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Dimethyl Adenosine Transferase (KsgA) Deficiency in *Salmonella enterica* Serovar Enteritidis Confers Susceptibility to High Osmolarity and Virulence Attenuation in Chickens

Kim Lam Chiok,[a] Tarek Addwebi,[a] Jean Guard,[b] Devendra H. Shah[a]

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA[a]; Egg Quality and Safety Research Unit, Agriculture Research Service, United States Department of Agriculture, Athens, Georgia, USA[b]

Dimethyl adenosine transferase (KsgA) performs diverse roles in bacteria, including ribosomal maturation and DNA mismatch repair, and synthesis of KsgA is responsive to antibiotics and cold temperature. We previously showed that a *ksgA* mutation in *Salmonella enterica* serovar Enteritidis results in impaired invasiveness in human and avian epithelial cells. In this study, we tested the virulence of a *ksgA* mutant (the *ksgA*::Tn*5* mutant) of *S.* Enteritidis in orally challenged 1-day-old chickens. The *ksgA*::Tn*5* mutant showed significantly reduced intestinal colonization and organ invasiveness in chickens compared to those of the wild-type (WT) parent. Phenotype microarray (PM) was employed to compare the *ksgA*::Tn*5* mutant and its isogenic wild-type strain for 920 phenotypes at 28°C, 37°C, and 42°C. At chicken body temperature (42°C), the *ksgA*::Tn*5* mutant showed significantly reduced respiratory activity with respect to a number of carbon, nitrogen, phosphate, sulfur, and peptide nitrogen nutrients. The greatest differences were observed in the osmolyte panel at concentrations of ≥6% NaCl at 37°C and 42°C. In contrast, no major differences were observed at 28°C. In independent growth assays, the *ksgA*::Tn*5* mutant displayed a severe growth defect in high-osmolarity (6.5% NaCl) conditions in nutrient-rich (LB) and nutrient-limiting (M9 minimum salts) media at 42°C. Moreover, the *ksgA*::Tn*5* mutant showed significantly reduced tolerance to oxidative stress, but its survival within macrophages was not impaired. Unlike *Escherichia coli*, the *ksgA*::Tn*5* mutant did not display a cold-sensitivity phenotype; however, it showed resistance to kasugamycin and increased susceptibility to chloramphenicol. To the best of our knowledge, this is the first report showing the role of *ksgA* in *S.* Enteritidis virulence in chickens, tolerance to high osmolarity, and altered susceptibility to kasugamycin and chloramphenicol.

In bacteria, the *ksgA* gene encodes a dimethyl adenosine transferase (KsgA) protein that belongs to the KsgA/Dim1 family of universally conserved methyltransferases. According to Harris et al. (1), the KsgA/Dim1 family is one of the 50 factors conserved in all kingdoms of life and probably the only one of its kind that was part of the genetic core of the last universal ancestor. Despite being highly conserved, KsgA mediates diverse functions in bacteria. For example, in *Escherichia coli*, KsgA acts as a 16S rRNA adenine methyltransferase by adding two methyl groups to the two highly conserved adenine residues located at positions 1518 and 1519 (numbered in the *E. coli* system) within the universally conserved helix 45 at the 3′ end of the translationally inactive form of the 16S rRNA subunit (2). These methyl groups are donated by *S*-adenosylmethionine, which is also highly conserved among bacteria, to produce N^6,N^6-dimethyladenosine bases (3). Methylation of 16S rRNA is important in ribosomal biogenesis and impacts ribosome functions during translation initiation and elongation phases (4). Deficiency of KsgA in *E. coli* results in altered ribosome profiles characterized by accumulation of free immature small ribosomal subunits (SSU) that are unable to enter the translation cycle. Current models indicate that the KsgA-mediated 30S rRNA methylation is a conserved maturation signal that enables release of KsgA from mature SSUs, resulting in conformational changes that permit SSUs to join the large subunit and IF3 to initiate translation (5).

KsgA also possesses a DNA glycosylase/AP lyase activity that prevents chromosomal mutations by repairing mismatched DNA strands. More specifically, KsgA excises mismatched cytosine bases opposing oxidatively damaged thymine bases by a β-excision mechanism in *E. coli* (6). Lack of RNA methylase activity caused by mutations within the *ksgA* locus in *E. coli* and *Neisseria gonorrhoeae* results in resistance to the aminoglycoside antibiotic kasugamycin (KSG) (3, 7). KSG inhibits translation initiation in bacteria by blocking tRNA binding to the 30S ribosomal subunit, mimicking the mRNA molecule and occupying its place in the peptidyl (P) and exit (E) sites of the ribosome, which eventually disturbs the mRNA-tRNA-ribosome spatial interaction (8). Exogenous supplementation of wild-type KsgA can rescue KSG sensitivity in KSG-resistant strains of *E. coli* (3). In addition, *E. coli* strains lacking KsgA also show a 4-fold reduction in the MIC of gentamicin (9). In contrast, a *ksgA* mutant of *Staphylococcus aureus* was more sensitive to kanamycin and paromomycin, probably due to the conformational changes distal to the aminoglycoside binding site in the SSU, which are further propagated from the KsgA methylation site (10). Recently, disruption of *ksgA* in a clarithromycin-resistant *Mycobacterium tuberculosis* strain resulted in abolishment of resistance (11), suggesting that KsgA-mediated drug resistance is likely to be strain and species dependent.

Depending on the bacterial system, lack of methylation of the 16S rRNA subunit due to KsgA deficiency also leads to a temper-

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Address correspondence to Devendra H. Shah, dshah@vetmed.wsu.edu.

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ature-sensitive phenotype. Connolly et al. (5) showed that *E. coli* mutant strains lacking KsgA display growth defects at suboptimal temperatures (25°C and 20°C). This phenotype was characterized by less efficient ribosome biogenesis as fewer mature and translationally active ribosomes were available at low temperature and immature ribosomal subunits accumulate in these cells (5). In contrast, a *Bacillus subtilis* KsgA-deficient mutant showed a significant growth disadvantage at 37°C when grown in competition assays against *E. coli*, *Streptomyces coelicolor*, and *Mycobacterium smegmatis* (12). Unlike *E. coli*, *S. aureus* displays a mild cold-sensitive phenotype that is not characterized by differential accumulation of free immature 30S ribosomal subunits, suggesting that KsgA may not be critical for ribosome biogenesis in this organism (10). Interestingly, overexpression of wild-type KsgA at low temperatures (25°C) can rescue the cold-sensitive phenotype in *E. coli*; however, its overexpression at 37°C exerts a negative impact on growth in both the wild type and the KsgA-deficient mutant strain (5). In concordance, overexpression of the chlamydial KsgA ortholog, in a *ksgA E. coli* deletion mutant, inhibited the growth of *E. coli* at 37°C (13). While this phenotype has not been observed in *S. aureus* at either low or high temperatures, overexpression of catalytically inactive KsgA in *S. aureus* at 37°C had a negative effect on growth (10). Similarly, overexpression of a catalytically inactive form of KsgA resulted in significant growth defects in KsgA-deficient and wild-type *E. coli* strains at both 37°C and 25°C. This catalytically inactive form, produced by an alanine substitution at position 66 of KsgA, does not methylate its adenine targets, although it remains attached to them, thereby blocking binding sites for other ribosomal factors and preventing ribosomal maturation from entering the translation cycle, resulting in accumulation of free SSUs (2). In *Saccharomyces cerevisiae*, growth assays comparing catalytically inactive Dim1 (KsgA) mutants versus their wild-type counterparts showed no difference in growth rate of yeast at 18, 25, 30, or 37°C (14). In general, the above-mentioned studies show that KsgA deficiency may confer temperature sensitivity; however, the dependence on KsgA may not be similar among different microorganisms.

The role of KsgA in bacterial virulence has been recently recognized in at least one bacterial model. A KsgA-deficient mutant of *Yersinia pseudotuberculosis* was attenuated after oral infection in BALB/c mice. This mutant was significantly impaired in its survival in the intestine, in its invasiveness in internal organs, such as liver and spleen, and in cultured HeLa cells (15, 16). We recently reported that KsgA deficiency in *Salmonella enterica* serovar Enteritidis, one of the most important food-borne pathogens, results in multiple phenotypes, including (i) KSG resistance, (ii) reduced invasiveness in cultured human intestinal epithelial cells (Caco-2) and chicken liver cells (LMH), and (iii) reduced survival in egg albumen (17). In this study, we hypothesized that KsgA plays a role in virulence of *S.* Enteritidis in chickens. Consequently, we tested the virulence of a *ksgA* mutant of *S.* Enteritidis (*ksgA*::Tn*5*) in a model of orally infected day-old chickens and demonstrated that the mutant was attenuated. We also found that, unlike *E. coli*, the KsgA-deficient mutant of *S.* Enteritidis does not display temperature sensitivity; however, it does confer resistance to kasugamycin and, in striking contrast, increased susceptibility to chloramphenicol. Finally, we also demonstrate that KsgA deficiency in *S.* Enteritidis confers susceptibility to oxidative stress and high osmolarity. To the best of our knowledge, this is the first report showing the role of *ksgA* in *S.* Enteritidis virulence in chickens, tolerance to high osmolarity, and increased susceptibility to chloramphenicol.

MATERIALS AND METHODS

Bacterial strains. The *S.* Enteritidis G1 Nal^r (phage type 4) strain, which is invasive in human intestinal epithelial cells (Caco-2) and virulent in orally infected mice and chickens (17, 18), was used as a wild-type (WT) parental strain. A Caco-2 cell invasion-attenuated *S.* Enteritidis mutant (*ksgA*::Tn*5*), kanamycin and nalidixic acid resistant, was identified previously (17). A complemented mutant (*ksgA*::Tn*5*-pACYC184-*ksgA*) was generated by cloning the full-length *ksgA* gene into the tetracycline resistance site of a low-copy-number plasmid, pACYC184 (New England BioLabs, USA), bearing a chloramphenicol resistance cassette, as described previously (17). Unless otherwise stated, strains were cultured in Luria-Bertani (LB) broth, on LB agar plates containing 30 μg/ml of nalidixic acid (WT and *ksgA*::Tn*5*), or in 30 μg/ml of chloramphenicol (*ksgA*::Tn*5*-pACYC184-*ksgA*).

Chicken virulence assay. Specific-pathogen-free (SPF) fertile eggs were obtained from Sunrise Farms (Catskill, NY) and hatched in isolation at an animal facility at Washington State University. One-day-old birds were distributed in three groups of 9 birds each (experiment 1) or three groups of 15 birds each (experiment 2). Cloacal swabs were taken before placement in environmentally controlled isolation cages to screen for *Salmonella* by enrichment in tetrathionate broth (TTB) and plating onto xylose-lysine deoxycholate (XLD; Difco) agar. Antibiotic-free flock raiser diet (Purina, St. Louis, MO) and water were provided *ad libitum* throughout the experimental period. Bacterial inoculum was prepared from an overnight culture grown at 37°C and diluted in maximum recovery diluent (MRD; 1 g/liter peptone, 8.5 g/liter NaCl, pH 7.0) to obtain the desired concentration. Chicks were orally infected with 200 μl of LB broth containing ~10^8 (experiment 1) or ~10^9 (experiment 2) CFU of the *ksgA*::Tn*5* mutant or the G1 Nal^r WT parent strain. Negative-control groups in both experiments were mock inoculated with 200 μl of LB broth. Three birds per group were sacrificed at 4 days, 8 days, and 12 days postinfection (p.i.; experiment 1) or 24 h, 48 h, 4 days, 8 days, and 12 days p.i. (experiment 2). Small intestine, cecum, liver, and spleen were aseptically collected and homogenized in sterile phosphate-buffered saline (PBS), and serial dilutions were plated onto XLD agar (Difco) to obtain the number of viable colonies per gram of each tissue. Animal experiments were performed according to protocols approved by the WSU Institutional Animal Care and Use Committee. Data were analyzed by two-way analysis of variance (ANOVA) and a Tukey-Kramer test (NCSS 2007).

Growth assays. Single colonies of the WT *S.* Enteritidis G1 Nal^r strain, the *ksgA*::Tn*5* mutant, and a *ksgA*::Tn*5*-pACYC184-*ksgA* complemented mutant were inoculated separately in 5 ml of LB broth with the appropriate antibiotics and incubated overnight (16 h) at 37°C with shaking at 200 rpm. Approximately 100 CFU of each strain was inoculated in 5 ml of LB broth with the appropriate antibiotics and incubated at 42°C, 37°C, 25°C, and 20°C for temperature effect experiments. Ten-fold dilutions of each strain were prepared in MRD at 24, 48, 72, and 96 h postincubation and spotted in triplicate on LB agar plates to determine CFU at each time point. Additionally, turbidity was measured at each time point (optical density at 600 nm [OD_{600}]) by using a BioTek EL808 spectrophotometer (BioTek Instruments, USA). Each strain was tested in duplicates in three independent experiments. Results were transformed to $\log_{10}$ units, and independent replicates were analyzed by ANOVA followed by a Tukey-Kramer test using NCSS 2007 statistical software (NCSS, Kaysville, UT).

Phenotype microarray. A total of 10 96-well phenotype microarray (PM) plates constituting eight metabolic panels (PM1 to PM8) and 2 sensitivity panels (PM9 and PM10) were used according to published protocols (19). To assess the altered phenotypes of the *ksgA*::Tn*5* mutant, the respiratory activity (RA) units of the mutant were compared with those of its WT parent at 42°C, 37°C, and 28°C. Cell respiration is measured by reduction of tetrazolium violet dye, which turns purple upon

reduction caused by respiration processes along the electron transport chain and accumulates irreversibly within the cells, allowing for colorimetric detection (20). A mean RA threshold of >50 was considered a significant difference (21). The data were further confirmed by a Student *t* test. Selected results of the phenotype microarray were confirmed by culture in LB and M9 media (3.4 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.85 mM NaCl, 0.93 mM NH_4Cl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 25 mM sodium pyruvate). To assess effects of osmolarity, 100 CFU of each strain was inoculated into 5 ml of LB or M9 medium supplemented with 6.5% NaCl and incubated at 42°C. Ten-fold dilutions of cultures were plated on LB agar plates at 24 h, 48 h, 72 h, 96 h, and 6 days postincubation. Growth was also monitored by measuring OD_{600}. Each strain was tested in two independent experiments. An unpaired Student *t* test was used to assess differences ($P < 0.05$) in growth between strains by NCSS 2007 software.

Antibiotic resistance assay. The emergence of chloramphenicol-resistant spontaneous mutants was assessed using an agar dilution method. Briefly, an average of approximately 3.8×10^2 CFU of each strain (WT and *ksgA*::Tn*5* mutant) was inoculated in duplicate on LB agar plates containing 10 μg/ml of chloramphenicol and incubated for 48 h at 37°C. Colonies were counted in three independent experiments to obtain an average number of CFU/ml. The frequency of resistant colonies was calculated by dividing the recovered colony number by the initial inoculum and multiplying by 100. Data were analyzed by conducting a Z test between two independent proportions (NCSS 2007).

Oxidative stress responses in KsgA-deficient *S.* Enteritidis. A single colony of each strain was inoculated in 5 ml of LB broth with appropriate antibiotics at 37°C and incubated overnight (16 h) with shaking at 200 rpm. An aliquot of overnight culture was diluted to determine initial CFU followed by centrifugation at 5,000 rpm for 10 min at 25°C. Oxidative stress was tested by resuspending bacterial pellets in 5 ml normal saline (0.9% NaCl, pH 7.2) preheated to 42°C followed by the addition of H_2O_2 to a final concentration of 15 mM and incubation at 42°C for 30 min with constant agitation (200 rpm). At the end of exposure, suspensions were diluted 10-fold in MRD and plated on LB agar. Each strain was tested in duplicate in three independent experiments. Percent survival was calculated as follows: (CFU at 30 min/initial CFU) × 100. Data were analyzed by conducting a Z test between two independent proportions (NCSS 2007).

Infection assays in chicken macrophages. The uptake and survival within chicken macrophages (HD-11 cells) was tested using a gentamicin protection assay as described previously with minor modifications (17). Briefly, HD-11 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) in two 12-well plates at a density of 1×10^6 cells per well and incubated for 2 days at 37°C with 5% CO_2. Bacterial inoculum was prepared by culturing strains overnight at 37°C in LB broth with the appropriate antibiotics. Cells were inoculated with the bacterial preparations at a multiplicity of infection (MOI) of approximately 20, centrifuged for 3 min at 1,000 rpm, and incubated at 42°C with 5% CO_2 for 30 min to allow bacterial uptake by the cells. Next, plates were washed three times in PBS (pH 7.4) to remove extracellular bacteria followed by treatment with gentamicin (200 μg/ml for 30 min) in IMDM with 10% fetal bovine serum (FBS) to kill any remaining extracellular bacteria. Subsequently, gentamicin was removed by washing cells three times in PBS. At this point, cells in one plate were lysed with the addition of 0.5% (vol/vol) Triton X-100 for 10 min at 42°C, and dilutions of cell lysates were plated on LB agar to determine bacterial uptake (30 min). The second plate was incubated for 8 h and treated as described above to determine intramacrophage survival. Bacterial uptake was calculated using the following formula: (intracellular CFU at 30 min/inoculum CFU) × 100. The intramacrophage survival of the *ksgA*::Tn*5*, *ksgA*::Tn*5*-pACYC184-*ksgA*, and G1 Nal^r strains was calculated using the following formula: (CFU at 24 h/CFU at 30 min) × 100. Data were analyzed by conducting a Z test for difference of proportions (NCSS 2007).

RESULTS AND DISCUSSION

KsgA-deficient *S.* Enteritidis is virulence attenuated in chickens. We recently reported that KsgA deficiency significantly reduced invasiveness of *S.* Enteritidis in cultured human intestinal epithelial cells (Caco-2), impaired growth of *S.* Enteritidis in egg albumen at 25 ± 2°C, and resulted in moderate reduction in the invasiveness in chicken liver (LMH) cells compared to that of the WT parent (17). These finding led us to our hypothesis that *ksgA* plays a role in virulence of *S.* Enteritidis in the target host chicken. Consequently, we conducted two experiments to test this hypothesis. In experiment 1, we inoculated 1-day-old chickens with the *ksgA* mutant and WT strain at an initial dose of 10^8 CFU per bird and monitored kinetics of *Salmonella* infection in the small intestine, cecum, liver, and spleen by determining viable colonies at 4 days, 8 days, and 12 days p.i. Significant differences in the CFU of the *ksgA* mutant and WT were found throughout the experimental period (Fig. 1). The *ksgA* mutant showed a 3- to 4-log reduction in CFU in the small intestine and cecum at any given time compared with the WT parent, indicating that the *ksgA* mutant was significantly attenuated in its ability to colonize the chicken gut. Unlike the WT, the mutant strain was not recovered from internal organs, such as liver and spleen, from any of the infected birds at any time points (Fig. 1), suggesting that the mutant was significantly attenuated in its organ invasiveness.

In the second experiment, we increased the infection dose to 10^9 CFU per bird, included a group of chickens challenged with a strain expressing KsgA in *trans* (pACYC184-*ksgA*), and sacrificed infected birds early during the infection process (i.e., 24 h and 48 h). With these modifications, the number of viable colonies of the WT parent and mutant strain were similar at 24 and 48 h p.i. ($P > 0.05$, ANOVA) in the small intestine. After this period, the numbers of CFU of the mutant strain were consistently lower than those of the WT parent strain ($P < 0.02$), reaching a maximum difference of 2.4 log at 12 days p.i. (Fig. 1a). A similar trend was observed in the ceca of birds infected with the high dose (10^9 CFU); however, these differences were statistically significant only at 12 days p.i. ($P = 0.000014$) (Fig. 1b). While these results demonstrate an infectious dose response, the number of mutant bacteria recovered tapered off later during infection, consistent with attenuation. In liver, the *ksgA* mutant showed significantly lower numbers of CFU at 48 h (2.11 ± 0.21), 8 days (3.35 ± 0.04), and 12 days (1.96 ± 0.03) p.i. when a 10^9 CFU dose was used ($P < 0.05$), yielding a maximum average difference of 5.10 log at 48 h ($P = <0.05$), although this difference was not significant ($P = 0.054$) at 4 days p.i. (Fig. 1c), which also coincided with increased numbers of CFU in the small intestine and ceca (Fig. 1a and b). At a high dose, the CFU counts in spleen also showed the same decreasing trends, both for the WT parent and the mutant strain (Fig. 1d). The mutant strain showed significantly lower CFU counts than the WT parent at 48 h, 8 days, and 12 days ($P < 0.05$). The most remarkable difference between the WT and the mutant was observed at 48 h p.i., reaching up to 5.5 log ($P = 0.003$). In *trans* complementation of KsgA on a low-copy-number plasmid, pACYC184, carrying a chloramphenicol resistance cassette was unable to rescue the virulence phenotype in the KsgA-deficient strain (Fig. 1). The numbers of CFU of the complemented mutant were consistently lower than those of the wild type and the KsgA-deficient mutant throughout the experimental period in all organs tested ($P < 0.05$).

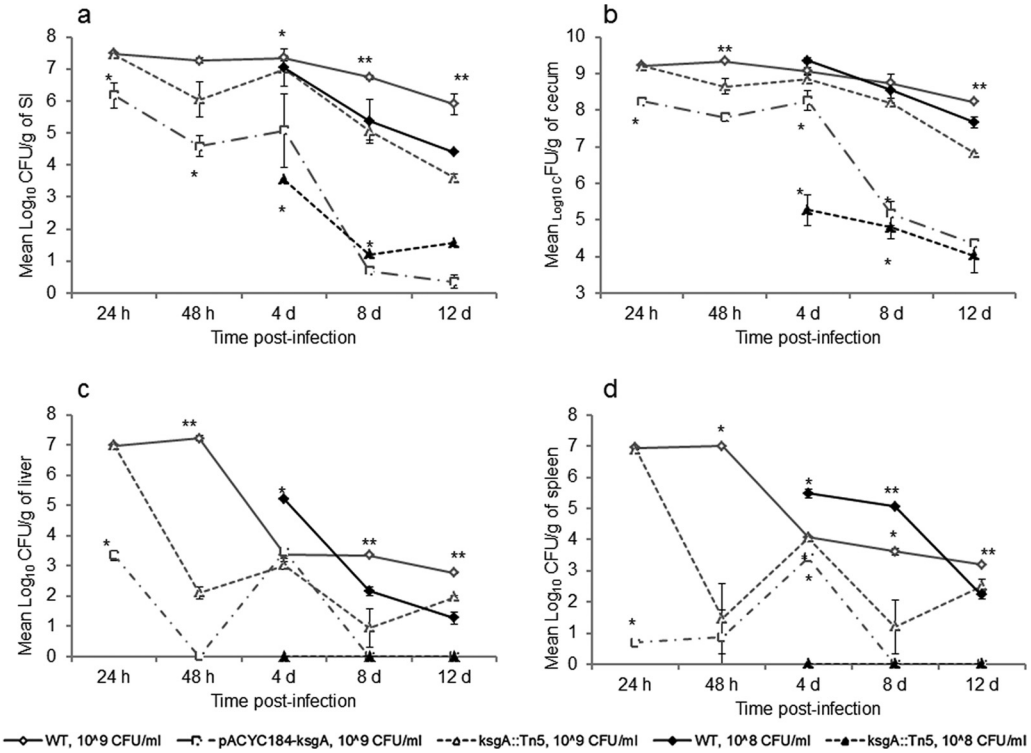

FIG 1 KsgA deficiency decreases the ability of *S.* Enteritidis to survive within chicken small intestine (SI) (a), cecum (b), liver (c), and spleen (d). One-day-old chicks were orally infected with 10^8 or 10^9 CFU of the *ksgA*::Tn*5* mutant or the WT strain. Mean $\log_{10}$ CFU values ± standard error (SE) per gram of tissue were determined on XLD agar at different time points. *, significant difference, $P < 0.05$; **, significant difference between all groups at a specific time point, $P < 0.05$.

The role for *ksgA* in virulence has been previously examined only for *Y. pseudotuberculosis* in a murine model. Oral inoculation of BALB/c mice with 5×10^8 CFU of a KsgA-deficient mutant of *Y. pseudotuberculosis* resulted in a bacterial burden significantly lower than that of its wild-type parent in small intestine, Peyer's patches, spleen, and liver until 10 days p.i., which also translated in higher survival rates (15, 22). More importantly, this mutant was able to confer protection against the WT *Y. pseudotuberculosis* challenge (16). Similar to that in *Y. pseudotuberculosis*, KsgA deficiency in *S.* Enteritidis impacts the bacterial burden in intestinal and extraintestinal tissues, rendering possible attenuation in this bacterium. The molecular mechanism underlying this attenuation is currently unknown. Given the role of KsgA in ribosomal maturation, translational initiation, and protein synthesis, it is possible that KsgA deficiency may have pleotropic effects in *S.* Enteritidis. While dissecting molecular mechanism of virulence attenuation is beyond the scope of this study, we performed a comprehensive phenotypic characterization to identify other altered phenotypes in the *ksgA* mutant strain of *S.* Enteritidis.

KsgA deficiency does not confer growth defects in *S.* Enteritidis. Growth defects caused by *ksgA* deletion, particularly at low temperatures, have been reported in *E. coli* and other bacterial systems (5); the extent of this effect, however, remains unclear due to differential dependence on KsgA (10). Consequently, we tested whether lack of KsgA activity in a *ksgA* mutant might confer growth defects in *S.* Enteritidis at a wide range of temperatures. In contrast to the aforementioned reports, we did not observe a significant difference ($P > 0.05$, Tukey's test) in bacterial growth between the *ksgA*::Tn*5* mutant, a WT strain, and a complemented mutant (data not shown) cultured in LB broth at 20°C, 25°C, 37°C, or 42°C (Fig. 2). These data suggest that unlike in *E. coli*, KsgA deficiency does not significantly alter the growth of *S.* Enteritidis at either optimal or suboptimal temperature for growth *in vitro*, nor does it display a cold-sensitive phenotype observed in other bacterial systems.

Deficiency of KsgA significantly alters respiratory activity of *S.* Enteritidis. Phenotype microarray (PM) technology was used to assess respiratory activity (RA) of a *ksgA* mutant and its WT parent at 42°C, 37°C, and 28°C for up to 48 h, testing a total of 920 different phenotypes arranged in 10 96-well microplates (PM1 to PM10) (Table 1). At low temperatures (28°C), the RA of the mutant was not significantly different from that of the WT parent strain with the exception of three (0.33%) out of the 920 phenotypes, all within the carbon panel. The *ksgA* mutant had an RA significantly higher than that of the wild type in the presence of L-asparagine, whereas it showed significantly lower RA in the presence of D,L-α-glycerol phosphate and D-glucuronic acid as carbon sources (Table 1). The largest numbers of differences were found at avian body temperature (42°C), comprising 15 (1.63%) out of 920 phenotypes tested. The *ksgA* mutant showed significantly impaired utilization of D-alanine (PM1-A09) and Tween 20 (PM1-C05) as carbon sources, glucuronamide (PM3B-E06) as the nitrogen source, and pyrophosphate (PM4A-A03) as the phosphorus source (Table 1). The RA was also impaired for five different peptide-nitrogen combinations (Table 1). The greatest differences were observed under multiple high-osmolarity panels (4 out of 15 phenotypes) composed of 6% or 6.5% NaCl with and without supplementation with a variety of osmoprotectants (Table 1).

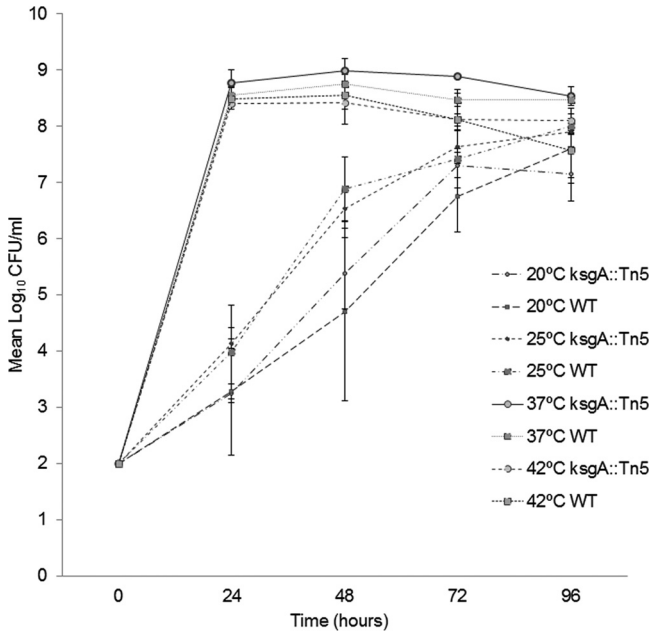

FIG 2 KsgA deficiency does not impair growth in *S.* Enteritidis *in vitro*. Growth of the *S.* Enteritidis *ksgA*::Tn*5*, *ksgA*::Tn*5*-pACYC184-*ksgA* (not shown), and WT parent strains was assessed in LB broth with 30 μg/ml of nalidixic acid for up to 96 h at 20°C, 25°C, 37°C, and 42°C. Mean $\log_{10}$ CFU values ± standard deviation (SD) per ml were determined on XLD agar at different experimental time points for three independent replicates.

Similar results were observed at 37°C, at which at least 4 out of 8 differences were observed in osmolyte panels (Table 1), indicating that KsgA deficiency in *S.* Enteritidis alters susceptibility to high osmolarity. As expected, no significant differences in the RA were observed when the principle compatible solutes, such as glycine-betaine, carnitine, proline, ectoine, and trehalose, were supplemented as osmoprotectants (see Table 1), further confirming the reduced tolerance of the mutant strain to high osmolarity. When mild osmoprotectants, such as choline, trimethylamine, and trigonelline, were supplemented, the RA of the *ksgA* mutant was significantly lower than that of the WT parent (Table 1). This is not surprising, because choline is not directly used as an osmoprotectant. It is taken up by cells either by the common ProU transporter or by the specific BetT system and must be enzymatically processed to betaine by the BetA and BetB enzymes induced during osmotic stress (23). Similarly, trigonelline is a cationic betaine and is usually accumulated by Gram-negative bacteria, such as *E. coli*, but it does not function as an osmoprotectant in *Salmonella* (24, 25). Finally, trimethylamine is an osmoprotectant in many marine invertebrates and some vertebrates (26), where it induces refolding of thermodynamically misfolded proteins (27), while plant microorganisms obtain this compound from vegetable sources, such as alfalfa seeds (28). Based on these data, it is reasonable to suggest that transport systems for osmoprotectants in the *ksgA* mutant remain viable, although internal enzymatic machinery might be impaired, potentially due to impaired protein synthesis as a result of accumulation of unmethylated immature ribosomes. Alternatively, these differences might also reflect the poor osmoprotectant activity of choline, trimethylamine, and trigonelline under high-osmolarity conditions tested in this study. Nonetheless, these results indicate that RA of the *ksgA* mutant was significantly impaired in the presence of high-osmolarity conditions.

Utilization of D-glucuronic acid (PM1-B05), a structural element of the repeating unit of the colanic acid capsule (29), was significantly impaired in the *ksgA* mutant at all temperatures tested. Liver is considered a rich source of D-glucuronic acid, in which it acts as a precursor for a glucuronidation reaction that is involved in metabolic conversion of endogenous and exogenous substances to more water-soluble compounds that are excreted into urine and bile (30). Several enzymes are involved in D-glucuronic acid metabolism to yield glyceraldehyde-3-phosphate and pyruvate that is used in the Embden-Meyerhof-Parnas pathway in the liver (31). Moreover, *Salmonella* can use glucuronic acid as a sole source of carbon (32). Therefore, it is possible that deficiency of KsgA may impair the ability of *S.* Enteritidis to use D-glucuronic acid as the sole carbon source in certain tissues, such as liver, thereby diminishing its ability to invade and survive within such tissues. This effect is consistent with our chicken experiment, in which we were not able to recover viable colonies in liver or spleen when 10^8 CFU was used for oral infection (Fig. 1c) and the bacterial burden was fairly diminished even when chickens were infected with a high dose (10^9 CFU).

At 42°C, the *ksgA* mutant showed significantly lower RA than the WT parent in the presence of leucine-arginine or leucine-phenylalanine as the peptide nitrogen source (Table 1). Leucine plays an important role in protein synthesis, in which more than 90% of intracellular leucine is incorporated for protein production, implying protein synthesis may be impaired at 42°C in the *ksgA* mutant strain. Deficiency of KsgA may result in production of immature unmethylated ribosomes, which may in turn lead to impaired protein synthesis during initiation and elongation steps and stimulate translation errors (33). It is possible that reduced tolerance to osmotic stress at avian physiological temperature (42°C) along with the likelihood of impaired protein synthesis could impact expression of genes necessary for intestinal invasion and colonization of extraintestinal tissues in chickens. Peptide nitrogen source utilization was impaired only at 42°C when five different peptide-nitrogen sources were used. These results indicate potential pleiotropic effects of KsgA deficiency in *S.* Enteritidis that are more obvious at the physiological temperature of its natural reservoir host. Interestingly, these defects did not seem to affect growth *in vitro* (Fig. 2), highlighting the importance of using physiological appropriate models when testing bacterial strains with genetic mutations.

KsgA deficiency confers susceptibility to high osmolarity in *S.* Enteritidis. The PM analysis indicated that high osmolarity (6 to 6.5% NaCl) negatively impacted the *ksgA* mutant. Therefore, we compared the WT, *ksgA* mutant, and complemented mutant strains for their ability to grow in nutrient-rich LB broth containing 6.5% NaCl (equivalent to 1.1 M) incubated at 42°C for up to 6 days (34). The mutant strain failed to grow at any of the time points tested, whereas both the WT parent and the complemented mutant strain were detectable at 72 h postincubation (Fig. 3). The average doubling times were similar between all three strains when cultured in LB alone at 42°C, being close to an average of 34 min for the three strains. In the presence of 6.5% NaCl, the WT doubling time increased to 273 min (4.5 h), whereas the complement had an average doubling time of 500 min (8.33 h), indicating a partial ability of in *trans* complementation to rescue this specific phenotype. Increased doubling time, from 1 h to 2.6 h, in response

TABLE 1 Differences in the RA between the WT and *ksgA*-deficient *S.* Enteritidis mutant grown at different temperatures as tested by phenotype microarray (Biolog, USA)[a]

Temp	Plate type	PM well	Phenotype	RA ± SE		Mean difference	*P* value
				WT	*ksgA* mutant		
42°C	Carbon source	PM1-B05	D-Glucuronic acid	258 ± 3	179 ± 1	78.5	0.023
	Carbon source	PM1-A09	D-Alanine	237 ± 1	181 ± 0.3	56	0.023
	Carbon source	PM1-C05	Tween 20	207 ± 10	125 ± 10	81.5	0.004
	Nitrogen source	PM3-E06	Glucuronamide	199 ± 0.3	119 ± 1	79.5	0.020
	Phosphorus and sulfur source	PM4A-A03	Pyrophosphate	209 ± 3	156 ± 1	53.5	0.042
	Peptide-nitrogen source	PM6-C08	Asn-Val	197 ± 0.3	146 ± 3	51	0.050
	Peptide-nitrogen source	PM6-G07	Ile-His	177 ± 2	125 ± 0.3	52.5	0.042
	Peptide-nitrogen source	PM6-H05	Leu-Arg	165 ± 0	91 ± 1	74.5	0.021
	Peptide-nitrogen source	PM6-H12	Leu-Phe	150 ± 3	97 ± 2	53	0.024
	Peptide-nitrogen source	PM8-E09	Val-Lys	120 ± 2	63 ± 2	57.5	0.006
	Osmolyte	PM9-A08	6.5% NaCl	170 ± 12	107 ± 13	63	0.020
	Osmolyte	PM9-B02	6% NaCl + betaine	198 ± 8	177 ± 15	21	0.335
	Osmolyte	PM9-B03	6% NaCl + *N*,*N*-dimethyl glycine	189 ± 3	151 ± 4	38	0.034
	Osmolyte	PM9-B07	6% NaCl + ectoine	197 ± 7	112 ± 11	85.5	0.231
	Osmolyte	PM9-B08	6% NaCl + choline	189 ± 1	129 ± 1	60	0.011
	Osmolyte	PM9-B12	6% NaCl + L-carnitine	193 ± 3	144 ± 1	50	0.032
	Osmolyte	PM9-C02	6% NaCl + L-proline	192 ± 3	160 ± 8	32	0.156
	Osmolyte	PM9-C08	6% NaCl + trehalose	173 ± 4	125 ± 5	49	0.033
	Osmolyte	PM9-C10	6% NaCl + trimethylamine	189 ± 3	123 ± 0	66	0.048
	Osmolyte	PM9-C12	6% NaCl + trigonelline	188 ± 3	121 ± 3	67.5	0.005
	Osmolyte	PM9-D04	6% potassium chloride	226 ± 5	167 ± 6	59.5	0.027
37°C	Carbon source	PM1-B05	D-Glucuronic acid	264 ± 11	156 ± 9	108	0.02
	Carbon source	PM1-H06	L-Lyxose	64 ± 1	132 ± 5	−68	0.004
	Osmolyte	PM9-A07	6% NaCl	221 ± 1	164 ± 8	57	0.020
	Osmolyte	PM9-B01	6% NaCl	224 ± 3	171 ± 6	53	0.016
	Osmolyte	PM9-B05	6% NaCl + dimethyl sulfonyl propionate	114 ± 7	62 ± 4	52	0.020
	Osmolyte	PM9-C05	6% NaCl + γ-amino-*N*-butyric acid	208 ± 3	158 ± 8	51	0.025
	Osmolyte	PM9-E03	3% sodium formate	166 ± 8	110 ± 8	56	0.037
	Osmolyte	PM9-F09	9% sodium lactate	181 ± 1	109 ± 7	73	0.008
28°C	Carbon source	PM1-B05	D-Glucuronic acid	229 ± 5	140 ± 9	89	0.013
	Carbon source	PM1-B07	D,L-α-glycerol phosphate	225 ± 6	164 ± 4	61	0.011
	Carbon source	PM1-D01	L-Asparagine	62 ± 4	146 ± 3	−85	0.003

[a] Phenotypes with a mean difference in RA of >50 and a *P* value of <0.05 were considered significantly different. RA, respiratory activity; WT, wild type.

to high osmolarity (7.5% NaCl) has also been observed in *Listeria monocytogenes* (35). While complementation of *ksgA* completely rescued the *ksgA* mutant's inability to grow in high osmolarity at 6 days postincubation, this effect was also partially observed at 72 and 96 h, suggesting that *ksgA* is indeed involved in tolerance of *S.* Enteritidis to high osmolarity. We also tested tolerance of the *ksgA* mutant to high osmolarity in M9 medium containing 6.5% (1.1 M) NaCl for up to 4 days (96 h) at 42°C. In this assay, sodium pyruvate was provided as the carbon source, and viable colonies were counted in LB agar at time points of 24, 48, 72, and 96 h postincubation. Similar to the growth assay in LB medium, the mutant strain did not grow in M9 medium up to 96 h; however, 5.64 ± 0.27 $\log_{10}$ CFU/ml of the WT strain was detected at 24 h postincubation, confirming susceptibility of the mutant to high osmolarity. Unlike with growth assay in LB, we were unable to rescue this phenotype by in *trans* complementation of *ksgA* in M9 minimal salts medium.

To our knowledge, this is the first report showing that *ksgA* is required for survival under high osmolarity in any bacterial model. Similar to other Gram-negative bacteria, *Salmonella* has evolved two major mechanisms that function biphasically to respond to this type of stress. A primary response involves an inducible high-affinity system (Kdp) and two low-affinity systems (Trk, Kup) that stimulate uptake of potassium (36). This system tolerates up to 0.5 M of NaCl before inducing a dramatic increase in cytoplasmic concentration (by uptake or synthesis) of osmoprotective compounds, such as glycine-betaine, carnitine, ectoine, proline, trehalose, and amino acids, that are part of the secondary response (37). Additionally, outer membrane porins OmpC and OmpF also play a role in bacterial response to osmotic shock by modulating pore size and number, thus regulating permeability (38). It remains unclear which mechanism is specifically affected in the *ksgA* mutant. The osmolarity condition (6.5% NaCl) used in this study is well above the theoretical cutoff for the potassium uptake system. It is also important to note that the WT parent and mutant strain showed similar growth under moderate-osmolarity (LB with 300 mM NaCl, data not shown) conditions encountered in the intestinal environment (39). Consequently, we surmise that uptake of potassium is less likely to be affected in the mutant strain.

Deficiency of KsgA alters sensitivity to chloramphenicol. Deficiency of KsgA or its homologs is known to promote resistance to

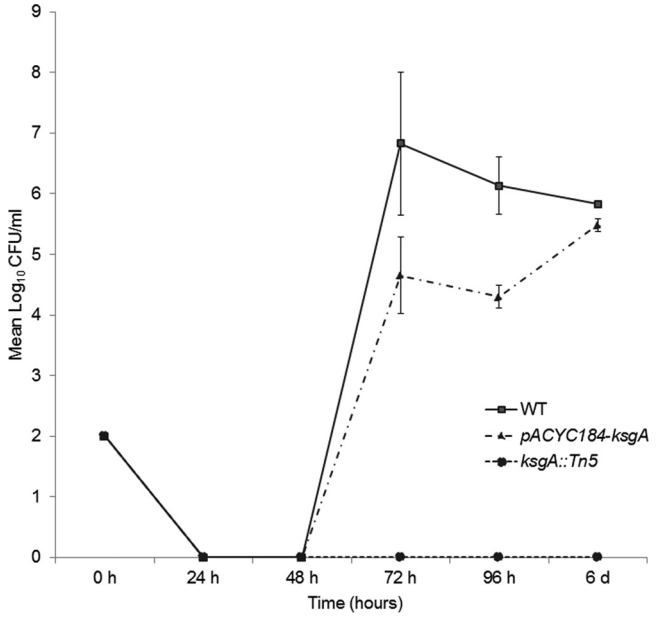

FIG 3 KsgA deficiency confers susceptibility to high osmolarity in *S.* Enteritidis. Growth of the *S.* Enteritidis *ksgA*::Tn*5*, *ksgA*::Tn*5*-pACYC184-*ksgA*, and WT parent strains was assessed in LB broth and 1.1 M NaCl with 30 μg/ml of nalidixic acid for up to 6 days at 42°C. Mean $\log_{10}$ CFU values ± standard deviation (SD) per ml were determined on XLD agar at different time points for two independent replicates.

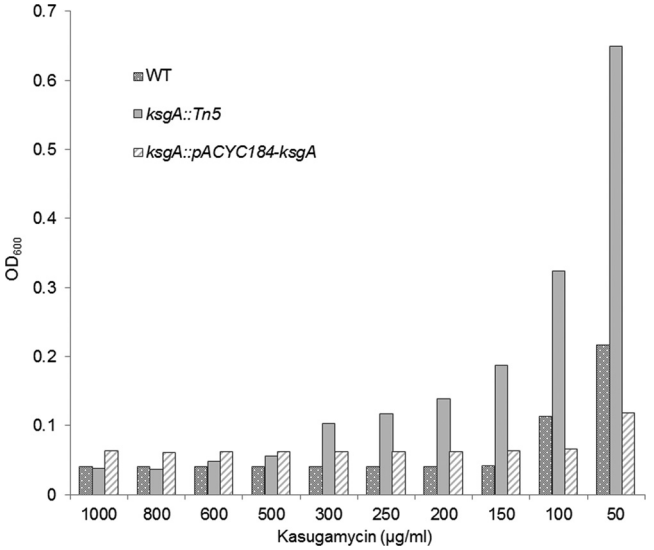

FIG 4 KsgA deficiency confers resistance to kasugamycin (KSG) in *S.* Enteritidis. Growth was monitored under increasing concentrations of KSG (50 to 1,000 μg/ml) in LB broth by measuring OD_{600}. The average MIC of KSG was 500 μg/ml, 150 μg/ml, and 100 μg/ml for the *ksgA*::Tn*5*, *ksgA*::Tn*5*-pACYC184-*ksgA*, and WT strains, respectively.

the aminoglycoside KSG in *E. coli*, *Chlamydia trachomatis*, and *Neisseria gonorrheae* (13). KsgA deficiency in the *S.* Enteritidis mutant strain used in this study was confirmed by demonstration of resistance to the antibiotic KSG by monitoring growth under increasing concentrations of KSG (from 50 to 1,000 μg/ml). The average MIC of KSG for the *ksgA* mutant was 500 μg/ml, whereas for the WT and complemented strains, the MIC was 150 μg/ml and 100 μg/ml, respectively, confirming that deficiency of KsgA indeed conferred resistance to the antibiotic KSG (Fig. 4). KSG is a potent inhibitor of translation initiation which acts by preventing binding of the initiator fMet-tRNA to the P site of the 30S ribosomal subunit (40). Resistance is caused indirectly because of unstable interactions between helix 45 (where A1519 and A1518 are located) and helix 44 (primary binding site of kasugamycin) (8). Recent data suggest a new role for KsgA in resistance of *M. tuberculosis* to the macrolide clarithromycin (11). While the molecular basis of *ksgA*-mediated macrolide resistance is unknown, macrolides bind to the 50S subunit, causing premature detachment of incomplete polypeptide chains, resulting in impaired protein synthesis (41). Chloramphenicol also binds to the 50S ribosomal subunit, thereby inhibiting protein synthesis by preventing growth of the polypeptide chain (42). Interestingly, one binding site of chloramphenicol lies at the entrance of the peptide exit tunnel (E site) overlapping partially the macrolide erythromycin binding site (43). Therefore, we examined the frequency of resistant (breakout) colonies to moderate doses of the antibiotic chloramphenicol by means of an agar dilution method. The complemented strain was excluded from this assay because the pACYC184 carrier plasmid contains a chloramphenicol resistance cassette for appropriate selection. In this assay, the *S.* Enteritidis mutant showed significantly increased susceptibility to chloramphenicol (10 μg/ml) at 48 h of incubation. The frequency of appearance of breakouts for the KsgA-deficient mutant was 21.6 ± 11.3 per 10^8 colonies, whereas the WT strain had an average of 149.3 ± 31.5 breakouts per 10^8 colonies ($P < 0.05$) (Fig. 5). These differences indicate that KsgA deficiency in *S.* Enteritidis confers increased susceptibility to chloramphenicol. Dimethylation caused by KsgA occurs within the loop conformation of helix 45. This loop consists of bases G1516, G1517, A1518, and A1519, which compress the con-

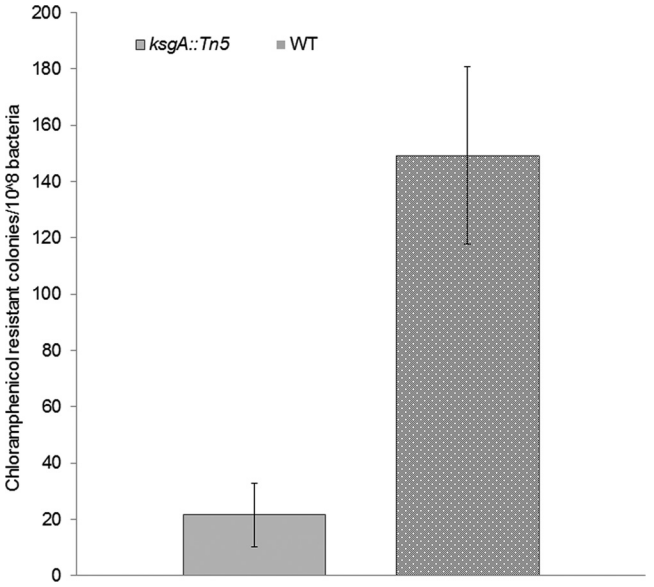

FIG 5 KsgA deficiency reduces tolerance to chloramphenicol in *S.* Enteritidis. Frequency of resistant colonies to chloramphenicol (10 μg/ml) was assessed through the agar dilution method in wild-type (WT) and KsgA-deficient mutant (*ksgA*::Tn*5*) strains. Results from three independent experiments are expressed as average numbers of resistant colonies per 10^8 bacteria ± SD.

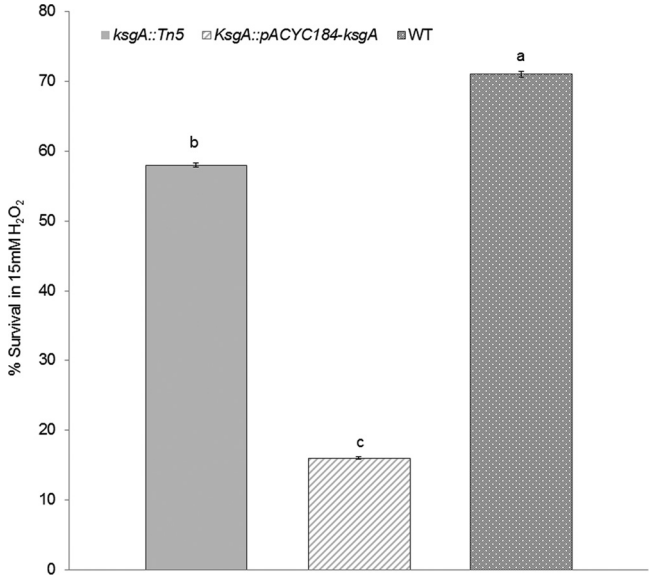

FIG 6 KsgA deficiency reduces tolerance to oxidative stress in *S.* Enteritidis. Oxidative stress response was assessed as average percentage of survival ± SD after treatment with 15 mM H_2O_2 for 30 min at 42°C in the *S.* Enteritidis *ksgA*::Tn*5*, *ksgA*::Tn*5*-pACYC184-*ksgA*, and wild-type (WT) parent strains. Three independent experiments were performed; different letters (a, b, and c) represent statistical significant difference ($P < 0.05$).

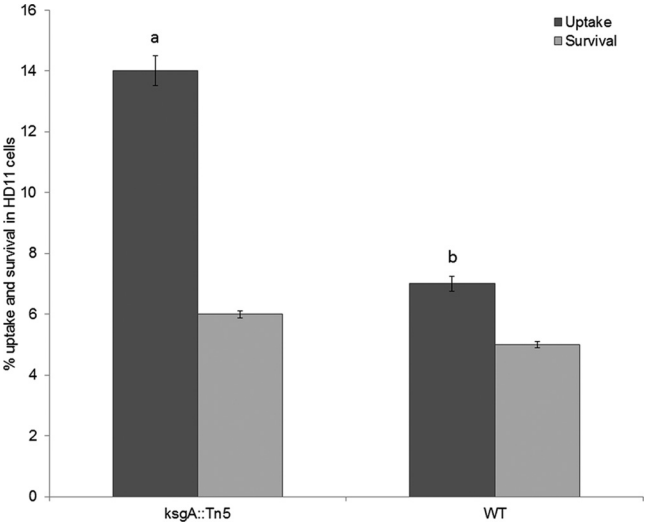

FIG 7 KsgA-deficient *S.* Enteritidis reduces the ability to survive within chicken macrophage HD11 cells. A gentamicin protection assay with an MOI of 20 was performed at 42°C. Mean $\log_{10}$ CFU values ± standard deviation (SD) per ml were determined at 30 min (invasion) and 8 h (survival) postinfection. The uptake of the *ksgA*::Tn*5* mutant (a) was significantly higher than that of the wild-type parent strain (b) ($P < 0.05$). Three independent replicates were included in these experiments.

served GNRA tetraloop where the second to fourth bases stack toward the 3′ end of the loop. Structurally, the fully methylated 30S lacks the hydrogen bonding between N2 of G1516 and N7 of A1519, so the loop is wider and allows binding of 50S and IF3 and direct contact of helix 45 and helix 44. Lack of methylation provides a tighter loop that inhibits these functions and impairs correct folding and stabilization of the mature ribosome (33). This structural defect might account for the difference in sensitivity to antibiotics that target ribosome sites near helixes 44 and 45 since such unstable interaction might spread throughout the ribosomal P and E sites. Further studies that uncover the mechanism of *ksgA*-mediated altered drug susceptibility in *Salmonella* and other bacterial models may provide clues to develop antimicrobials that can specifically target KsgA to render the bacterium more susceptible to specific therapeutic interventions.

KsgA deficiency reduces tolerance of *S.* Enteritidis to oxidative stress and impacts uptake by avian macrophages. Hydrogen peroxide produced by chicken macrophages and heterophils can penetrate cell membranes and act on intracellular targets, thereby playing a crucial role in *Salmonella* killing. We tested whether the KsgA deficiency would alter the oxidative stress response of *S.* Enteritidis to treatment with 15 mM H_2O_2 for 30 min at 42°C. Interestingly, 58 ± 2.8% of the *ksgA* mutant survived the H_2O_2 treatment, whereas 71% ± 4% of the WT strain survived this treatment ($P < 0.05$) (Fig. 6). We were unable to rescue this phenotype by in *trans* complementation of *ksgA*, for which the proportion of survivors for the complemented mutant was 16% ± 1% ($P < 0.05$). Nevertheless, these results corroborate with those of Shah et al. (17), who demonstrated that stress-sensitive *S.* Enteritidis strains treated with 15 mM H_2O_2 show a survival proportion as low as 66%. In addition, uptake and intramacrophage survival assays showed that the avian macrophages were able to engulf a significantly higher proportion (14%) of the *ksgA* mutant than the WT parent (7%) (Fig. 7). However, the intramacrophage survival was similar for both strains, suggesting that mechanisms other than intramacrophage killing may be responsible for reduced virulence of the *ksgA* mutant in chicken.

It has been reported that the *ksgA* gene has a weak promoter, lacks a recognizable ribosomal binding site (44), and displays an autogenous regulation during translation (45). Additionally, overexpression of *ksgA* in *E. coli* results in growth defects, suggesting that the controlled expression of this enzyme may be critical to overcome such defects (5). Therefore, while constructing a complementation plasmid, we incorporated a ribosomal binding site upstream of the *ksgA* gene to enable a heterologous gene to be controlled by the promoter of the tetracycline resistance gene (17). Apparently, this strategy, combined with the use of a low-copy-number pACYC184 plasmid vector, was suitable, as it did not impact growth *in vitro* at a range of temperatures and also successfully restored the sensitivity of the *ksgA* mutant to KSG and resistance to high osmolarity in LB medium. However, we were unable to restore other phenotypes, such as resistance to high osmolarity in M9 medium, oxidative stress, uptake by cultured macrophages, and virulence in orally challenged chickens (Fig. 1). While the pACYC plasmid has been successfully used as a low-copy-number plasmid to complement gene function in *Salmonella* (46), it is possible that unlike for the WT parent strain, the expression levels of *ksgA* in our complemented strain are not tightly regulated, which may have resulted in detrimental effects on the mutant in some cases. In addition, some reports indicate that either carriage of pACYC184 or the chloramphenicol resistance cassette may also impact invasion efficiency of *S.* Typhimurium in human epithelial (HeLa) and phagocytic (RAW 264.7) cells and can suppress expression of *Salmonella* pathogenicity island 1 genes involved in intestinal pathogenesis (47, 48). Therefore, one or more of the above-mentioned factors may have impacted the ability of the complemented strain to restore certain

phenotypes. In *cis* complementation may circumvent some of these difficulties.

In summary, our results clearly show that *ksgA* contributes to intestinal colonization and organ invasiveness of *S.* Enteritidis in chickens. Deficiency of KsgA in *S.* Enteritidis confers no apparent growth defects *in vitro* at a wide range of temperatures under nutrient-rich conditions. KsgA deficiency does, however, confer increased susceptibility to (i) high osmolarity, (ii) chloramphenicol, and (iii) oxidative stress, suggesting potential pleotropic effects on *Salmonella* physiology. Given the impaired kinetics of infection of the *ksgA* mutant in the target host, it appears that the sum of all these defects might become evident within the host environment, where *Salmonella* must outgrow the local microbiota and also overcome antimicrobial defenses produced by the host (49). For instance, exposure of *Salmonella* to intestinal high osmolarity and bile salts serves as a cue to modulate its own gene expression (50). The effects of bile salts include DNA damage, secondary structure formation in RNA, and misfolding or denaturation of proteins. *Salmonella* uses multiple mismatch repair proteins, such as MutH, MutL, and MutS, to prevent DNA damage from bile activity (51). It is important to note that KsgA performs a DNA glycosylase/AP lyase activity to prevent such mutations in *E. coli*, and KsgA deficiency results in increased spontaneous mutations in *mutM* and *mutY*, which also contribute to DNA repair (6). Similar conditions might be encountered in chicken intestinal lumen, which may result in virulence attenuation and lower numbers of physiologically adjusted bacteria within the host.

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REFERENCES

1. **Harris JK, Kelley ST, Spiegelman GB, Pace NR.** 2003. The genetic core of the universal ancestor. Genome Res. **13:**407–412.
2. **O'Farrell HC, Pulicherla N, Desai PM, Rife JP.** 2006. Recognition of a complex substrate by the KsgA/Dim1 family of enzymes has been conserved throughout evolution. RNA **12:**725–733.
3. **Helser TL, Davies JE, Dahlberg JE.** 1972. Mechanism of kasugamycin resistance in *Escherichia coli*. Nat. New Biol. **235:**6–9.
4. **van Buul CPJJ, Visser W, van Knippenberg PH.** 1984. Increased translational fidelity caused by the antibiotic kasugamycin and ribosomal ambiguity in mutants harbouring the *ksgA* gene. FEBS Lett. **177:**119–124.
5. **Connolly K, Rife JP, Culver G.** 2008. Mechanistic insight into the ribosome biogenesis functions of the ancient protein KsgA. Mol. Microbiol. **70:**1062–1075.
6. **Zhang-Akiyama QM, Morinaga H, Kikuchi M, Yonekura S, Sugiyama H, Yamamoto K, Yonei S.** 2009. KsgA, a 16S rRNA adenine methyltransferase, has a novel DNA glycosylase/AP lyase activity to prevent mutations in *Escherichia coli*. Nucleic Acids Res. **37:**2116–2125.
7. **Duffin PM, Seifert HS.** 2009. *ksgA* mutations confer resistance to kasugamycin in *Neisseria gonorrhoeae*. Int. J. Antimicrob. Agents **33:**321–327.
8. **Schluenzen F, Takemoto C, Wilson DN, Kaminishi T, Harms JM, Hanawa-Suetsugu K, Szaflarski W, Kawazoe M, Shirouzu M, Nierhaus KH, Yokoyama S, Fucini P.** 2006. The antibiotic kasugamycin mimics mRNA nucleotides to destabilize tRNA binding and inhibit canonical translation initiation. Nat. Struct. Mol. Biol. **13:**871–878.
9. **Zarubica T, Baker MR, Wright HT, Rife JP.** 2011. The aminoglycoside resistance methyltransferases from the ArmA/Rmt family operate late in the 30S ribosomal biogenesis pathway. RNA **17:**346–355.
10. **O'Farrell HC, Rife JP.** 2012. *Staphylococcus aureus* and *Escherichia coli* have disparate dependences on KsgA for growth and ribosome biogenesis. BMC Microbiol. **12:**244.
11. **Phunpruch S, Warit S, Suksamran R, Billamas P, Jaitrong S, Palittapongarnpim P, Prammananan T.** 2013. A role for 16S rRNA dimethyltransferase (*ksgA*) in intrinsic clarithromycin resistance in *Mycobacterium tuberculosis*. Int. J. Antimicrob. Agents **41:**548–551.
12. **Ochi K, Kim JY, Tanaka Y, Wang G, Masuda K, Nanamiya H, Okamoto S, Tokuyama S, Adachi Y, Kawamura F.** 2009. Inactivation of KsgA, a 16S rRNA methyltransferase, causes vigorous emergence of mutants with high-level kasugamycin resistance. Antimicrob. Agents Chemother. **53:**193–201.
13. **Binet R, Maurelli AT.** 2009. The chlamydial functional homolog of KsgA confers kasugamycin sensitivity to *Chlamydia trachomatis* and impacts bacterial fitness. BMC Microbiol. **9:**279.
14. **Pulicherla N, Pogorzala LA, Xu Z, O'Farrell HC, Musayev FN, Scarsdale JN, Sia EA, Culver GM, Rife JP.** 2009. Structural and functional divergence within the Dim1/KsgA family of rRNA methyltransferases. J. Mol. Biol. **391:**884–893.
15. **Mecsas J, Bilis I, Falkow S.** 2001. Identification of attenuated *Yersinia pseudotuberculosis* strains and characterization of an orogastric infection in BALB/c mice on day 5 postinfection by signature-tagged mutagenesis. Infect. Immun. **69:**2779–2787.
16. **Bergman MA, Loomis WP, Mecsas J, Starnbach MN, Isberg RR.** 2009. CD8(+) T cells restrict *Yersinia pseudotuberculosis* infection: bypass of anti-phagocytosis by targeting antigen-presenting cells. PLoS Pathog. **5:**e1000573. doi:10.1371/journal.ppat.1000573.
17. **Shah DH, Zhou X, Kim HY, Call DR, Guard J.** 2012. Transposon mutagenesis of *Salmonella enterica* serovar Enteritidis identifies genes that contribute to invasiveness in human and chicken cells and survival in egg albumen. Infect. Immun. **80:**4203–4215.
18. **Shah DH, Zhou X, Addwebi T, Davis MA, Orfe L, Call DR, Guard J, Besser TE.** 2011. Cell invasion of poultry-associated *Salmonella enterica* serovar Enteritidis isolates is associated with pathogenicity, motility and proteins secreted by the type III secretion system. Microbiology **157:**1428–1445.
19. **Morales CA, Porwollik S, Frye JG, Kinde H, McClelland M, Guard-Bouldin J.** 2005. Correlation of phenotype with the genotype of egg-contaminating *Salmonella enterica* serovar Enteritidis. Appl. Environ. Microbiol. **71:**4388–4399.
20. **Bochner BR, Gadzinski P, Panomitros E.** 2001. Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Res. **11:**1246–1255.
21. **Zhang J, Biswas I.** 2009. A phenotypic microarray analysis of a *Streptococcus mutans liaS* mutant. Microbiology **155:**61–68.
22. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM.** 2011. Foodborne illness acquired in the United States—major pathogens. Emerg. Infect. Dis. **17:**7–15.
23. **Lamark T, Rokenes TP, McDougall J, Strom AR.** 1996. The complex bet promoters of *Escherichia coli*: regulation by oxygen (ArcA), choline (BetI), and osmotic stress. J. Bacteriol. **178:**1655–1662.
24. **Peddie BA, Lever M, Randall K, Chambers ST.** 1999. Osmoprotective activity, urea protection, and accumulation of hydrophilic betaines in *Escherichia coli* and *Staphylococcus aureus*. Antonie Van Leeuwenhoek **75:**183–189.
25. **Ly A, Henderson J, Lu A, Culham DE, Wood JM.** 2004. Osmoregulatory systems of *Escherichia coli:* identification of betaine-carnitine-choline transporter family member BetU and distributions of *betU* and *trkG* among pathogenic and nonpathogenic isolates. J. Bacteriol. **186:**296–306.
26. **Kempf B, Bremer E.** 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch. Microbiol. **170:**319–330.
27. **Baskakov I, Bolen DW.** 1998. Forcing thermodynamically unfolded proteins to fold. J. Biol. Chem. **273:**4831–4834.
28. **Perroud B, Le Rudulier D.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. **161:**393–401.
29. **Stevenson G, Andrianopoulos K, Hobbs M, Reeves PR.** 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J. Bacteriol. **178:**4885–4893.

30. **Watkins JB, Gregus Z, Thompson TN, Klaassen CD.** 1982. Induction studies on the functional heterogeneity of rat liver UDP-glucuronosyltransferases. Toxicol. Appl. Pharmacol. **64:**439–446.
31. **Shulami S, Gat O, Sonenshein AL, Shoham Y.** 1999. The glucuronic acid utilization gene cluster from *Bacillus stearothermophilus* T-6. J. Bacteriol. **181:**3695–3704.
32. **Gutnick D, Calvo JM, Klopotowski T, Ames BN.** 1969. Compounds which serve as the sole source of carbon or nitrogen for *Salmonella typhimurium* LT-2. J. Bacteriol. **100:**215–219.
33. **Demirci H, Murphy F, IV, Belardinelli R, Kelley AC, Ramakrishnan V, Gregory ST, Dahlberg AE, Jogl G.** 2010. Modification of 16S ribosomal RNA by the KsgA methyltransferase restructures the 30S subunit to optimize ribosome function. RNA **16:**2319–2324.
34. **Warriss PD, Knowles TG, Brown SN, Edwards JE, Kettlewell PJ, Mitchell MA, Baxter CA.** 1999. Effects of lairage time on body temperature and glycogen reserves of broiler chickens held in transport modules. Vet. Rec. **145:**218–222.
35. **Patchett RA, Kelly AF, Kroll RG.** 1992. Effect of sodium chloride on the intracellular solute pools of *Listeria monocytogenes*. Appl. Environ. Microbiol. **58:**3959–3963.
36. **Alvarez-Ordonez A, Begley M, Prieto M, Messens W, Lopez M, Bernardo A, Hill C.** 2011. *Salmonella* spp. survival strategies within the host gastrointestinal tract. Microbiology **157:**3268–3281.
37. **Galinski EA.** 1995. Osmoadaptation in bacteria. Adv. Microb. Physiol. **37:**272–328.
38. **Sleator RD, Hill C.** 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. **26:**49–71.
39. **Tran QT, Gomez G, Khare S, Lawhon SD, Raffatellu M, Baumler AJ, Ajithdoss D, Dhavala S, Adams LG.** 2010. The *Salmonella enterica* serotype Typhi Vi capsular antigen is expressed after the bacterium enters the ileal mucosa. Infect. Immun. **78:**527–535.
40. **Schuwirth BS, Day JM, Hau CW, Janssen GR, Dahlberg AE, Cate JH, Vila-Sanjurjo A.** 2006. Structural analysis of kasugamycin inhibition of translation. Nat. Struct. Mol. Biol. **13:**879–886.
41. **Giguere S.** 2006. Macrolides, azalides, and ketolides, 4th ed. Blackwell Publishing Professional, Hoboken, NJ.
42. **Dowling P.** 2006. Chloramphenicol, thiamphenicol, and florfenicol, 4th ed. Blackwell Publishing Professional, Hoboken, NJ.
43. **Long KS, Porse BT.** 2003. A conserved chloramphenicol binding site at the entrance to the ribosomal peptide exit tunnel. Nucleic Acids Res. **31:**7208–7215.
44. **van Gemen B, Koets HJ, Plooy CA, Bodlaender J, Van Knippenberg PH.** 1987. Characterization of the *ksgA* gene of *Escherichia coli* determining kasugamycin sensitivity. Biochimie **69:**841–848.
45. **van Gemen B, Twisk J, van Knippenberg PH.** 1989. Autogenous regulation of the *Escherichia coli ksgA* gene at the level of translation. J. Bacteriol. **171:**4002–4008.
46. **Deiwick J, Nikolaus T, Shea JE, Gleeson C, Holden DW, Hensel M.** 1998. Mutations in *Salmonella* pathogenicity island 2 (SPI2) genes affecting transcription of SPI1 genes and resistance to antimicrobial agents. J. Bacteriol. **180:**4775–4780.
47. **Knodler LA, Bestor A, Ma C, Hansen-Wester I, Hensel M, Vallance BA, Steele-Mortimer O.** 2005. Cloning vectors and fluorescent proteins can significantly inhibit *Salmonella enterica* virulence in both epithelial cells and macrophages: implications for bacterial pathogenesis studies. Infect. Immun. **73:**7027–7031.
48. **Clark L, Martinez-Argudo I, Humphrey TJ, Jepson MA.** 2009. GFP plasmid-induced defects in *Salmonella* invasion depend on plasmid architecture, not protein expression. Microbiology **155:**461–467.
49. **Baumler AJ, Winter SE, Thiennimitr P, Casadesus J.** 2011. Intestinal and chronic infections: *Salmonella* lifestyles in hostile environments. Environ. Microbiol. Rep. **3:**508–517.
50. **Prouty AM, Brodsky IE, Falkow S, Gunn JS.** 2004. Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*. Microbiology **150:**775–783.
51. **Prieto AI, Ramos-Morales F, Casadesus J.** 2006. Repair of DNA damage induced by bile salts in *Salmonella enterica*. Genetics **174:**575–584.