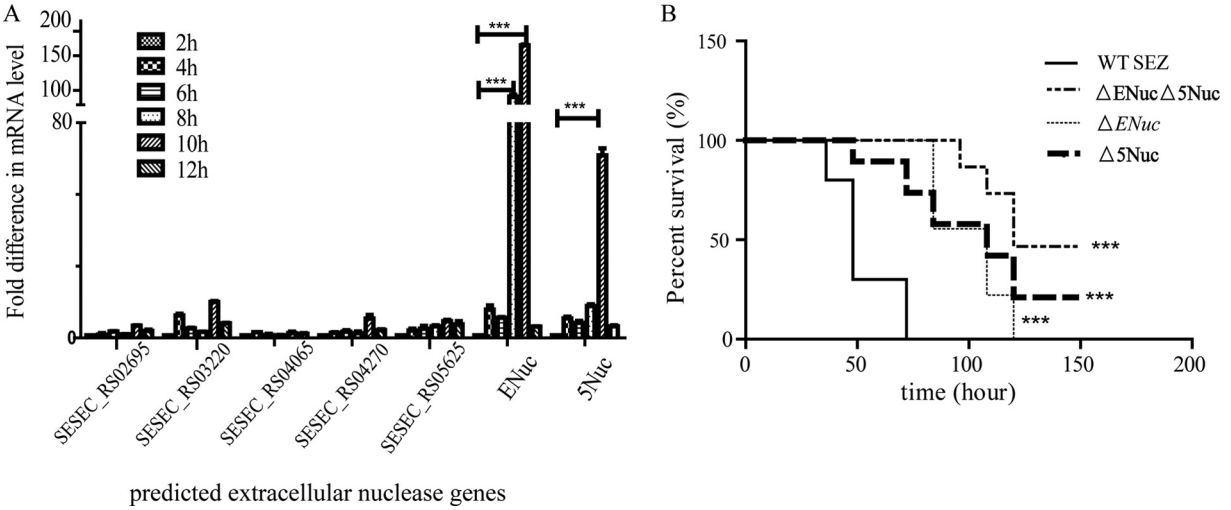

FIG 1 Major nucleases and their virulence as determined by qRT-PCR and in a mouse survival assay. (A) Changes in the transcription of predicted extracellular nuclease genes measured by qRT-PCR during different growth phases of wild-type *S. equi* subsp. *zooepidemicus* (SEZ). *S. equi* subsp. *zooepidemicus* glyceraldehyde-3-phosphate dehydrogenase (GADPH) 16S rRNA was chosen as the internal control. The results are depicted as means ± standard deviations (SD) ($n = 3$). (B) Survival rates of mice challenged with wild-type *S. equi* subsp. *zooepidemicus* and 3 gene deletion mutants. During the first 72 h, all mice challenged with wild-type *S. equi* subsp. *zooepidemicus* died. At 84 h, mice challenged with the Δ*ENuc* single-mutant strain began to die, and all mice were dead by 120 h. Mice challenged with the Δ*5Nuc* single-mutant strain began to die at 48 h postinfection, and almost 20% of mice survived by 148 h. Mice challenged with the Δ*ENuc* Δ*5Nuc* double-mutant strain showed low mortality; approximately 50% survived by 148 h. The virulences of the three mutants and wild-type *S. equi* subsp. *zooepidemicus* were compared. ***, $P < 0.001$.

genes encoding a nuclease and a 5′-nucleotidase, and their corresponding proteins are designated ENuc and 5Nuc, respectively. ENuc contains a Gram-positive coccus surface protein LPXTG motif, a signal peptide, and three predicted extracellular nuclease domains, including two motifs similar to the OB fold of *Bacillus subtilis* YhcR: a sugar-nonspecific nuclease (YhcR_OBF_like; accession, cd04486; E value = 2.72e−13 and 2.13e−12) and an exonuclease-endonuclease-phosphatase domain (accession, cl00490; E value = 4.48e−30). 5Nuc contains an LPXTG motif, a peptide, and two 5′-nucleotidase metallophosphatase domains in the amino-terminal region (accession, cl13995; E value = 1.26e−121) and a 5′-nucleotidase domain (accession, pfam02872; E value = 3.18e−32) in the carboxyl-terminal region. All data available from the NCBI were analyzed.

Virulence comparison. The virulence of the three gene deletion mutants decreased to various degrees compared with that of WT *S. equi* subsp. *zooepidemicus* ($P < 0.001$). Half of the mice challenged with the Δ*ENuc* Δ*5Nuc* double-mutant strain survived for 7 days postinfection. All mice in the WT *S. equi* subsp. *zooepidemicus* group died between 24 h and 72 h postinfection. In contrast, mice challenged with the Δ*ENuc* single-mutant strain and the Δ*ENuc* Δ*5Nuc* double-mutant strain exhibited no obvious symptoms during the first 3 days postinfection; however, all mice challenged with the Δ*ENuc* single-mutant strain eventually died between 84 h and 120 h postinfection, suggesting that ENuc contributes to bacterial survival during the early stage of infection. Mice challenged with the Δ*5Nuc* single-mutant strain began to die at 48 h postinfection, and 20% of mice in the Δ*5Nuc* single-mutant strain group survived, indicating an important role for 5Nuc in bacterial proliferation during infection (Fig. 1B). Clearly, ENuc and 5Nuc are important virulence factors during *S. equi* subsp. *zooepidemicus* infection, and ENuc may play a role in early infection, while 5Nuc appears to be more active than ENuc. All mice challenged with phosphate-buffered saline (PBS) survived (these data are not shown in Fig. 1B).

Roles of ENuc and 5Nuc in NET entrapment evasion. To study the functions of ENuc and 5Nuc, we cloned their two genes from the genome of WT *S. equi* subsp. *zooepidemicus* and ligated them into the vectors pET-32a(+) and pET-28a(+), respectively. Recombinant proteins were purified from *Escherichia coli* Rosetta. ENuc recom-

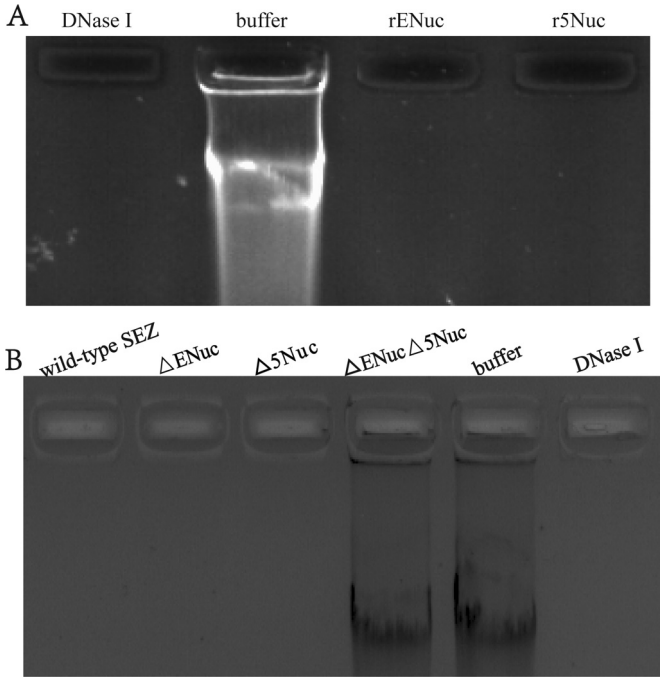

FIG 2 DNase activities of ENuc and 5Nuc. (A) ENuc and 5Nuc degrade calf thymus DNA. Banding indicates the remaining DNA, and lanes from left to right represent DNase I, reaction buffer, rENuc, and r5Nuc incubated with calf thymus DNA. (B) Wild-type *S. equi* subsp. *zooepidemicus* and the 3 mutants degrade calf thymus DNA. Lanes from left to right indicate the remaining DNA after calf thymus DNA was incubated with wild-type *S. equi* subsp. *zooepidemicus*, the Δ*ENuc* and Δ*5Nuc* single-mutant strains, the Δ*ENuc* Δ*5Nuc* double-mutant strain, reaction buffer, and DNase I.

binant protein (rENuc) and 5Nuc recombinant protein (r5Nuc) both degraded calf thymus DNA within 1 h at 37°C. Notably, the Δ*ENuc* Δ*5Nuc* double-mutant strain lost its ability to degrade calf thymus DNA compared with those of WT *S. equi* subsp. *zooepidemicus* and the Δ*ENuc* and Δ*5Nuc* single-mutant strains (Fig. 2). Thus, the expression levels of *ENuc* and *5Nuc* allow WT *S. equi* subsp. *zooepidemicus* to acquire the ability to degrade extracellular DNA.

Deoxyadenosine derived from the degradation of the NET DNA backbone is toxic to host immune cells. To determine whether ENuc and 5Nuc degrade DNA into deoxyadenosine, deoxyadenosine production was analyzed using reverse-phase high-performance liquid chromatography (rpHPLC). Recombinant proteins rENuc and r5Nuc degraded calf thymus DNA, which mimics the DNA backbone of NETs, and NET DNA and produced deoxyadenosine (Fig. 3A to G). WT *S. equi* subsp. *zooepidemicus* and the Δ*ENuc* and Δ*5Nuc* single-mutant strains also produced deoxyadenosine following NET interaction, but the Δ*ENuc* Δ*5Nuc* double-mutant strain did not (Fig. 3H to K). Additionally, the WT and the Δ*ENuc* and Δ*5Nuc* single-mutant strains degraded calf thymus DNA to produce deoxyadenosine (see Fig. S1 in the supplemental material). Thus, ENuc and 5Nuc, which possess both nuclease activity and 5′-nucleotidase activity, appear to be the primary nucleases that allow *S. equi* subsp. *zooepidemicus* to evade entrapment by NETs.

Recombinant proteins rENuc and r5Nuc both exhibited 5′-nucleotidase activity and hydrolyzed ATP, ADP, and AMP to produce adenosine (Fig. 4). Adenosine is an immunomodulatory molecule that suppresses neutrophil functions such as degranulation. Therefore, the ability of ENuc and 5Nuc to generate adenosine enhances the virulence of *S. equi* subsp. *zooepidemicus*.

Survival in different organs. NET degradation contributes to *S. equi* subsp. *zooepidemicus*-induced bacteremia and spread to the organs from the blood. In particular, WT *S. equi* subsp. *zooepidemicus* showed a greater tendency to break through

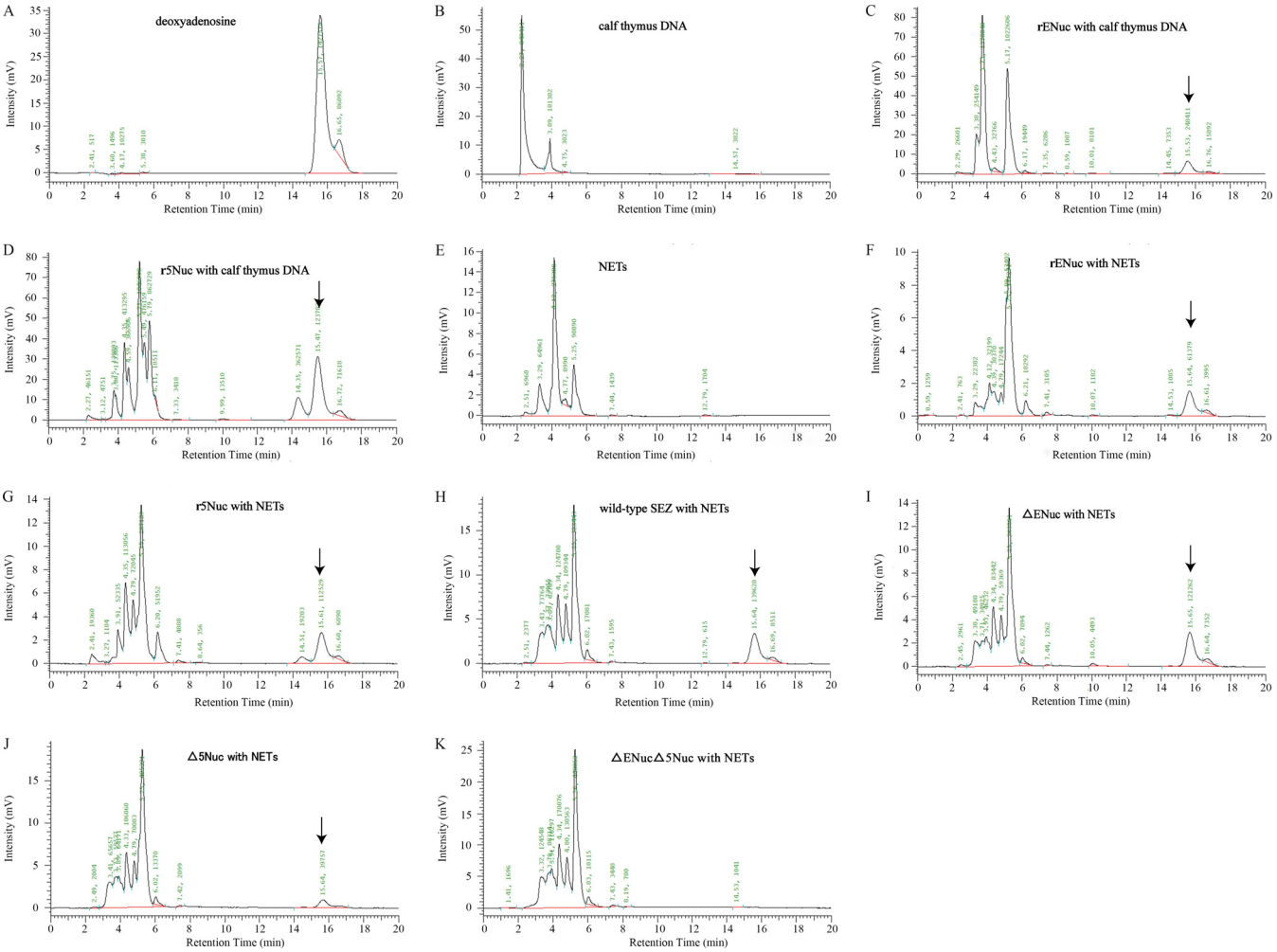

FIG 3 Production of deoxyadenosine detected by rpHPLC. (A) A standard deoxyadenosine sample (Sigma-Aldrich) was evaluated by rpHPLC. (B) Calf thymus DNA was evaluated by rpHPLC. (C and D) Deoxyadenosine production following the incubation of rENuc and r5Nuc with calf thymus DNA. (E) NETs derived from neutrophils induced by PMA were measured by rpHPLC. (F to K) Deoxyadenosine production after the incubation of NETs with rENuc, r5Nuc, wild-type *S. equi* subsp. *zooepidemicus*, the Δ*ENuc* and Δ*5Nuc* single-mutant strains, and the Δ*ENuc* Δ*5Nuc* double-mutant strain was determined by rpHPLC. The arrows indicate deoxyadenosine.

the blood barrier and cause bacteremia. At 24 h postinfection, only mice challenged with WT *S. equi* subsp. *zooepidemicus* demonstrated bacterial loads in the blood, lung, spleen, and liver, indicating that the mutant strains were less able to survive and cross the blood barrier. Mice challenged with the Δ*ENuc* single-mutant strain showed bacterial loads at 48 h and 72 h, but mice challenged with the Δ*5Nuc* single-mutant strain only showed bacteria loads at 48 h, suggesting an increased tendency for single-mutant Δ*ENuc* cells to spread to the organs from the blood. The nuclease activity of 5Nuc appears to be more potent than that of ENuc, which is consistent with the virulence of the Δ*ENuc* and Δ*5Nuc* single-mutant strains. Importantly, at 72 h postinfection, mice challenged with the Δ*ENuc* single-mutant strain demonstrated bacterial loads equivalent to those in mice challenged with WT *S. equi* subsp. *zooepidemicus*; however, at 96 h postinfection, WT *S. equi* subsp. *zooepidemicus* was better able to survive in organs such as the lung, liver, and spleen. Thus, WT *S. equi* subsp. *zooepidemicus* not only demonstrates a superior ability to transfer to organs but also impedes clearance by the host. Mice challenged with the Δ*ENuc* Δ*5Nuc* double-mutant strain did not exhibit bacterial loads from 24 h to 96 h, suggesting that bacteria were killed by the host immune system at an early stage in the blood due to the lack of ENuc and 5Nuc (Fig. 5).

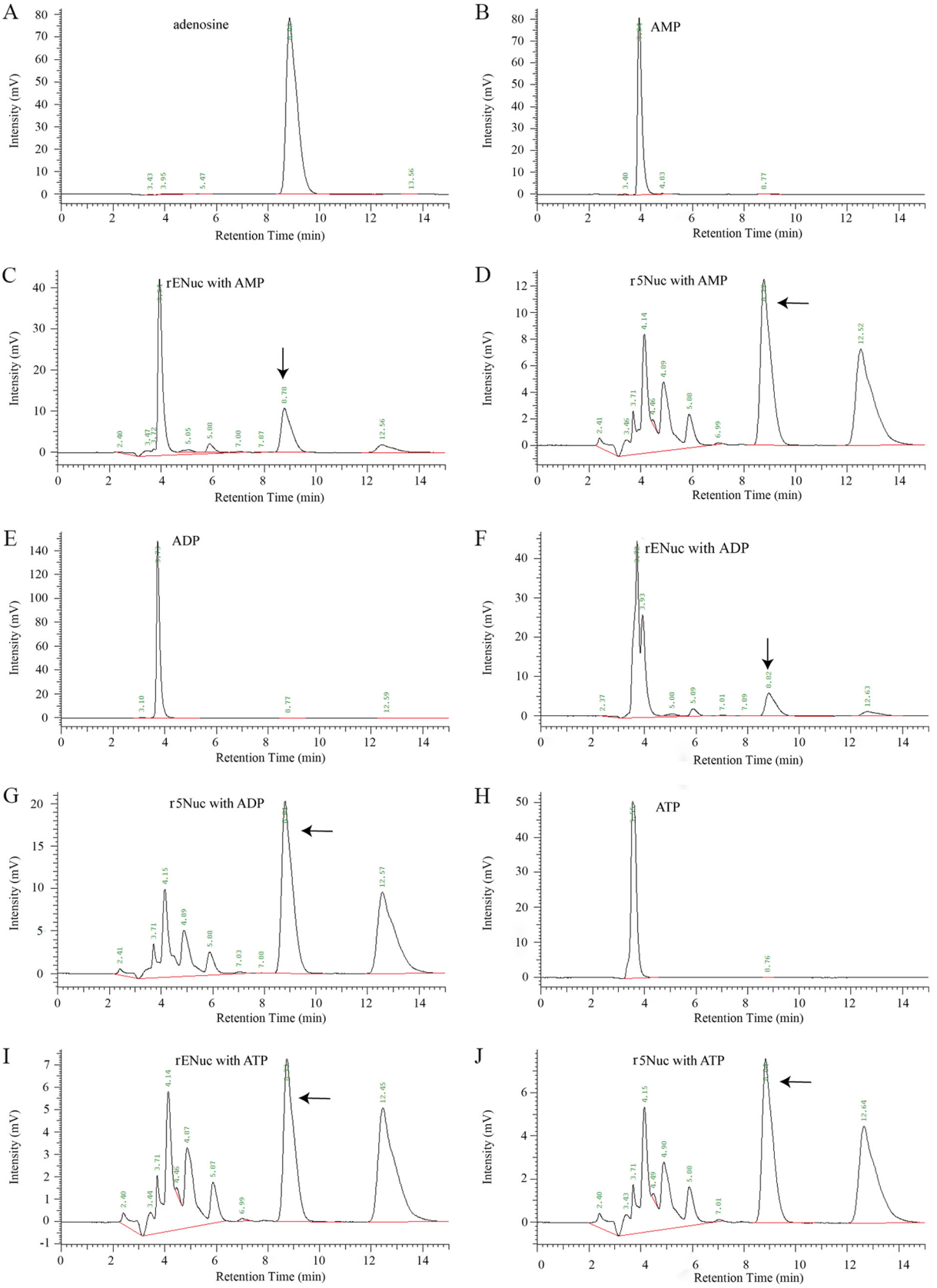

FIG 4 Hydrolysis of AMP, ADP, and ATP by ENuc and 5Nuc. (A and B) Standard adenosine and AMP samples were evaluated by rpHPLC. (C and D) AMP was hydrolyzed by rENuc and r5Nuc to generate adenosine. (E) Standard ADP sample. (F and G) Adenosine evaluated by rpHPLC as a product of rENuc and r5Nuc following incubation with ADP. (H) Standard ATP sample. (I and J) ATP was hydrolyzed by rENuc and r5Nuc to generate adenosine. The arrows indicate the adenosine produced.

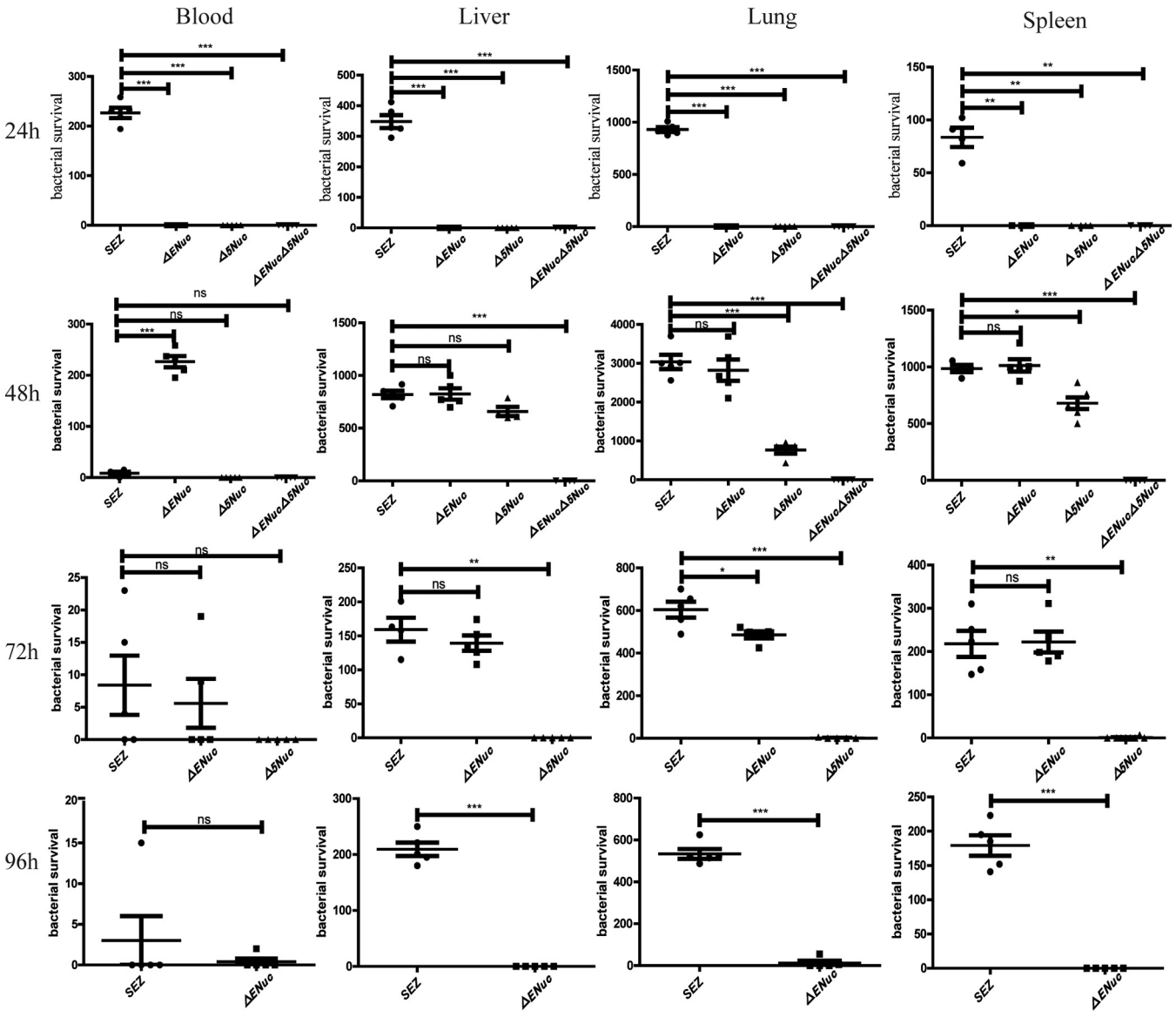

FIG 5 Survival of wild-type *S. equi* subsp. *zooepidemicus* and the 3 mutant strains in different organs. Rows represent the time points postinfection for sample collection. Columns represent organs collected from infected mice. The results are depicted as means ± SD ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no difference between groups.

Bactericidal capacity of NETs. WT *S. equi* subsp. *zooepidemicus* resists killing by NETs. The bactericidal activity of NETs after incubation with WT *S. equi* subsp. *zooepidemicus* was negative, facilitating bacterial replication and increased numbers of WT *S. equi* subsp. *zooepidemicus* bacteria. However, approximately 30% of the Δ*ENuc* single-mutant, 50% of the Δ*5Nuc* single-mutant, and 60% of the Δ*ENuc* Δ*5Nuc* double-mutant cells were killed following incubation with NETs for 90 min (Fig. 6). In addition, the fibrous structure of the NET DNA backbone was only found in gene deletion mutant strains and NET concentrations induced by the Δ*ENuc* and Δ*5Nuc* single-mutant strains were 2 and 5 times higher, respectively, than that induced by WT *S. equi* subsp. *zooepidemicus*. Importantly, NET concentrations induced by the Δ*ENuc* Δ*5Nuc* double-mutant strain were more than 7 times higher than that induced by WT *S. equi* subsp. *zooepidemicus* (see Fig. S2 in the supplemental material). A weaker ability to induce NET formation by WT *S. equi* subsp. *zooepidemicus* than by gene deletion mutants may be attributed to the DNA degradation activities of ENuc and 5Nuc, which contribute to bacterial survival in NETs. Therefore, ENuc and 5Nuc play important roles in protecting

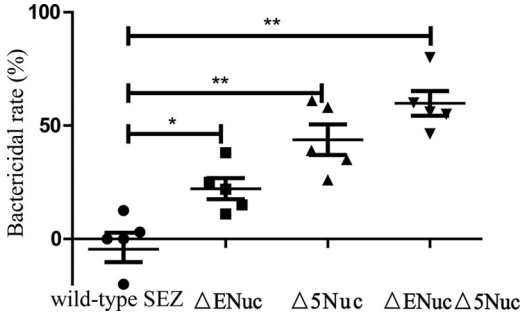

FIG 6 NET survival capacity of wild-type *S. equi* subsp. *zooepidemicus* and its 3 mutants. Nearly all wild-type *S. equi* subsp. *zooepidemicus* cells survived and proliferated in the presence of NETs, whereas 30%, 50%, and 60% of the Δ*ENuc* single-mutant, Δ*5Nuc* single-mutant, and Δ*ENuc* Δ*5Nuc* double-mutant cells, respectively, were killed in the presence of NETs. The results are depicted as the means ± SD (n = 5). *, $P < 0.05$; **, $P < 0.01$.

bacteria against killing by NETs, aiding *S. equi* subsp. *zooepidemicus* proliferation and spread to deep tissues.

Effects of deoxyadenosine on RAW 264.7 phagocytosis. To evaluate the cytotoxic effects of the NET degradation product deoxyadenosine on immune cells, RAW 264.7 cells and neutrophils and monocytes obtained from blood were employed. Phagocytosis and the bactericidal capacity of RAW 264.7 pretreated with deoxyadenosine decreased by almost 60% (Fig. 7A). Surprisingly, in an intracellular killing assay, the intracellular killing activity of RAW 264.7 cells pretreated with deoxyadenosine was equivalent to that of RAW 264.7 cells without pretreatment (Fig. 7B). Thus, deoxyadenosine primarily impairs the phagocytosis of macrophages but does not affect the intracellular killing process. Furthermore, deoxyadenosine did not influence the bactericidal activity of monocytes (Fig. 7C).

Effects of deoxyadenosine on neutrophil function. To evaluate the effects of deoxyadenosine on neutrophils, two assays were designed. In the first, we tested the influence of deoxyadenosine on bacterial killing activity, and in the second, we tested the effects of deoxyadenosine on NET formation induced by phorbol myristate acetate (PMA). Based on the results of both assays, deoxyadenosine did not affect neutrophil function (Fig. 8). Purified neutrophils comprised greater than 90% of total cells, and viability was nearly 90% over 4 h *in vitro* in this study (see Fig. S3 in the supplemental material). Thus, our experiments were carried out with active and pure neutrophils, lending credibility to the results.

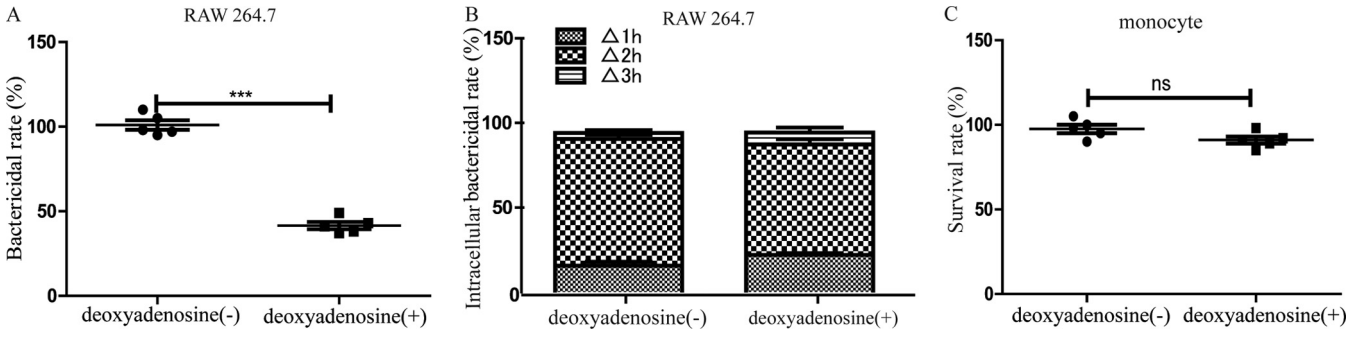

FIG 7 Effects of deoxyadenosine on RAW 264.7 phagocytosis. Deoxyadenosine(−) indicates control groups that were not treated with deoxyadenosine; deoxyadenosine(+) indicates groups that were pretreated with deoxyadenosine. (A) The percent bactericidal activity of the control group was set to 100%. The bactericidal activity of RAW 264.7 cells treated with deoxyadenosine decreased to 40%. (B) △1 h, △2 h, and △3 h indicate the intracellular bactericidal rates of RAW 264.7 cells between 1 and 2 h, 2 and 3 h, and 3 and 4 h after extracellular bacteria were killed. Bactericidal activities for the time ranges were compared. After 4 h, all intracellular bacteria were killed in both the control and treatment groups. (C) The bactericidal activity of monocytes was evaluated by determining the CFU of viable bacteria after incubation. The CFU of viable bacteria in the control group was set to 100%. The results are depicted as means ± SD (n = 5). ***, $P < 0.001$; ns, no differences between groups.

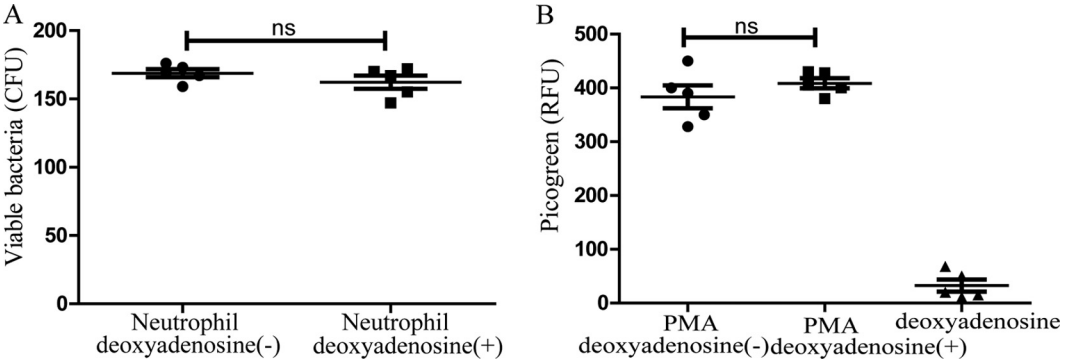

FIG 8 Measurement of the effects of deoxyadenosine on neutrophil function. Deoxyadenosine(−) indicates control groups that were not treated with deoxyadenosine; deoxyadenosine(+) indicates treatment groups that were pretreated with deoxyadenosine. (A) The bactericidal activity of neutrophils was evaluated by determining the CFU of surviving bacteria after incubation. (B) PMA-stimulating NET formation by neutrophils was measured as the concentration of extracellular DNA, which was assessed with PicoGreen and is represented as relative fluorescence units (RFU). Resulted are depicted as means ± SD (n = 5). ns, no differences between groups.

DISCUSSION

This study was initially designed to identify the functions of the extracellular nucleases of *S. equi* subsp. *zooepidemicus* in the context of immune evasion and provide new ideas for future studies examining bacterial zoonosis. We assessed the interactions between *S. equi* subsp. *zooepidemicus* and NETs and observed the ability of *S. equi* subsp. *zooepidemicus* bacteria to evade entrapment and killing by NETs. Importantly, *S. equi* subsp. *zooepidemicus* possesses a potent ability to degrade NETs into deoxyadenosine, negatively influencing macrophage phagocytic activity. The production of extracellular nucleases by bacteria is a significant mechanism utilized by bacteria to escape NETs (13). In this work, we confirmed the identities of two primary extracellular nucleases by conducting a literature search of the NCBI database and performing qRT-PCR. Interestingly, recombinant proteins rENuc and r5Nuc demonstrated both nuclease and nucleotidase activities, allowing both to degrade NETs and produce deoxyadenosine. To further study the functions of these two enzymes, three gene deletion mutant strains, the Δ*ENuc* and Δ*5Nuc* single-mutant strains and the Δ*ENuc* Δ*5Nuc* double-mutant strain, were constructed. These mutants exhibited lower virulence and a weaker ability to spread from the blood to organs than the WT *S. equi* subsp. *zooepidemicus* strain; this was particularly the case for the two-gene deletion mutant Δ*ENuc* Δ*5Nuc* strain. Half of the mice challenged with the Δ*ENuc* Δ*5Nuc* double-mutant strain survived, while all of the mice in the WT *S. equi* subsp. *zooepidemicus* group were dead within 3 days. Thus, it appears difficult for Δ*ENuc* Δ*5Nuc* double-mutant cells to exit the bloodstream. Despite the degradation of NETs by *S. equi* subsp. *zooepidemicus*, deoxyadenosine, a NET degradation product, may contribute to bacterial survival in the host; according to our results, deoxyadenosine suppresses macrophage phagocytosis. These two characteristics of *S. equi* subsp. *zooepidemicus* strongly contribute to its pathogenicity and proliferation in the host.

NETs constitute an important and novel innate immune defense system that influences pathogen invasion. Recently, this phenomenon was noted in several bacteria, including *Staphylococcus aureus* and *Streptococcus pyogenes* (14). In this study, when the genes *ENuc* and *5Nuc* were knocked down simultaneously, *S. equi* subsp. *zooepidemicus* was less able to survive in NETs and better able to stimulate neutrophils to produce NETs, which indicated that ENuc and 5Nuc influenced *S. equi* subsp. *zooepidemicus* virulence. PMA is generally acknowledged to be an appropriate positive control when neutrophils are stimulated to produce NETs and was employed in this study *in vitro* (15). In previous studies examining the interactions between pathogens and NETs in the context of DNase, extracellular deoxyribonucleases protected pathogens by allowing them to evade entrapment by NETs; these include EndA produced by *Streptococcus pneumoniae* and Sda1 produced by *Streptococcus pyogenes* (16–19). The

recombinant rENuc and r5Nuc proteins both degraded calf thymus DNA, a common substitute for the NET DNA backbone in laboratory research. Thus, ENuc and 5Nuc likely protect *S. equi* subsp. *zooepidemicus* from NET entrapment. In addition, high bacterial loads were only observed in mice challenged with WT *S. equi* subsp. *zooepidemicus* at 24 h postinfection; this may be attributable to the functions of ENuc and 5Nuc such that WT *S. equi* subsp. *zooepidemicus* not only survived in the host but also was able to spread to organs better than the mutant strains. Moreover, the Δ*5Nuc* single-mutant and Δ*ENuc* Δ*5Nuc* double-mutant cells demonstrated difficulty in spreading to other organs from the blood, probably due to the deletion of 5Nuc. Thus, 5Nuc may be more active than ENuc. Importantly, mice challenged with the Δ*ENuc* Δ*5Nuc* double-mutant strain demonstrated no bacterial loads, highlighting the importance and virulence of ENuc and 5Nuc. In summary, *S. equi* subsp. *zooepidemicus* degrades NETs and protects itself by evading entrapment by the host immune system, which may account for the differences in virulence between WT *S. equi* subsp. *zooepidemicus*, the Δ*ENuc* and Δ*5Nuc* single-mutant strains, and the Δ*ENuc* Δ*5Nuc* double-mutant strain.

Extracellular 5′-nucleotidases, such as AdsA of *Staphylococcus aureus*, produce adenosine, which is an immunomodulatory molecule (20, 21). However, Thammavongsa and colleagues noted cooperation between AdsA and a nuclease, facilitating the production of deoxyadenosine through the degradation of NET DNA. Deoxyadenosine induces macrophage apoptosis via caspase-3 activation, thereby restricting macrophage survival and promoting the establishment of infection (12). In this study, *S. equi* subsp. *zooepidemicus* degraded the NET DNA backbone and produced deoxyadenosine to suppress the phagocytosis of macrophages but did not negatively influence neutrophils and monocytes. Unexpectedly, both ENuc and 5Nuc demonstrated the ability to convert calf thymus DNA and NET DNA into deoxyadenosine as directly detected by rpHPLC. This mechanism differs from that observed in other pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes* because these pathogens possess NET-cleaving nucleases in addition to 5′-nucleotidases (20, 22, 23). In this study, *S. equi* subsp. *zooepidemicus* secreted two major extracellular enzymes, ENuc and 5Nuc, that degraded the DNA backbone of NETs and produced deoxyadenosine. Even with the deletion of either of their two genes, *S. equi* subsp. *zooepidemicus* was also able to degrade NETs into deoxyadenosine. However, when both genes were knocked out, *S. equi* subsp. *zooepidemicus* virulence decreased, and its ability to degrade NETs and generate deoxyadenosine was lost. Additionally, ENuc and 5Nuc possess the ability to hydrolyze AMP, ADP, and ATP to produce the immunomodulatory substrate adenosine, similar to Nt5e produced by *Streptococcus sanguinis* and AdsA produced by *Staphylococcus aureus* but unlike S5nA produced by *Streptococcus pyogenes* and NudP produced by *Streptococcus agalactiae*, which demonstrate limited specificity for AMP and ADP (21, 24, 25). Adenosine suppresses neutrophil activation by occupying the A2a receptor on neutrophils and promoting bacterial survival in the host bloodstream (26). Our data reveal a novel and potent mechanism in which extracellular nucleases degrade NETs and produce deoxyadenosine.

Deoxyadenosine is cytotoxic to macrophages. During infection with *Staphylococcus aureus*, deoxyadenosine production is sufficient to restrict macrophage survival and promote the establishment of persistent infection (12). The production of deoxyadenosine through the degradation of the DNA backbone of NETs is an important mechanism enabling bacterial survival. However, only a few studies have examined the functions of deoxyadenosine in immune cells. In this study, we investigated the influence of deoxyadenosine on macrophages, neutrophils, and monocytes and only observed negative effects on macrophage phagocytosis; however, deoxyadenosine did not affect the bactericidal activity of macrophages. Additionally, deoxyadenosine exerted no influence on the functions of neutrophils and monocytes. The molecular mechanism of deoxyadenosine underlying immune cell impairment will be explored in a future study.

In summary, *S. equi* subsp. *zooepidemicus* secretes two potent extracellular enzymes, ENuc and 5Nuc, each demonstrating nuclease and 5′-nucleotidase activities, which

TABLE 1 Strains and plasmids used in this study

Bacterial strain or plasmid	Notable characteristic(s)[a]	Source or reference
S. equi subsp. *zooepidemicus* strains		
S. equi subsp. *zooepidemicus* ATCC 35246	Wild type isolated from infected pig	Purchased from ATCC
Δ*ENuc* single mutant	*SESEC_RS04165* (*ENuc*) deletion mutant of *S. equi* subsp. *zooepidemicus* ATCC 35246	This study
Δ*5Nuc* single mutant	*SESEC_RS05720* (*5Nuc*) deletion mutant of *S. equi* subsp. *zooepidemicus* ATCC 35246	This study
Δ*ENuc* Δ*5Nuc* double mutant	*ENuc* and *5Nuc* double gene deletion mutant of *S. equi* subsp. *zooepidemicus* ATCC 35246	This study
E. coli strains		
E. coli DH5a	Plasmid cloning host and Gram-negative replicon	Purchased from Invitrogen
E. coli Rosetta	*E. coli* plasmid-expressing host	Purchased from Invitrogen
E. coli Rosetta 32a-*ENuc*	*E. coli* Rosetta carrying plasmid pET32a-*ENuc*, Amp^r	This study
E. coli Rosetta 28a-*5Nuc*	*E. coli* Rosetta carrying plasmid pET28a-*5Nuc*, Km^r	This study
Plasmids		
pSET4s	*Streptococcus* thermosensitive suicide vector, Spc^r	Takamatsu et al. (29)
pSET4s-Δ*ENuc*	pSET4s containing the upstream and downstream sequences surrounding *ENuc* for deleting *ENuc*, Spc^r	This study
pSET4s-Δ*5Nuc*	pSET4s containing the upstream and downstream sequences surrounding *5Nuc* for deleting *5Nuc*, Spc^r	This study
pET-32a(+)	*E. coli* gene-expressing vector, Amp^r	Purchased from Invitrogen
pET32a-*ENuc*	pET-32a containing *ENuc* gene, Amp^r	This study
pET-28a(+)	*E. coli* gene-expressing vector, Km^r	Purchased from Invitrogen
pET28a-*5Nuc*	pET-28a containing *5Nuc* gene, Km^r	This study

[a]Amp^r, ampicillin resistance cassette; Km^r, kanamycin resistance cassette; Spc^r, spectinomycin resistance cassette.

allow it to evade entrapment by degrading NETs, and producing the cytotoxic substrate deoxyadenosine to impair macrophage phagocytosis. This mechanism differs from a previously reported mechanism in which bacteria utilize nuclease in collaboration with 5′-nucleotidase to degrade NET DNA into deoxyadenosine. Our novel mechanism represents an ingenious method for bacteria to utilize host components for survival and simultaneously perturb host immune defenses. In addition, the two enzymes hydrolyze ATP, ADP, and AMP to produce the immunomodulatory substrate adenosine. Thus, ENuc and 5Nuc are novel and important virulence factors for *S. equi* subsp. *zooepidemicus*, and their identification will aid in the further study of immune evasion by zoonotic pathogens and contribute to the design of therapeutic agents.

MATERIALS AND METHODS

Bacterial strains, cells, and experimental animals. The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type *S. equi* subsp. *zooepidemicus* strain ATCC 35246 (here referred to as WT *S. equi* subsp. *zooepidemicus*) was purchased from the American Type Culture Collection (ATCC) and grown in Todd-Hewitt broth (THB) medium (Difco; BD, Franklin, NJ, USA) at 37°C on a gentle rocking shaker. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C. When necessary, antibiotics were used at the following concentrations to screen transformants: 100 μg/ml spectinomycin (Sigma-Aldrich, St. Louis, MO, USA) for *S. equi* subsp. *zooepidemicus*, 50 μg/ml spectinomycin for *E. coli*, 50 μg/ml ampicillin (Sigma-Aldrich) for *E. coli*, and 50 μg/ml kanamycin (Sigma-Aldrich) for *E. coli*. RAW 264.7 cells (ATCC TIB-71) were purchased from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent, Canada) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2.

Four-week-old female ICR (Institute of Cancer Research) specific-pathogen-free mice were purchased from the Comparative Medicine Center of Yangzhou University. All experimental protocols were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province. Animals showing signs of illnesses were humanely euthanized with 100% CO_2.

qRT-PCR analysis. Total RNA was extracted from WT *S. equi* subsp. *zooepidemicus* in the logarithmic phase using TRIzol reagent (Gibco-BRL; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the bacteria were washed 3 times with phosphate-buffered saline (PBS). One milliliter of TRIzol solution was added to the bacterial precipitate, which was shaken for 15 s to lyse the bacterial cells; after that, 200 μl of chloroform was added. The mixture stood at room temperature for 2 min and was then centrifuged at 12,000 × *g* for 15 min. Two hundred microliters of the upper phase (avoiding the middle layer containing DNA) was collected, and 500 μl of isopropyl alcohol was added to precipitate the RNA. The suspension was centrifuged to collect the RNA and washed twice with 75% ethanol. Seven genes predicted to encode extracellular nucleases were chosen based on genomic information for *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246 (NC_017582.1) in the NCBI database. qRT-PCR was performed to determine the transcript concentrations of these 7 predicted extracellular nuclease genes: *ENuc*,

TABLE 2 Predicted genes encoding extracellular nucleases in *S. equi* subsp. *zooepidemicus*

Gene	Product[a]	Abbreviation
SESEC_RS04165	Nuclease	*ENuc*
SESEC_RS05720	5′-Nucleotidase	*5Nuc*
SESEC_RS02695	CRISP-associated protein Cas4	
SESEC_RS03220	DNA/RNA-nonspecific endonuclease	
SESEC_RS04065	DNase	
SESEC_RS04270	Competence-associated endonuclease	
SESEC_RS05625	DNA/RNA-nonspecific endonuclease	

[a]Data information refers to the genomic information for *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246 (NC_017582.1) in the NCBI database.

5Nuc, *SESEC_RS02695*, *SESEC_RS03220*, *SESEC_RS04065*, *SESEC_RS04270*, and *SESEC_RS05625* (Table 2). All primers used are listed in Table 3. The results were analyzed using the $2^{-\Delta\Delta CT}$ method.

Cloning and purification of recombinant ENuc and 5Nuc. Cloning and purification of the recombinant protein ENuc (rENuc) were performed as previously described (27). *ENuc* was cloned by PCR using the primers ENucF and ENucR (Table 3) and the WT *S. equi* subsp. *zooepidemicus* genome as a template. The cloned *ENuc* was digested with BamHI and XhoI and cloned into the pET-32a(+) vector (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). After sequencing (Shanghai Sunny Biotechnology Co., Ltd., China), the recombinant plasmid pET32a-*ENuc* was transformed into *E. coli* Rosetta cells with ampicillin selection. The recombinant *E. coli* was incubated at 37°C until the exponential growth phase was reached, and then protein expression was induced for 6 h with the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich). Cells were harvested, washed, and suspended in ice-cold binding buffer (20 mM Tris, 200 mM NaCl [pH 7.4]).

Protein purification procedures were performed at 4°C. Bacteria were homogenized by ultrasonication for 30 min. The resulting crude extract was centrifuged at 10,000 × *g* for 30 min. Recombinant rENuc were purified using a 1-ml Ni Sepharose 6 Fast Flow column (GE Healthcare, Uppsala, Sweden). *5Nuc* was cloned into the pET-28a(+) vector (Invitrogen) to improve its expression; purification of 5Nuc recombinant protein (r5Nuc) was performed as mentioned above.

Construction of gene deletion mutants. The construction of *ENuc* and *5Nuc* single-gene deletion mutants (here called Δ*ENuc* and Δ*5Nuc*, respectively) and double-gene deletion mutant (Δ*ENuc* Δ*5Nuc*) was performed as previously described (28). Briefly, the upstream and downstream sequences surrounding *ENuc* were amplified from WT *S. equi* subsp. *zooepidemicus* chromosomal DNA using the primer combinations ENuc-L-F/ENuc-L-R and ENuc-R-F/ENuc-R-R (Table 3). The upstream and downstream flanking sequences of *ENuc* were ligated between the SalI and BamHI sites of the thermosensitive suicide vector pSET4s to yield the recombinant plasmid pSET4s-Δ*ENuc*, which was electroporated

TABLE 3 Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Restriction enzyme
Fl-ENucF	TCACTGGCTGCTGAGTTTATT	
Fl-ENucR	CGGCTGAACACGTCCATATAA	
Fl-5NucF	TCAGTCAAGGTGGGCTTTATC	
Fl-5NucR	CCACTCTAGGCAACCATCTTC	
Fl-Nuc1F	GCTTGGGTCTGTATGGGATTAT	
Fl-Nuc1R	CCACTCTAGGCAACCATCTTC	
Fl-Nuc2F	CTGGAGTGCTTTCGTCTGTAATA	
Fl-Nuc2R	TACCAATATGGCGGCATCAG	
Fl-Nuc3F	GCATTCCTGTGCGGTAATCTA	
Fl-Nuc3R	CAAATTGGGAGGCAACAAAGAA	
Fl-Nuc4F	CTTCTAGGCTATGCGCTTGTT	
Fl-Nuc4R	CTCTCGTTAGCTGCTTGGTTAG	
Fl-Nuc5F	ATTACCAATCCAGCCAGAAGG	
Flu-Nuc5R	CAGGTCGCAAGAGGAACAATA	
ENucF	CGCGGATCCCAGTCTTCAGCTCAGCATGTTTATG	BamHI
ENucR	GCCCTCGAGGCTTCCTTTCCTGACGTTTCCTA	XhoI
ENuc-L-F	GCCGGATCCAGCTTAGCTCGTTATGGAAGAGTGT	BamHI
ENuc-L-R	AAAGAAATCCTTTACTTCCTTTCCTTTGACATAAAAACTCCTTTTTAATG	
ENuc-R-F	CATTAAAAAGGAGTTTTTATGTCAAAGGAAAGGAAGTAAAGGATTTCTTT	
ENuc-R-R	GCCGTCGACTCTACCACGTTTGTAATATGCTTGG	SalI
5NucF	CGCGGATCCGATCAGGTAGACGTGCAATTTCTTG	BamHI
5NucR	GCCCTCGAGTTATAAGGCAGCATTAGCTTGCTTT	XhoI
5Nuc-L-F	GCCGGATCCGGAATAATAAAAGCAATCATACCAC	BamHI
5Nuc-L-R	GCCGGTTTTATTGGCCTTCCTTTTTAAACAAAATGCTTTTTCATAAACGC	
5Nuc-R-F	GCGTTTATGAAAAAGCATTTTGTTTAAAAAGGAAGGCCAATAAAACCGGC	
5Nuc-R-R	GCCGTCGACTCATCGATTGTATGACATCATGAAG	SalI

[a]Restriction enzyme sites are underlined.

into WT *S. equi* subsp. *zooepidemicus* (29). The plasmid pSET4s-Δ*ENuc* and the bacterial genome exchanged genetic fragments twice via intermolecular recombination. Putative deletion mutants were screened by PCR performed with the primer combination ENucF/ENucR (Table 3). The Δ*5Nuc* single-mutant strain was constructed on the WT *S. equi* subsp. *zooepidemicus* background using the process mentioned above, and the Δ*ENuc* Δ*5Nuc* double-mutant strain was constructed using the same process on the Δ*ENuc* background.

Isolation and purification of neutrophils. Murine neutrophils were isolated from fresh, heparinized venous blood of healthy ICR mice as previously described, with some modifications (30). Briefly, whole blood was centrifuged at 400 × *g* for 30 min, and then the top layer of plasma was discarded. Red blood cells were treated with lysis buffer (Tris-NH_4Cl). White blood cells were suspended in 0.85% NaCl. Three milliliters of 65% Percoll was gently overlaid in a 15-ml tube (BD Falcon), followed by a 3-ml layer of 55% Percoll; finally, the cell suspension was carefully overlaid as the top layer. The Percoll gradient was centrifuged at 1,000 × *g* for 30 min, and the cells at the plasma interface were carefully removed and discarded. The cells between the 55% and 65% Percoll layers were collected, washed in RPMI 1640 medium (Gibco), and resuspended in RPMI 1640 medium. Neutrophil purity was confirmed by performing flow cytometry, and cell viability was detected by performing trypan blue staining and counting cells in a chamber slide (Invitrogen).

Isolation and purification of monocytes from blood. Blood from the ICR mice was diluted in PBS and overlaid on a Histopaque 1077 solution (Sigma-Aldrich) via gradient centrifugation at 400 × *g* for 20 min (31). The upper layer was aspirated, and monocytes were enriched in the interphase. Then, the cells were washed with PBS, suspended in RPMI 1640, and seeded into 24-well plates for further study.

Mouse survival assay. A mouse survival assay was carried out to assess the virulence of WT *S. equi* subsp. *zooepidemicus*, the Δ*ENuc* and Δ*5Nuc* single-mutant strains, and the Δ*ENuc* Δ*5Nuc* double-mutant strain. Fifty ICR mice were divided into 5 groups, with 10 mice in each group. The positive group was challenged with WT *S. equi* subsp. *zooepidemicus* at a dose of 10^5 CFU via intraperitoneal injection. The experimental groups were challenged with 10^5 CFU of the Δ*ENuc* and Δ*5Nuc* strains and the Δ*ENuc* Δ*5Nuc* double-mutant strain via intraperitoneal injection. The negative-control group was challenged with PBS. The symptoms of the mice were documented for 7 days postinfection.

Bacterial dissemination assay. Bacterial dissemination was evaluated as described previously (32). Mice were inoculated with 10^4 CFU of WT *S. equi* subsp. *zooepidemicus*, the Δ*ENuc* and Δ*5Nuc* single-mutant strains, and the Δ*ENuc* Δ*5Nuc* double-mutant strain, with 5 mice in each group, via intravenous injection into the caudal vein to ensure the direct interaction of bacteria and neutrophils; this dose of bacteria causes asymptomatic infection. Bacterial burdens were enumerated in the blood, liver, lung, and spleen at 24 h, 48 h, 72 h, and 96 h to evaluate bacterial proliferation. At each time point, mice were euthanized. Livers, spleens, lungs, and blood were harvested, weighed, and homogenized in 1 ml of PBS. After that, the mixture was serially diluted and plated on THB agar plates.

NET bactericidal assays. Bactericidal assays were carried out to compare the survival abilities of WT *S. equi* subsp. *zooepidemicus*, the Δ*ENuc* and Δ*5Nuc* single-mutant strains, and the Δ*ENuc* Δ*5Nuc* double-mutant strain in the presence of NETs, as described elsewhere (33). For the NET bactericidal assays, neutrophils were activated with phorbol myristate acetate (PMA) (Sigma-Aldrich) for 3 h, after which cytochalasin B (Sigma-Aldrich) was added to a final concentration of 10 μg/ml to suppress cell phagocytosis. WT *S. equi* subsp. *zooepidemicus* bacteria and the three mutant strains were grown to the logarithmic phase in THB, washed 3 times with sterile PBS, and added to neutrophils at an multiplicity of infection (MOI) of 100 at 37°C under a 5% CO_2 atmosphere. After incubation for 90 min, the remaining neutrophils were lysed with 0.02% Triton X-100 (Sigma-Aldrich), and the contents were serially diluted and plated on THB agar plates to determine the number of viable bacteria. For the blank control, the same numbers of CFU for each bacterial strain were plated without incubation. The percent bactericidal activity was calculated as ([CFU per milliliter in the blank control] − [CFU per milliliter in the experimental group])/[CFU per milliliter in the blank control] × 100%.

Nuclease activity assay. To detect the function of ENuc and 5Nuc, nuclease assays were performed as described previously (34, 35). Briefly, 10 μl of calf thymus DNA (Sigma-Aldrich) at a dose of 100 ng/μl was mixed with reaction buffer (300 mM Tris, 3 mM $MgCl_2$, 3 mM $CaCl_2$ [pH 7.0]) in a total volume of 200 μl. The reaction was initiated by addition of 0.1 μM rENuc or r5Nuc protein and DNase I (Sigma-Aldrich). DNase I and reaction buffer were used as a positive control and a negative control, respectively. After incubation at 37°C for 1 h, the reaction was halted with the addition of EDTA at a final concentration of 50 mM. The remaining DNA was visualized using GoldView nucleic acid stain (Vazyme Biotech Co., Ltd., Nanjing, China) under UV light. To further study the function of ENuc and 5Nuc in *S. equi* subsp. *zooepidemicus*, WT *S. equi* subsp. *zooepidemicus* and the three mutant strains were collected in the logarithmic phase of growth and washed 3 times with PBS. Thereafter, 10 μl of bacterial suspension at a dose of 10^6 CFU/ml was incubated with 1 μg of calf thymus DNA in reaction buffer in a total volume of 50 μl at 37°C for 3 h. The remaining DNA was detected as described above. DNase I and reaction buffer were used as a positive control and a negative control, respectively.

Nucleotidase activity and rpHPLC. To digest nucleotides and DNA, reaction buffer (300 mM Tris, 3 mM $MgCl_2$, 3 mM $CaCl_2$ [pH 7.0]) was mixed with 1 mM nucleotides or 1 μg of calf thymus DNA (Sigma-Aldrich) and 0.1 μM rENuc, r5Nuc, or bacteria at doses of 10^6 CFU/ml in a total volume of 500 μl, followed by incubation at 37°C for 1 h. The enzymatic reaction was stopped by addition of EDTA at a final concentration of 50 mM. To assess NET digestion, 0.5 μM rENuc, r5Nuc, or bacteria at a dose of 10^7 CFU/ml was incubated with NETs for 3 h. The solution was centrifuged at 800 × *g* for 10 min, and the supernatant was collected.

The production of deoxyadenosine and adenosine was determined by rpHPLC. Samples underwent chromatography on a 250- by 4.6-mm column (BDS Hypersil C_{18}, 5-μm particle size; Thermo Fisher Scientific). The mobile phase was a solution of 70% 65 mM K_3PO_4 mixed with 30% methanol (pH 6.8). The reaction samples were eluted with the mobile-phase solution for 20 min for deoxyadenosine or 15 min for adenosine at a flow rate of 0.8 ml/min. Peaks were detected via UV absorbance at 260 nm. Commercial nucleosides (Sigma-Aldrich) were used as a standard sample.

Phagocytosis assay. A phagocytosis assay was performed according to a previously described method with modifications (36). RAW 264.7 cells in 24-well plates were pretreated with 50 μM deoxyadenosine for 1 h. WT *S. equi* subsp. *zooepidemicus* was cultured to the mid-exponential phase and washed 3 times with PBS. The RAW 264.7 cells were then washed 3 times with DMEM without fetal bovine serum. Semiconfluent monolayers were infected with 100-μl suspensions containing 10^6 CFU/ml WT *S. equi* subsp. *zooepidemicus*. The control group comprised RAW 264.7 cells without deoxyadenosine pretreatment. Each 24-well plate was concentrated at 800 $\times$ *g* for 10 min and incubated for 2 h at 37°C in 5% CO_2. Cells were washed 3 times with DMEM, followed by treatment with penicillin-streptomycin at a concentration of 100 μg/ml for 1 h to kill extracellular bacteria. The cells were again washed 3 times with DMEM, and 100 μl of trypsin and 900 μl of sterile deionized water were added to release all bacteria. CFU numbers were determined by plating serial dilutions. WT *S. equi* subsp. *zooepidemicus* without incubation was plated on THB agar as the initial concentration, and the percent bactericidal activity in the control group was set as 100%. The percent bactericidal activity in the treatment group was calculated as (bactericidal CFU in the treatment group)/(bactericidal CFU in the control group) $\times$ 100%.

To study the effects of deoxyadenosine on macrophages, we designed the following assay. Briefly, cells were pretreated with or without 50 μM deoxyadenosine for 1 h and then incubated with WT *S. equi* subsp. *zooepidemicus* for 2 h; following this, extracellular bacteria were killed with 100 μg/ml penicillin-streptomycin for 1 h, 2 h, 3 h, and 4 h, and the number of viable bacteria on THB agar was calculated for each time point. The number of surviving bacteria after antibiotic killing for 1 h was considered the total number of viable intracellular bacteria. RAW 264.7 cells without treatment were used as controls. The bactericidal percentage was calculated as follows: ([CFU per milliliter at *n* hours] $-$ [CFU per milliliter at $n + 1$ h])/(CFU per milliliter at 1 h) $\times$ 100%.

Neutrophil and monocyte bactericidal assays. Cells isolated from murine blood were pretreated with or without 50 μM deoxyadenosine; following this, WT *S. equi* subsp. *zooepidemicus* bacteria cultured to the mid-exponential growth phase were washed 3 times with PBS and added to murine blood cells. The mixture was incubated for 90 min and lysed with 0.02% Triton X-100 on ice. The CFU of viable bacteria were determined by plating serial dilutions on THB agar.

Statistical analysis. All experiments were repeated at least 3 times. The Prism 5 software package (GraphPad Software, La Jolla, CA, USA) was used to perform statistical analyses. *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02468-16.

TEXT S1, PDF file, 2.3 MB.

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