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RESEARCH ARTICLE

SodA Contributes to the Virulence of Avian Pathogenic *Escherichia coli* O2 Strain E058 in Experimentally Infected Chickens

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ABSTRACT Strains of avian pathogenic *Escherichia coli* (APEC), the common pathogen of avian colibacillosis, encounter reactive oxygen species (ROS) during the infection process. Superoxide dismutases (SODs), acting as antioxidant factors, can protect against ROS-mediated host defenses. Our previous reports showed that the *sodA* gene (encoding a Mn-cofactor-containing SOD [MnSOD]) is highly expressed during the septicemic infection process of APEC. *sodA* has been proven to be a virulence factor of certain pathogens, but its role in the pathogenicity of APEC has not been fully identified. In this study, we deleted the *sodA* gene from the virulent APEC O2 strain E058 and examined the *in vitro* and *in vivo* phenotypes of the mutant. The *sodA* mutant was more sensitive to hydrogen peroxide in terms of both its growth and viability than was the wild type. The ability to form a biofilm was weakened in the *sodA* mutant. The *sodA* mutant was significantly more easily phagocytosed by chicken macrophages than was the wild-type strain. Chicken infection assays revealed significantly attenuated virulence of the *sodA* mutant compared with the wild type at 24 h postinfection. The virulence phenotype was restored by complementation of the *sodA* gene. Quantitative real-time reverse transcription-PCR revealed that the inactivation of *sodA* reduced the expression of oxidative stress response genes *katE*, *perR,* and *osmC* but did not affect the expression of *sodB* and *sodC*. Taken together, our studies indicate that SodA is important for oxidative resistance and virulence of APEC E058.

IMPORTANCE Avian colibacillosis, caused by strains of avian pathogenic *Escherichia coli*, is a major bacterial disease of severe economic significance to the poultry industry worldwide. The virulence mechanisms of APEC are not completely understood. This study investigated the influence of an antioxidant protein, SodA, on the phenotype and pathogenicity of APEC O2 strain E058. This is the first report demonstrating that SodA plays an important role in protecting a specific APEC strain against hydrogen peroxide-induced oxidative stress and contributes to the virulence of this pathotype strain. Identification of this virulence factor will enhance our knowledge of APEC pathogenic mechanisms, which is crucial for designing successful strategies against associated infections and transmission.

KEYWORDS avian pathogenic *Escherichia coli*, mutant, *sodA*, virulence

As a subset of extraintestinal pathogenic *Escherichia coli* (ExPEC), avian pathogenic *Escherichia coli* (APEC) strains cause a wide range of localized and systemic infections collectively called avian colibacillosis, leading to great economic losses in poultry (1–3). APEC strains use virulence factors for colonizing and invading the host, including adhesins, invasins, outer membrane proteins, iron acquisition systems, and others (4–11).

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APEC strains usually enter the host by crossing the respiratory epithelia and penetrate deeply into the mucosa and submucosa to reach the bloodstream, causing septicemia (12, 13). Phagocytic cells, such as macrophages and heterophils, are elicited into the respiratory tract and play a key role in the initial host defense against APEC (14, 15). An important killing mechanism of phagocytes involves the production of highly microbicidal reactive oxygen metabolites during the so-called oxidative burst, which is generally induced by the engulfment of bacteria (16). Thus, within the host, APEC faces reactive oxygen species (ROS) from the oxidative burst of macrophages. ROS, including superoxide anions (O_2^-), hydroxyl radicals ($OH\cdot^-$), and hydrogen peroxide (H_2O_2), are known to damage DNA, RNA, proteins, and lipids (16, 17).

Superoxide dismutases (SODs) are metalloenzymes that can directly convert deleterious superoxide to molecular oxygen and hydrogen peroxide; hydrogen peroxide is in turn metabolized by catalases and/or peroxidases (18). Hence, these enzymes play an important role in the pathogenesis of some pathogens (19–22). SODs are classified into the following three types depending on the metal cofactor used: Cu,ZnSOD (SodC), MnSOD (SodA), and FeSOD (SodB). Previously, we used an analysis of global gene expression profiles to show that the expression levels of the *sodA* gene in APEC strain E058 were clearly increased during infection *in vivo* (23). However, the role of *sodA* in the pathogenesis of APEC is not fully understood. Therefore, in this work, we constructed a *sodA* deletion mutant of the virulent APEC O2 strain E058 and evaluated its phenotype. The effects of *sodA* on serum and environmental stress resistance, biofilm formation, intracellular survival, virulence, and gene expression were evaluated to fully identify the contribution of SodA to the virulence of APEC O2 strain E058.

RESULTS

Bactericidal effects of serum and environmental stress on *sodA* mutant. Serum resistance and environmental stress adaptation correlate with the virulence of APEC during infection *in vivo* (5, 24). In a serum bactericidal assay, the bacterial numbers of the *sodA* mutant inoculated in serial serum dilutions were similar to those of wild-type strain E058. The control strain DH5α was sensitive to serum, which demonstrated that the serum was active and bactericidal (Fig. 1A). Thus, the deletion of *sodA* did not appear to affect serum resistance.

To identify whether SodA plays a role in adapting to environmental stresses, including alkalinity, acidity, and high osmolarity, we compared the resistance phenotype of the *sodA* mutant and its wild-type strain under these stressful conditions. The results showed that the survival of the *sodA* mutant resembled that of the wild-type strain (Fig. 1B), indicating that SodA was not required for resistance to these environmental stresses.

Effect of *sodA* mutation on sensitivity to oxidative stress. We examined the protective effects of SodA in APEC E058 against the oxidant hydrogen peroxide. Growth rate and survival in the presence of 10 mM H_2O_2 were tested in the *sodA* mutant, complementation, and wild-type strains. The growth of the *sodA* mutant was similar to that of the wild-type in Luria-Bertani broth (LB) medium but was severely inhibited in LB medium containing 10 mM H_2O_2 (Fig. 2A). The *sodA* mutant was poorly viable during exposure to 10 mM H_2O_2 compared to the wild-type and complementation strains (Fig. 2B).

Ability of *sodA* mutant to form biofilm. To determine the effect of *sodA* deletion on biofilm production, the biofilm formation ability of the *sodA* mutant, complementation, and wild-type E058 strains was examined using the microtiter plate method. Crystal violet staining of bacteria grown in LB rich or M9 minimum medium showed that the *sodA* mutant produced a decreased amount of biofilm compared with the wild-type and the complementation strains (Fig. 3), even though their growth rates were similar. Higher production of biofilm was observed under conditions using M9 minimal medium (Fig. 3). These results indicate that *sodA* is involved in biofilm formation by APEC E058.

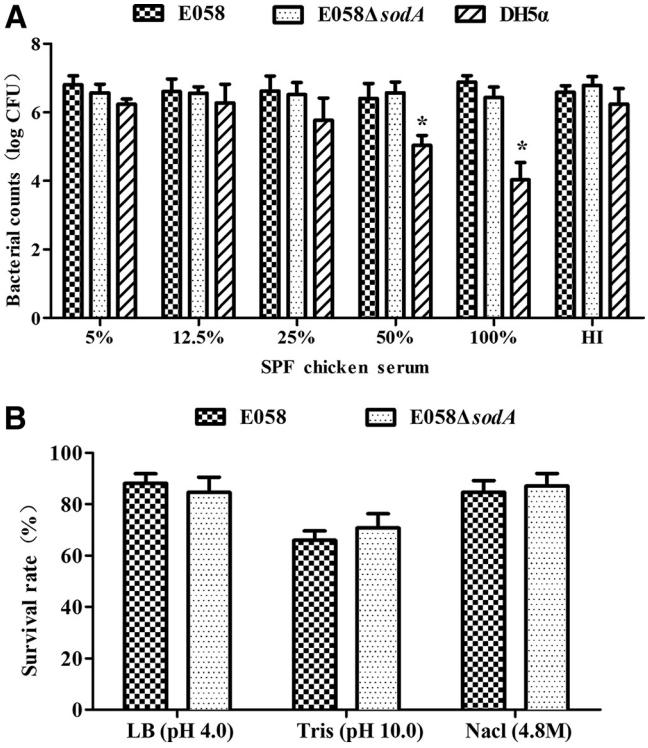

FIG 1 Bacterial resistance to SPF chicken serum and environmental stress. (A) Resistance to SPF chicken serum. Bacteria were incubated with the diluted SPF chicken serum at 37°C for 30 min. Bacteria were counted by plating on LB agar at serial dilutions. HI, heat-inactivated SPF chicken serum. (B) Resistance to environmental stress. Bacteria were examined for adaptation to environmental stresses, including alkalinity, acidity, and high osmolarity. Survival was calculated as the number of bacteria remaining after the stress exposure divided by the initial number of bacteria. Data represent averages of the results from three independent assays (*, $P < 0.05$).

SodA promotes the ability of APEC E058 to survive within chicken macrophages. An ingestion assay was used to analyze the ingestion of the *sodA* mutant by avian macrophage HD-11 cells. The inactivation of *sodA* rendered the bacteria more vulnerable to macrophages. The *sodA* mutant showed a highly increased ingestion ratio compared to the wild-type and complementation strains ($P < 0.01$) (Fig. 4A).

To determine whether SodA affects bacterial survival within macrophages, we compared the bacterial yields of *sodA* mutant, complementation, and wild-type strains recovered at 2, 4, 6, 8, and 12 h postinfection (beginning at time zero [T_0]). The proportions of wild-type bacteria recovered from macrophages were 0.19%, 0.18%, 0.22%, 0.17%, and 0.15% of the inoculum at 2, 4, 6, 8, and 12 h postinfection (h p.i.), which were 1.1- to 1.4-fold higher than those of the primary ingested bacteria (T_0) (Fig. 4B). However, the *sodA* mutant survived poorly in macrophages, with proportions of 0.17%, 0.16%, 0.12%, 0.07%, and 0.04% of the inoculum recovered at 2, 4, 6, 8, and 12 h p.i., which were 1.2- to 4.8-fold lower than those of the primary ingested bacteria (T_0) (Fig. 4B). Moreover, the survival ability within macrophages was restored for the complementation strain. Thus, it can be concluded that *sodA* is involved in the survival of APEC E058 within macrophages.

Competition between the wild-type strain E058 and the *sodA* mutant *in vitro* and *in vivo*. The results of competition experiments comparing mutant and wild-type strains reflect the effects of the mutation on the bacterial growth or virulence. *In vitro*, in LB medium, the *sodA* mutant competed equally with the wild-type strain (data not shown). However, during coinfection *in vivo*, the *sodA* mutant was weakened and showed a significantly reduced competitive index (CI), with mean decreases of 33-fold, 24-fold, 22-fold, and 36-fold compared to the wild-type strain in blood, liver, lung, and spleen, respectively (all $P < 0.01$) (Fig. 5). Meanwhile, complementation of the mutation

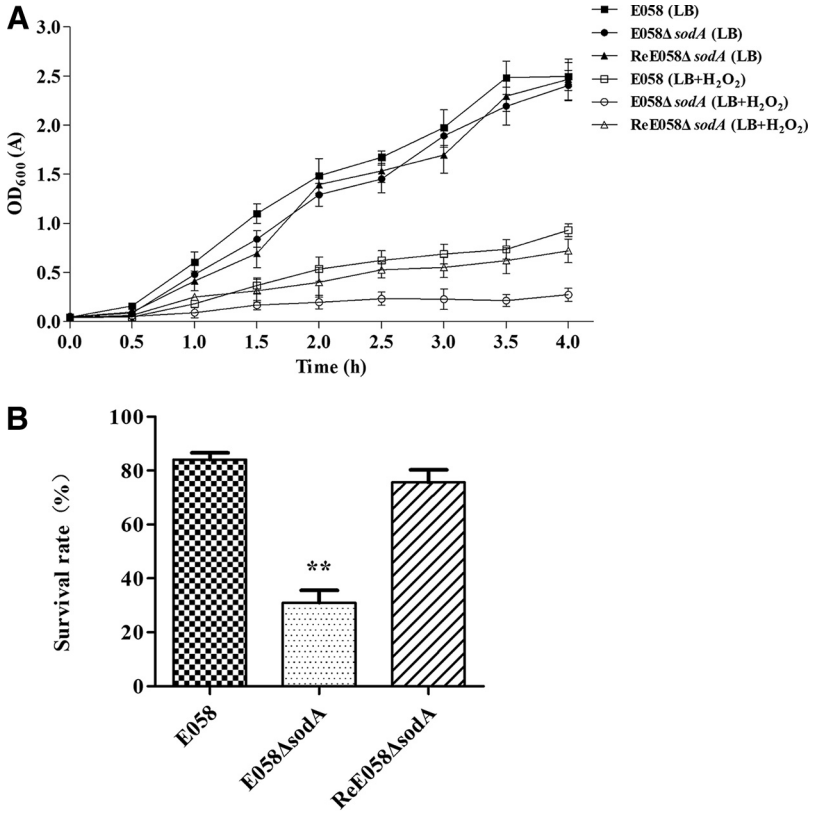

FIG 2 Effect of *sodA* deletion from APEC strain E058 on sensitivity to oxidative stress. (A) Growth curves of *sodA* mutant, complementation, and wild-type strains. Strains E058 (wild type), E058 Δ*sodA* (*sodA* deletion mutant), and ReE058 Δ*sodA* (complementation strain) were grown in Luria-Bertani broth with or without 10 mM H_2O_2 at 37°C, and their growth was determined by measurement of the optical density at 600 nm (OD_{600}). (B) The survival rates of strains in 10 mM H_2O_2 for 30 min at 37°C. Data represent the averages of the results from three independent assays (**, $P < 0.01$).

restored competitive fitness. These results indicate that SodA confers APEC E058 a survival advantage in an *in vivo* competitive colonization model.

SodA contributes to bacterial colonization during systemic infection *in vivo*. To investigate the involvement of *sodA* in APEC E058 virulence, colonization by the *sodA* mutant and its complementation strain, as well as the wild-type strain, was determined in challenged chickens. Bacterial loads in various tissues were compared between

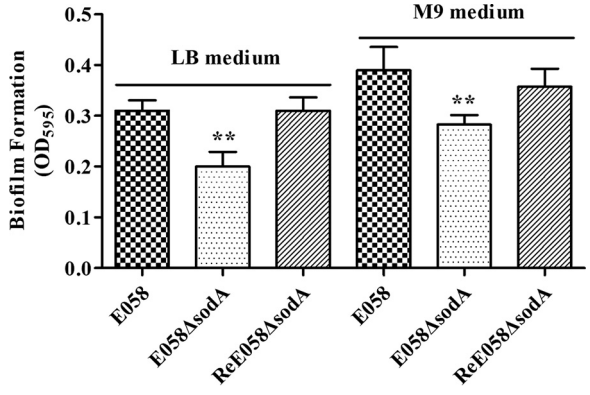

FIG 3 Quantification of the biofilms produced by the *sodA* mutant, the complementation strain, and wild-type APEC strain E058. Biofilm formation was measured for bacteria inoculated in LB rich and M9 minimum media in 96-well microtiter plates for 24 h. OD_{595} values for stained biofilm are the mean ± standard deviation (SD) of the results from three independent biological replicates (**, $P < 0.01$).

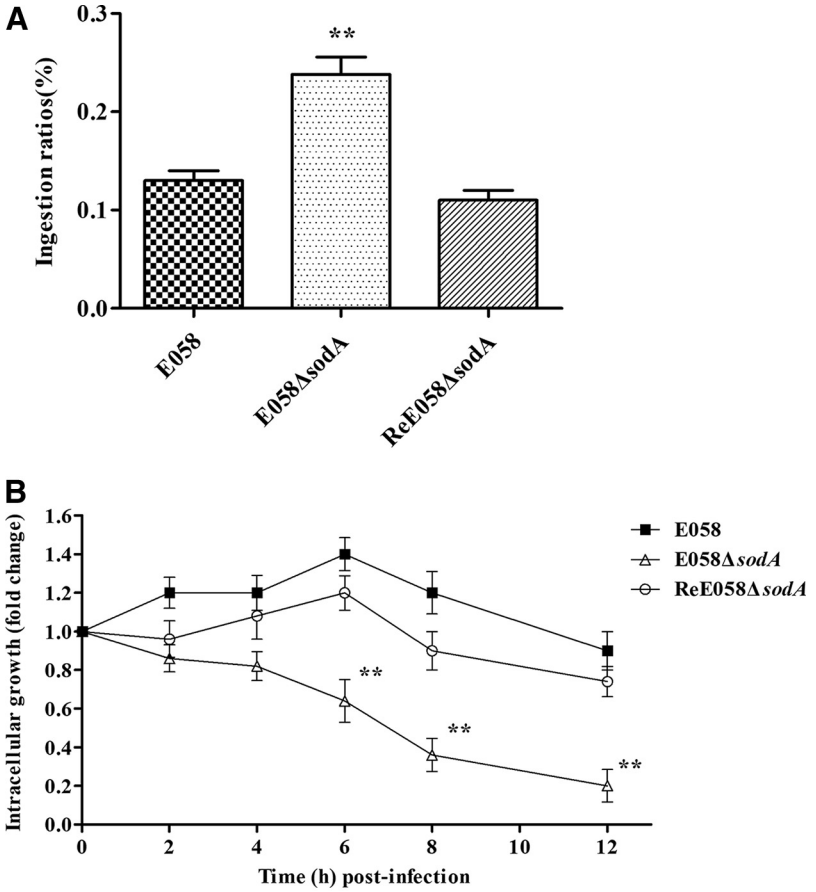

FIG 4 Survival of bacteria in HD-11 chicken macrophages. (A) Ingestion of wild-type strain E058, E058 Δ*sodA* isogenic mutant, and ReE058 Δ*sodA* complementation strain by HD-11 cells. The values represent the average data from three independent experiments. The error bars indicate standard deviations. (B) Intracellular growth of bacteria in chicken macrophage HD-11 cells. The intracellular growth of wild-type strain E058, the E058 Δ*sodA* isogenic mutant, and the ReE058 Δ*sodA* complementation strain were compared over a 12-h period. Standard errors of the mean for the results from three independent experiments are shown. The asterisks indicate statistically significant differences (**, $P < 0.01$).

groups of challenged chickens at 24 h postinfection. The *sodA* mutant colonized poorly *in vivo* in comparison to the corresponding parental strain, with the bacterial loads recovered significantly lower than those of the wild-type strain E058 ($P < 0.01$) (Fig. 6). Complementation of the *sodA* mutant restored bacterial colonization capacity, with bacterial loads reaching the level of the wild-type strain. These results showed that inactivation of *sodA* may lead to reduced bacterial numbers in the internal organs and limit systemic invasion, indicating that SodA is involved in APEC E058 virulence and contributes to survival during systemic infection in the chicken infection model.

Expression profile of genes involved in oxidant resistance. The transcription levels of several oxidant resistance genes were analyzed by quantitative real-time reverse transcription-PCR (qRT-PCR) in the *sodA* mutant. The expression of the oxidative stress response genes *katE*, *perR,* and *osmC* was significantly reduced in the *sodA* mutant strain, while the *sodB* and *sodC* genes were not affected compared to the wild-type strain. The expression levels of the tested genes were restored in the complementation strain (Fig. 7).

DISCUSSION

APEC, like most pathogens, may experience oxidative stress caused by ROS derived from aerobic metabolism, environmental sources, and the host immune response (16,

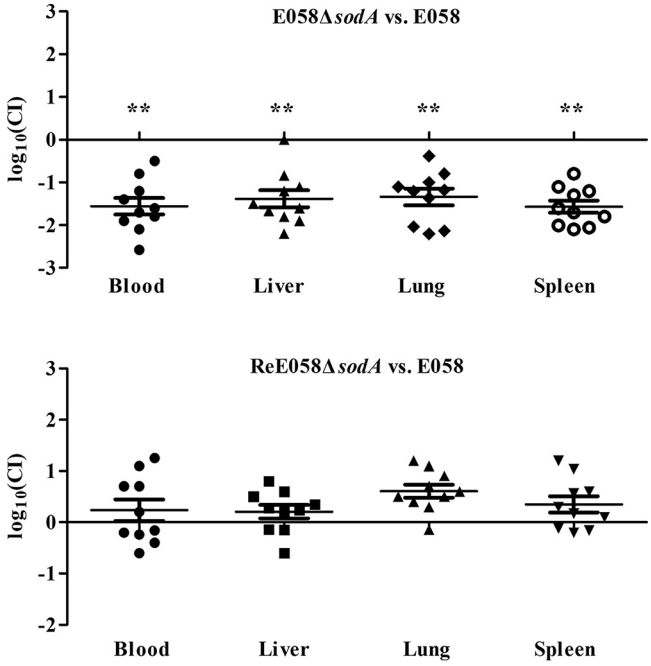

FIG 5 *In vivo* competition assays. Comparative colonization levels, presented as the $\log_{10}$ competitive index, in the blood, liver, lung, and spleen of SPF chickens 24 h after inoculation with a mixture of wild-type APEC strain E058 and the *sodA* mutant or complementation strain. Horizontal bars indicate the mean $\log_{10}$ CI values. Statistically significant decreases in CI values are indicated with asterisks (**, $P < 0.01$).

25). Bacterial antioxidants play a critical role in the detoxification of endogenously and host-derived oxidative radicals during host-pathogen interactions (26, 27). Mn-cofactor-containing superoxide dismutase SodA can destroy the toxic superoxide anion, which is produced both endogenously and within the oxidative burst of host phagocytes. For a comprehensive understanding of the influence of *sodA* on the phenotype and pathogenicity of APEC, the *sodA*-deficient mutant and complementation strains from the highly virulent APEC O2 strain E058 were constructed in this study. The deletion of *sodA* did not affect the growth or serum or environmental stress resistance of APEC E058. However, the *sodA* mutant displayed an increased susceptibility to killing by exogenous hydrogen peroxide (Fig. 2). The sensitivity of the *sodA* mutant to H_2O_2 may be due to increased cellular damage from hydroxyl radicals generated by iron-mediated Fenton reaction (28). This phenotype displayed by the *sodA* mutant is what would be expected of a bacterial strain that has lost an important cytoplasmic defense against oxidative stress and is similar to the phenotypes of other bacterial *sodA* mutants (19, 29).

Biofilms play an important role in protecting pathogens from the host immune system (30). Previous research found that a higher level of expression of the *sodA* gene was induced during the transition to biofilm growth both in *E. coli* and *Staphylococcus aureus* (31, 32). This prompted us to study the effect of the *sodA* deletion on biofilm production by APEC E058. We tested the biofilm formation ability of the bacteria under nutrient-rich or -poor culture conditions (i.e., LB rich or M9 minimum medium, respectively). The results showed that a mutation of *sodA* decreased biofilm formation in both LB and M9 media (Fig. 3). Additionally, biofilm formation in M9 minimal medium provides greater amounts of biofilm, which is probably caused by cell starvation, which triggers biofilm formation. These results suggest that the *sodA* gene involved in the bacterial oxidative stress responses may play a significant role in the development of biofilm.

In view of the role of *sodA* in bacterial resistance to the oxidative burst of host phagocytes, we tested the ability of the *sodA* mutant to survive within chicken

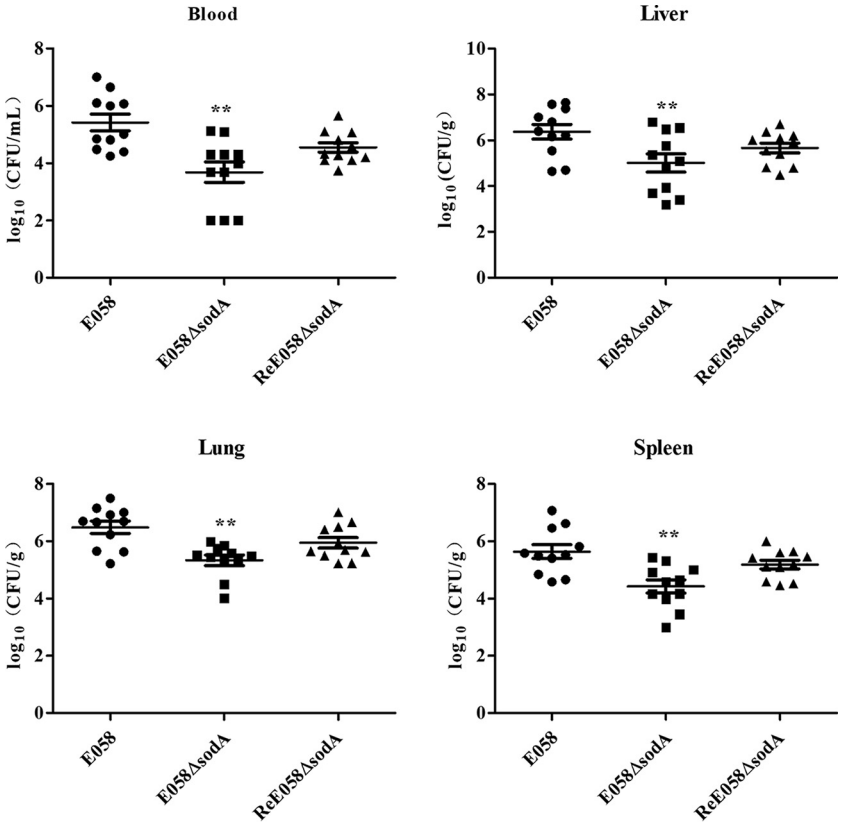

FIG 6 Colonization by the wild-type strain E058, E058 Δ*sodA* mutant, and ReE058 Δ*sodA* complementation strain during systemic infection of chickens. The data are presented as $\log_{10}$ CFU per milliliter of heart blood or $\log_{10}$ CFU per gram of tissue. The horizontal bars indicate the mean values. Each data point represents a sample from an individual chicken. Statistically significant differences, as determined by the Mann-Whitney test, are indicated by asterisks (**, $P < 0.01$).

macrophage HD-11 cells. The *sodA* mutant was more easily ingested by the macrophages, while the wild-type and complemented bacteria appeared to resist ingestion. The deletion of *sodA* also resulted in decreased intracellular growth of APEC E058 in macrophages (Fig. 4), while no differences were found between the survival within macrophages of the wild-type and complemented strains. Thus, the lack of a functional *sodA* gene alters the interaction of APEC E058 with macrophages. Our results indicate that Mn-cofactor-containing SOD (MnSOD), by its ability to scavenge the primary product of the oxidative burst, is able to effectively impair the

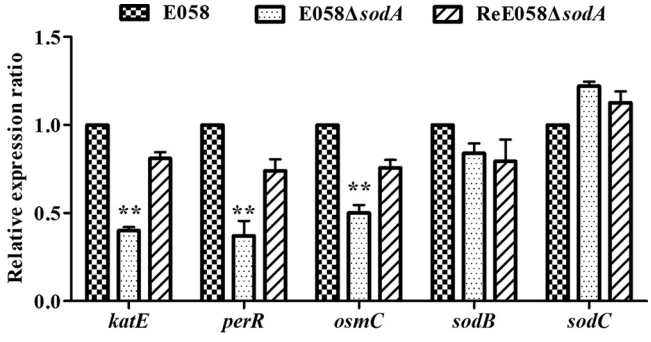

FIG 7 Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis of gene expression. Expression of genes involved in oxidative resistance in APEC was measured by qRT-PCR for each strain. Data were normalized to the housekeeping gene *gapA*. Results are relative expression ratios compared to wild-type strain E058 (**, $P < 0.01$). The error bars indicate standard deviations.

host response to APEC infection and thus may represent an important virulence factor in APEC E058.

To determine the contribution of *sodA* to APEC infection, we performed avian experimental infection tests. The *sodA* mutant was significantly outcompeted by the wild-type strain during *in vivo* coinfection (Fig. 5). Coinfection experiments can show fitness defects as well as decreased virulence, depending on the type of mutation. To test whether or not the drop in competitive fitness also occurred *in vitro* or if it was only seen during infection, we also conducted *in vitro* competition test. The *sodA* mutant competed equally with the wild-type strain in LB medium, which excluded the possibility of a fitness defect and demonstrated the decreased virulence phenotype of the mutant. As we have shown a potential role for SodA in survival in a macrophage cell line, we further investigated the survival of the mutant strain in the blood. The decreased numbers for the *sodA* mutant in the blood correlate with less of a capacity to survive in phagocytic cells. Moreover, the *sodA* mutant showed significantly decreased colonization compared with the wild-type strain in various organs tested in a single-strain challenge model (Fig. 6), implying that SodA plays an important role in the virulence of APEC E058 in chickens. The complementation strain showed restored virulence and colonized the internal organs of inoculated chickens to the same extent as the wild-type strain. Thus, the disruption of the *sodA* gene in APEC E058 leads to attenuation of virulence in systemic infection in chickens.

A previous report described a lack of a role for MnSOD in an extraintestinal pathogenic *E. coli* O18 strain in a newborn rat meningitis model (33). Their results indicated, however, that the elimination of *sodA* had little or no effect on either gastrointestinal tract colonization or the transition to bacteremia following oral inoculation in neonatal rats. Their results seem to be contradiction to ours, since APEC strains are closely related to the ExPEC strains that are associated with human neonatal meningitis. The contradiction might be due to the differences in the strains (APEC O2 versus NMEC O18) or the different inoculation routes (chickens infected via the air sac versus neonatal rat infected via oral administration). In their study, they determined the proportions of *E. coli* bacteremia in neonatal rats after oral inoculation but did not quantify the bacterial loads in the bloodstream, which may not fully reflect the virulence difference between the wild type and isogenic mutant. As the authors of that study indicated, their results did not rule out the possibility that SOD activity is important for survival in the bloodstream. Alternatively, previous work has shown that the virulence mechanisms of ExPEC strains can vary. Some may be invasive and internalize within cells and survive in phagocytes, whereas others may be highly refractory to phagocytosis and remain mainly extracellular. Such differences in pathogenic lifestyle could also result in differences in the importance of certain genes or pathways that would be dependent on the pathogenic mechanisms of the particular APEC strain or ExPEC strain. Thus, the previous results on ExPEC suggest that SodA may not always be important for certain types of ExPEC strains.

We also measured the effects of *sodA* deletion on the expression of other oxidation resistance genes. All of the *katE*, *perR,* and *osmC* genes, which are known to participate in the antioxidant defense mechanism against H_2O_2-induced stress (34–36), had lower transcriptional levels in the *sodA* mutant (Fig. 7) than in the wild type, which may be due to the loss of function of SodA that results in decreased production of H_2O_2 from intracellular superoxide radicals. The transcription of *sodB* or *sodC* was not affected by the disruption of *sodA*, suggesting that no compensation effect exists between these isozymes. Our previous array data showed that the *sodA* gene was highly expressed *in vivo*, while the other two metal-cofactor-containing SODs (*sodB* and *sodC*) were normally expressed (23), which implied that SodA may play a more important role than the other two SODs in the extraintestinal infectious process of APEC.

Taken together, our results suggest that *sodA* plays an important role in protecting against hydrogen peroxide (H_2O_2)-induced oxidative stress and that it contributes to the virulence of APEC strain E058.

TABLE 1 Bacterial strains and plasmids used in this study

Strain, plasmid, or cell line	Characteristics[a]	Source or reference
Strains		
E058	Wild-type avian *E. coli* serotype O2	37
E058 Δ*sodA*	E058 Δ*sodA*::*cat*	This study
ReE058 Δ*sodA*	Complementation of E058 Δ*sodA*	This study
DH5α	*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) RelA1 Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)M15]	Invitrogen
Plasmids		
pACYC184	Medium-copy-number vector, p15A *ori*, Cm^r Tc^r	Gifted
pACYC-*sodA*	pACYC184 carrying *sodA* ORF and its putative native promoter	This study
pKD46	Amp^r; expresses λ Red recombinase	38
pKD3	*cat* gene, template plasmid	38
Cell line HD-11	Chicken macrophage line, chicken myelomonocytic transformed by the myc-encoding MC29 virus	42

[a]Nal^r, nalidixic acid resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; ORF, open reading frame; Amp^r, ampicillin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Oligonucleotide primers obtained from Sangon (Shanghai, China) are listed in Table 2. APEC O2 serotype E058 (*iss*$^+$ *iut*$^+$ *iroN*$^+$ *tsh*$^+$ *irp*$^+$ *cvaC*$^+$ *sit*$^+$ *mntH*$^+$) is a highly virulent strain, isolated from a chicken with a respiratory infection (37). Bacteria were routinely grown at 37°C in Luria-Bertani broth (LB) with aeration. When necessary, ampicillin and chloramphenicol were added at 60 and 30 μg/ml, respectively.

Construction of the *sodA* mutant and its complementation strain. The *sodA* mutant was constructed using gene replacement methods based on the lambda Red recombinase system (38). The chloramphenicol resistance cassette, flanked by 5′ and 3′ sequences of *sodA,* was obtained by amplifying template plasmid pKD3 using primers AF and AR (Table 2). The PCR product was transformed into strain E058 containing pKD46. The mutant (E058 Δ*sodA*) was confirmed by PCR and verified by sequence analysis.

To construct the complementation strain, the *sodA* open reading frame and its upstream promoter were amplified and subcloned into pACYC184 using primer pairs RAF and RAR (Table 2). The purified recombinant plasmid pACYC-*sodA* was transformed into the *sodA* mutant strain to complement the *sodA* gene deletion to generate the ReE058 Δ*sodA* strain.

TABLE 2 Primers designed and used in this study

Primer by use	Primer sequence (5′–3′)	Target gene
Deletion		
AF	ATGAGCTATACCCTGCCATCCCTGCCGTATGCTTACGATGCCCTGGAACCGCACTTCGATAAGCAGACCAT TGTGTAGGCTGGAGCTGCT	*sodA*
AR	TTATTTTTTCGCCGCAAAACGTGCCGCTGCTTCGTCCCAGTTCACCACGTTCCAGAACTCTTTAATGTAGAT GGGAATTAGCCATGGTCC	
Complementation		
RAF	CTC**AAGCTT**CCATCGTAATCGCGTTAC[a]	*sodA*
RAR	CTC**TCTAGA**TTATTTTTTCGCCGCAAAACG[b]	
RT-qPCR		
katE RT-F	TTGTGGGAAGCCATTGAA	*katE*
katE RT-R	GCGATTGAGCACCATTTT	*katE*
perR RT-F	AGATGACGCCACCCAATA	*perR*
perR RT-R	GGGCATACCAGTTTACCG	*perR*
osmC RT-F	CGGGATTCACGCCAACAT	*osmC*
osmC RT-R	CGGCACCGCAACTTCACT	*osmC*
sodB RT-F	TATCACTACGGCAAGCACC	*sodB*
sodB RT-R	CAGGCAGTTCCAGTAGAAAGTA	*sodB*
sodC RT-F	ACATGAAGGGCCAGAAGG	*sodC*
sodC RT-R	ATATTATCGCCGCCAACG	*sodC*

[a]HindIII restriction site is in bold.
[b]XbaI restriction site is in bold.

Oxidative stress experiments. Overnight (18-h) cultures of the *sodA* mutant, complemented strain, and wild-type strain E058 were diluted 1:100 into LB broth and incubated until the optical density at 600 nm (OD_{600}) reached 0.4. The bacteria were challenged in LB containing 10 mM H_2O_2 for 30 min at 37°C. Viable cells were counted after the oxidative stress challenge, and the percent survival was calculated as the number of CFU per milliliter remaining after the oxidant treatment divided by the initial number of CFU per milliliter times 100.

To determine bacterial growth curves under oxidative stress, overnight cultures were washed twice in phosphate-buffered saline (PBS) and standardized to an OD_{600} of 1.0, and approximately 10^6 CFU were inoculated into 5 ml LB with or without 10 mM H_2O_2. Bacterial growth was measured every 30 min by spectrophotometry (OD_{600}). The experiments were performed in triplicate.

Adaptation to chicken serum and environmental stress. Complement-sufficient specific-pathogen-free (SPF) chicken serum was prepared and pooled from 10 SPF chickens. A bactericidal assay was performed in a 96-well plate, as described previously, but with the following modifications (39). SPF chicken serum was diluted to 5, 12.5, 25, 50, and 100% in PBS (pH 7.2). Bacteria (10 μl containing 10^6 CFU) were inoculated into wells containing 190 μl of the diluted SPF chicken serum and heat-inactivated SPF chicken serum and then incubated at 37°C for 30 min. Bacteria were counted by plating on LB agar at serial dilutions. *E. coli* DH5α was used as the control strain.

Bacterial adaptation to environmental stress was examined as described previously (5). The bacteria were suspended in PBS and diluted to 10^8 CFU/ml. For the acidic and alkaline adaptation, 100-μl bacterial suspensions were exposed to 900 μl LB broth (pH 4.0) or 100 mM Tris (pH 10.0) for 30 min. For high-osmolarity stress, bacteria were mixed equally with 4.8 M NaCl and incubated for 1 h at 37°C. After environmental stress exposure, bacteria were diluted serially and plated on LB agar. Survival was calculated as the number of bacteria remaining after the stress exposure divided by the initial number of bacteria.

Biofilm assay. The biofilm assay was carried out as previously described but with a slight modification (40). Overnight cultures were inoculated at 10^8 CFU/ml in LB or M9 minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ per liter of water [pH 7.0]) supplemented with 0.4% glucose. Then, 200 μl of bacterial cultures was incubated in 96-well plates at 37°C for 24 h. After three washes with PBS, the samples were stained for 30 min with 0.1% crystal violet, rinsed with sterile PBS, and air dried. Finally, the dye was dissolved in 95% ethanol. The solubilized biofilm was determined by measuring the OD_{595}. The experiment was performed in triplicate.

Ingestion and intracellular survival assay. Ingestion and intracellular survival assays were performed as previously described (41). The avian macrophage cell line HD-11 was cultured in Dulbecco's modified Eagle's medium (Gibco, NY) with 10% fetal bovine serum (PAA, Pasching, Australia). Cells were maintained at 37°C in a 5% CO_2 environment with 2×10^5 cells per well in 24-well cell culture plates. Bacteria were added to the cells with a multiplicity of infection of 100 for 1 h to allow ingestion. Wells were washed with PBS, and the appropriate cell culture medium containing 100 μg/ml gentamicin was added for 1.5 h to kill extracellular bacteria. At this time (T_0) and after different incubation periods (2, 4, 6, 8, and 12 h), the macrophages were washed with PBS, lysed with 0.1% Triton X-100, diluted in PBS, and plated onto LB agar plates for APEC CFU determination. The ingestion ratio was determined by dividing the number of ingested bacterial cells at T_0 by the initial bacterial inoculation number. Intracellular growth was expressed as the fold change in the bacterial number at an additional incubation time point (i.e., 2, 4, 6, 8, or 12 h) relative to the number of ingested bacteria at T_0.

***In vitro* and *in vivo* competition assays.** For the *in vitro* competition assay, cultures of wild-type strain E058 and the *sodA* mutant were mixed in a ratio of 1:1, incubated in LB broth for 4 h at 37°C, and then plated onto LB medium with or without chloramphenicol. For the *in vivo* competition assay, 5-week-old SPF chickens (White Leghorn; Jinan SPAFAS Poultry Co. Ltd., Jinan, China) were inoculated with cultures of wild-type strain E058 and its E058 Δ*sodA* mutant or ReE058 Δ*sodA* complemented strain, mixed in a ratio of 1:1 (1×10^8 CFU for each strain), via the left air sac. The chickens were provided with food and water *ad libitum* and treated in accordance with the regulations of the Administration of Affairs Concerning Experimental Animals; the protocol was approved by the Animal Care and Use Committee of Yangzhou University (approval ID SYXK [Su] 2007–0005, 21 September 2016). At 24 h after infection, cardiac blood samples (0.2 ml) were collected and suspended in 0.8 ml of PBS. Tissue samples (0.25 g) from livers, lungs, and spleens of the chickens were collected, weighed, triturated in 1 ml of PBS, and homogenized. Serial dilutions were plated on LB medium with or without chloramphenicol to select for the mutants and total bacteria, respectively. The results are shown as the $\log_{10}$ competitive index (CI). The CI was calculated for each mutant by dividing the output ratio (mutant/wild type) by the input ratio (mutant/wild type).

Bacterial colonization ability during systemic infection. Five-week-old SPF chickens were infected with the wild-type, mutant, and complementation strains via the left thoracic air sac, with each bacterial suspension containing 10^8 CFU. After 24 h, 10 chickens from each group were euthanized by carbon dioxide asphyxiation, and organs were removed aseptically. Cardiac blood samples (0.2 ml) were collected and suspended in 0.8 ml of PBS. Tissue samples (0.25 g) from livers, lungs, and spleens of the chickens were collected, weighed, triturated in 1 ml of PBS, and homogenized. Tenfold serial dilutions of homogenates were plated onto LB agar plates to determine the numbers of bacteria.

Quantitative real-time reverse transcription-PCR. To analyze how gene expression was affected by the disruption of *sodA*, we performed qRT-PCR analyses for various oxidation resistance genes in APEC E058. Overnight cultures of strains were diluted 1:100 in fresh LB medium and grown until logarithmic phase. Following RNA isolation and cDNA synthesis, qRT-PCR was performed to determine the transcrip-

tion levels of the selected genes using SYBR premix *Ex Taq* and gene-specific primers (Table 2), and the data were normalized to the *gapA* transcript.

Statistical analysis. Differences between groups were analyzed using the Statistical Package for the Social Sciences (SPSS version 15.0; SPSS, Chicago, IL). *P* values of <0.05 were considered significant.

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REFERENCES

1. Johnson TJ, Kariyawasam S, Wannemuehler Y, Mangiamele P, Johnson SJ, Doetkott C, Skyberg JA, Lynne AM, Johnson JR, Nolan LK. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J Bacteriol 189:3228–3236. https://doi.org/10.1128/JB.01726-06.
2. Younis G, Awad A, Mohamed N. 2017. Phenotypic and genotypic characterization of antimicrobial susceptibility of avian pathogenic *Escherichia coli* isolated from broiler chickens. Vet World 10:1167–1172. https://doi.org/10.14202/vetworld.2017.1167-1172.
3. Dziva F, Stevens MP. 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol 37:355–366. https://doi.org/10.1080/03079450802216652.
4. Kariyawasam S, Johnson TJ, Nolan LK. 2006. The pap operon of avian pathogenic *Escherichia coli* strain O1:K1 is located on a novel pathogenicity island. Infect Immun 74:744–749. https://doi.org/10.1128/IAI.74.1.744-749.2006.
5. Wang S, Bao Y, Meng Q, Xia Y, Zhao Y, Wang Y, Tang F, ZhuGe X, Yu S, Han X, Dai J, Lu C. 2015. IbeR facilitates stress-resistance, invasion and pathogenicity of avian pathogenic *Escherichia coli*. PLoS One 10:e0119698. https://doi.org/10.1371/journal.pone.0119698.
6. Hejair HMA, Ma J, Zhu Y, Sun M, Dong W, Zhang Y, Pan Z, Zhang W, Yao H. 2017. Role of outer membrane protein T in pathogenicity of avian pathogenic *Escherichia coli*. Res Vet Sci 115:109–116. https://doi.org/10.1016/j.rvsc.2017.01.026.
7. Hejair HMA, Zhu Y, Ma J, Zhang Y, Pan Z, Zhang W, Yao H. 2017. Functional role of *ompF* and *ompC* porins in pathogenesis of avian pathogenic *Escherichia coli*. Microb Pathog 107:29–37. https://doi.org/10.1016/j.micpath.2017.02.033.
8. Sabri M, Caza M, Proulx J, Lymberopoulos MH, Bree A, Moulin-Schouleur M, Curtiss R, III, Dozois CM. 2008. Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic *Escherichia coli* O78 strain χ7122. Infect Immun 76:601–611. https://doi.org/10.1128/IAI.00789-07.
9. Vidotto MC, Navarro HR, Gaziri LC. 1997. Adherence pili of pathogenic strains of avian *Escherichia coli*. Vet Microbiol 59:79–87. https://doi.org/10.1016/S0378-1135(97)00124-7.
10. Janben T, Schwarz C, Preikschat P, Voss M, Philipp HC, Wieler LH. 2001. Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. Int J Med Microbiol 291:371–378.
11. Caza M, Lepine F, Milot S, Dozois CM. 2008. Specific roles of the *iroBCDEN* genes in virulence of an avian pathogenic *Escherichia coli* O78 strain and in production of salmochelins. Infect Immun 76:3539–3549. https://doi.org/10.1128/IAI.00455-08.
12. Ackermann MR, Cheville NF. 1991. Ultrastructural studies of the lung of turkeys (Meleagris gallopavo) inoculated intratracheally with *Escherichia coli*. Vet Pathol 28:183–191. https://doi.org/10.1177/030098589102800301.
13. Pourbakhsh SA, Boulianne M, Martineau-Doizé B, Fairbrother JM. 1997. Virulence mechanisms of avian fimbriated *Escherichia coli* in experimentally inoculated chickens. Vet Microbiol 58:195–213. https://doi.org/10.1016/S0378-1135(97)00163-6.
14. Mellata M, Dho-Moulin M, Dozois CM, Curtiss R, III, Lehoux B, Fairbrother JM. 2003. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. Infect Immun 71:494–503. https://doi.org/10.1128/IAI.71.1.494-503.2003.
15. de Geus ED, Vervelde L. 2013. Regulation of macrophage and dendritic cell function by pathogens and through immunomodulation in the avian mucosa. Dev Comp Immunol 41:341–351. https://doi.org/10.1016/j.dci.2013.03.008.
16. Miller RA, Britigan BE. 1997. Role of oxidants in microbial pathophysiology. Clin Microbiol Rev 10:1–18. https://doi.org/10.1128/CMR.10.1.1.
17. Bayir H. 2005. Reactive oxygen species. Crit Care Med 33:S498–S501. https://doi.org/10.1097/01.CCM.0000186787.64500.12.
18. Hassan HM. 1984. Determination of microbial damage caused by oxygen free radicals, and the protective role of superoxide dismutase. Methods Enzymol 105:404–412. https://doi.org/10.1016/S0076-6879(84)05056-4.
19. Tang Y, Zhang X, Wu W, Lu Z, Fang W. 2012. Inactivation of the *sodA* gene of *Streptococcus suis* type 2 encoding superoxide dismutase leads to reduced virulence to mice. Vet Microbiol 158:360–366. https://doi.org/10.1016/j.vetmic.2012.02.028.
20. Esteve-Gassent MD, Elliott NL, Seshu J. 2009. *sodA* is essential for virulence of *Borrelia burgdorferi* in the murine model of Lyme disease. Mol Microbiol 71:594–612. https://doi.org/10.1111/j.1365-2958.2008.06549.x.
21. Bakshi CS, Malik M, Regan K, Melendez JA, Metzger DW, Pavlov VM, Sellati TJ. 2006. Superoxide dismutase B gene (*sodB*)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. J Bacteriol 188:6443–6448. https://doi.org/10.1128/JB.00266-06.
22. Battistoni A. 2003. Role of prokaryotic Cu, Zn superoxide dismutase in pathogenesis. Biochem Soc Trans 31:1326–1329. https://doi.org/10.1042/bst0311326.
23. Gao Q, Xia L, Liu J, Wang X, Gao S, Liu X. 2016. DNA microarray-mediated transcriptional profiling of avian pathogenic *Escherichia coli* O2 strain E058 during its infection of chicken. Microb Pathog 100:1–9. https://doi.org/10.1016/j.micpath.2016.08.032.
24. Mellata M, Dho-Moulin M, Dozois CM, Curtiss R, III, Brown PK, Arne P, Bree A, Desautels C, Fairbrother JM. 2003. Role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infect Immun 71:536–540. https://doi.org/10.1128/IAI.71.1.536-540.2003.
25. Fang FC. 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol 2:820–832. https://doi.org/10.1038/nrmicro1004.
26. Piddington DL, Fang FC, Laessig T, Cooper AM, Orme IM, Buchmeier NA. 2001. Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an

oxidative burst. Infect Immun 69:4980–4987. https://doi.org/10.1128/IAI.69.8.4980-4987.2001.
27. Edwards KM, Cynamon MH, Voladri RK, Hager CC, DeStefano MS, Tham KT, Lakey DL, Bochan MR, Kernodle DS. 2001. Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis*. Am J Respir Crit Care Med 164:2213–2219. https://doi.org/10.1164/ajrccm.164.12.2106093.
28. Takeda K, Sato J, Goto K, Fujita T, Watanabe T, Abo M, Yoshimura E, Nakagawa J, Abe A, Kawasaki S, Niimura Y. 2010. *Escherichia coli* ferredoxin-NADP$^+$ reductase and oxygen-insensitive nitroreductase are capable of functioning as ferric reductase and of driving the Fenton reaction. Biometals 23:727–737. https://doi.org/10.1007/s10534-010-9339-8.
29. Poyart C, Pellegrini E, Gaillot O, Boumaila C, Baptista M, Trieu-Cuot P. 2001. Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of *Streptococcus agalactiae*. Infect Immun 69:5098–5106. https://doi.org/10.1128/IAI.69.8.5098-5106.2001.
30. Valle J, Solano C, Garcia B, Toledo-Arana A, Lasa I. 2013. Biofilm switch and immune response determinants at early stages of infection. Trends Microbiol 21:364–371. https://doi.org/10.1016/j.tim.2013.05.008.
31. Resch A, Rosenstein R, Nerz C, Gotz F. 2005. Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. Appl Environ Microbiol 71:2663–2676. https://doi.org/10.1128/AEM.71.5.2663-2676.2005.
32. Schembri MA, Kjaergaard K, Klemm P. 2003. Global gene expression in *Escherichia coli* biofilms. Mol Microbiol 48:253–267. https://doi.org/10.1046/j.1365-2958.2003.03432.x.
33. Bloch CA, Thorne GM, Ausubel FM. 1989. General method for site-directed mutagenesis in *Escherichia coli* O18ac:K1:H7: deletion of the inducible superoxide dismutase gene, *sodA*, does not diminish bacteremia in neonatal rats. Infect Immun 57:2141–2148.
34. Lee JW, Helmann JD. 2006. The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. Nature 440:363–367. https://doi.org/10.1038/nature04537.
35. Jha V, Chelikani P, Carpena X, Fita I, Loewen PC. 2012. Influence of main channel structure on H_2O_2 access to the heme cavity of catalase KatE of *Escherichia coli*. Arch Biochem Biophys 526:54–59. https://doi.org/10.1016/j.abb.2012.06.010.
36. Saikolappan S, Das K, Sasindran SJ, Jagannath C, Dhandayuthapani S. 2011. OsmC proteins of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* protect against organic hydroperoxide stress. Tuberculosis (Edinb) 91:S119–S127. https://doi.org/10.1016/j.tube.2011.10.021.
37. Zhao L, Gao S, Huan H, Xu X, Zhu X, Yang W, Gao Q, Liu X. 2009. Comparison of virulence factors and expression of specific genes between uropathogenic *Escherichia coli* and avian pathogenic *E. coli* in a murine urinary tract infection model and a chicken challenge model. Microbiology 155:1634–1644. https://doi.org/10.1099/mic.0.024869-0.
38. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. https://doi.org/10.1073/pnas.120163297.
39. Dozois CM, Dho-Moulin M, Bree A, Fairbrother JM, Desautels C, Curtiss R, III. 2000. Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the tsh genetic region. Infect Immun 68:4145–4154. https://doi.org/10.1128/IAI.68.7.4145-4154.2000.
40. Luo G, Huang L, Su Y, Qin Y, Xu X, Zhao L, Yan Q. 2016. *flrA*, *flrB* and *flrC* regulate adhesion by controlling the expression of critical virulence genes in *Vibrio alginolyticus*. Emerg Microbes Infect 5:e85. https://doi.org/10.1038/emi.2016.82.
41. Gahring LC, Heffron F, Finlay BB, Falkow S. 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. Infect Immun 58:443–448.
42. Beug H, von Kirchbach A, Doderlein G, Conscience JF, Graf T. 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18:375–390. https://doi.org/10.1016/0092-8674(79)90057-6.