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The Global Regulator ArcA Controls Resistance to Reactive Nitrogen and Oxygen Intermediates in *Salmonella enterica* Serovar Enteritidis

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Salmonella enterica **serovar Enteritidis is a major cause of food-borne diseases associated with consumption of shell eggs. Clinical isolates of *S. enterica* serovar Enteritidis exhibit a wide spectrum of virulence in mice. A highly virulent isolate (SE2472) was previously shown to be more resistant in vitro than other clinical isolates to acidified sodium nitrite (ASN), a generator of reactive nitrogen and oxygen intermediates (RNI/ROI). SE2472 is also more resistant to *S*-nitrosoglutathione (GSNO) and hydrogen peroxide (H_2O_2) than an ASN-susceptible isolate of *S. enterica* serovar Enteritidis (SE8743). To investigate the molecular basis for the RNI/ROI resistance of *S. enterica* serovar Enteritidis, we transformed a genomic DNA library of SE2472 into SE8743. A plasmid clone conferred upon SE8743 enhanced resistance to ASN, GSNO, and H_2O_2. The DNA insert in the clone encoded ArcA, a global regulator. An *arcA* mutant of SE2472 was constructed and was found to be more susceptible to GSNO and hydrogen peroxide but not more susceptible to ASN than wild-type SE2472. The susceptibility of the *arcA* mutant to GSNO and H_2O_2 was complemented by a plasmid harboring *arcA*. The coding sequence of the *arcA* gene in SE2472 and the coding sequence of the *arcA* gene in SE8743 were identical, suggesting that the difference in resistance to RNI/ROI maybe due to the activity of genes regulated by ArcA. No significant difference in virulence between the wild type and the *arcA* mutant of SE2472 was observed in mice. These observations show that *arcA* is essential for resistance of *S. enterica* serovar Enteritidis to nitrosative and oxidative stress. However, additional genetic loci may contribute to the resistance to RNI/ROI and unusually high virulence for mice of SE2472.**

The facultatively intracellular pathogen *Salmonella* is a leading cause of food-borne diseases in the United States. Between 1993 and 1997, *Salmonella* caused more outbreaks and cases of food poisoning than all other bacteria combined (6), and *Salmonella enterica* serovar Enteritidis was responsible for more outbreaks, cases, and deaths than any other *Salmonella* serovar during this period. Most of the cases were associated with consumption of contaminated eggs laid by infected chickens (6, 33, 38). Chickens are probably infected on poultry farms by field mice, which serve as a reservoir for *S. enterica* serovar Enteritidis (10, 23, 25). Therefore, infection of mice is believed to be a link in the natural transmission cycle of *S. enterica* serovar Enteritidis.

S. enterica serovar Enteritidis causes systemic infections in mice. In orally infected mice, bacteria can be recovered from many organs, including the liver, spleen, intestine, ovaries, and blood. Death can occur rapidly (<12 h after infection), although the exact cause of death is not entirely clear. We have previously reported that clinical isolates of *S. enterica* serovar Enteritidis exhibit a wide range of 50% lethal doses (LD_{50}) after oral challenge in BALB/c mice (29). In contrast to *S. enterica* serovar Typhimurium isolates, which usually have an oral LD_{50} of approximately 10^5 organisms (S. Lu and L. W. Riley,

unpublished results), clinical *S. enterica* serovar Enteritidis isolates may have oral LD_{50} of less than 100 organisms. *S. enterica* serovar Enteritidis isolates are quite heterogeneous with respect to virulence-associated phenotypes determined in vitro, and these in vitro phenotypes correlate poorly with virulence in mice. However, one clinical isolate (SE2472) with an oral LD_{50} of 16 organisms was found to be significantly more resistant than other isolates to acidified sodium nitrite (ASN), which generates a variety of reactive nitrogen intermediate (RNI) and reactive oxygen intermediate (ROI) products (29). Isolate SE2472 was also found to be more resistant to other RNI/ROI generators, such as *S*-nitrosoglutathione (GSNO) and hydrogen peroxide (H_2O_2), than ASN-susceptible isolate SE8743.

Microbes have evolved a variety of strategies to defend themselves against toxic reactive oxygen and nitrogen products in aerobic and anaerobic environments. For pathogens, differences in the responses to such stresses in vivo may determine the clinical outcome for an infected mammalian host. Generation of RNI and ROI is essential for the defense against *Salmonella* in mice (12, 17, 37). De Groote et al. have observed oxygen-dependent and -independent antimicrobial activities of different redox forms of NO against isogenic wild-type and mutant *Salmonella* strains in vitro (11). In *Escherichia coli* and *Salmonella*, at least four global regulators are responsible for much of the response to molecular oxygen. These regulators include the OxyR, SoxRS, Fnr, and ArcAB systems (2, 28, 31). OxyR and SoxRS are activated by reactive oxygen species, such as hydrogen peroxide and superoxide, respectively (7, 22). Dis-

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Characteristics	Source or reference
E. coli DH5α	F⁻ φ80d *lacZΔM15* Δ(*lacZYA-argF*)U169 *deoR recA1 endA1* *hsdR17* (r$_k$⁻ m$_k$⁺) *phoA supE44*λ⁻ *thi-1 gyrA96 relA1*	Gibco/BRL
S. enterica serovar Enteritidis strains		
SE2472	Clinical isolate, mouse virulent and ASN resistant	29
SE2472arcA⁻	*arcA::kan* derivative of SE2472	This study
SE8743	Clinical isolate, ASN susceptible	29
S. enterica serovar Typhimurium LB5000	Restriction-deficient strain	5
Plasmids		
pKD4	Ap^r Kan^r oriR$_\gamma$	9
pKD46	Ap^r, containing the Red recombinase of λ phage	9
pRB3-273C	Ap^r, low- to medium-copy-number plasmid for *Salmonella*	3
pRB3arcA	Derivative of pRB3-273C containing *arcA*	This study

ruption of the transcriptional regulators *oxyR* and *soxS* affects the oxidative stress responses in vitro but does not appear to affect the virulence of *S. enterica* serovar Typhimurium in mice (19, 39). However, disruption of genes encoding bacterial products directly involved in detoxification or electron transport and energy transduction (*zwf* and *sodC*) has been shown to increase the susceptibility of *S. enterica* serovar Typhimurium to RNI and to killing by mice (18, 30). Insertion mutations in the *metL* gene rendered *S. enterica* serovar Typhimurium hypersusceptible to the nitric oxide (NO) donor compound GSNO, and such a strain became attenuated in mice (13). These observations indicate the importance of RNI in *Salmonella* infection control in vivo.

We investigated the molecular basis of the RNI/ROI resistance of *S. enterica* serovar Enteritidis isolate SE2472. In this report, we provide evidence that ArcA, the response regulator of the ArcAB global regulatory system, is essential for nitrosative and oxidative stress resistance in *S. enterica* serovar Enteritidis SE2472.

MATERIALS AND METHODS

Reagents. Growth media for bacteria were purchased from Difco Laboratories (Detroit, Mich.). Anaerobic peptone-yeast extract medium was obtained from Anaerobe Systems (Morgan Hill, Calif.). Chemicals and antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. GSNO was obtained from Alexis Co. (San Diego, Calif.). Restriction and modifying enzymes for manipulating DNA were obtained from New England Biolabs (Beverly, Mass.). Custom oligonucleotides were purchased from Operon (Alameda, Calif.) or Sigma Genosys (The Woodlands, Tex.).

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. *S. enterica* serovar Enteritidis isolates SE2472 and SE8743 were kindly provided by Duc Vugia and Sharon Abbott of the Department of Health Services, State of California, and have been described previously (29). *E. coli* DH5α (Gibco/BRL, Gaithersburg, Md.) was used as the host for all recombinant DNA manipulations. Plasmid vector pRB3-273C (3) was used to construct a genomic library from isolate SE2472. *S. enterica* serovar Typhimurium LB5000 is a restriction-deficient LT2 derivative (5). Bacteriophage P22 was used for generalized transduction. Plasmids pKD4 and pKD46 used for mutagenesis of *S. enterica* serovar Enteritidis were generously provided by Barry Wanner (Purdue University, West Lafayette, Ind.). Plasmid pKD4 contains the kanamycin resistance gene sequence, and pKD46 contains the Red recombinase gene. All bacterial strains were grown aerobically with shaking at 225 rpm unless otherwise stated. Anaerobic cultures were grown by using the Hungate method and peptone-yeast extract medium obtained from Anaerobe Systems.

Construction of *S. enterica* serovar Enteritidis genomic DNA library and screening of transformants for plasmids conferring ASN resistance. *Salmonella* genomic DNA was purified as described previously (32). Plasmid DNA was purified by the alkaline lysis method (36). DNA fragments used for cloning were purified by using Geneclean II obtained from Bio101 (Vista, Calif.) after gel electrophoresis. A genomic DNA library of SE2472 was constructed by partially digesting the SE2472 genomic DNA with *Sau*3AI and cloning the 0.7- to 2.5-kb DNA fragments into *Bam*HI-digested plasmid pRB3-273C. The library was introduced into *E. coli* DH5α by electroporation. Approximately 5×10^3 recombinant DH5α colonies were obtained, which were estimated to represent approximately 85% of the *S. enterica* serovar Enteritidis genome. Plasmid DNA was purified from the DH5α host and transformed into *S. enterica* serovar Typhimurium LB5000. Plasmid DNA was purified from LB5000 and transformed into SE8743 (15). The resulting library was plated onto Luria-Bertani (LB) agar with 100 μg of ampicillin per ml, and colonies were scraped from the plate and exposed to 15 mM sodium nitrite at pH 5.0 (ASN). After exposure to ASN, the *Salmonella* culture was plated onto LB agar supplemented with ampicillin and incubated overnight at 37°C. All colonies that survived the first round of ASN stress were scraped from the plates for another round of selection. After three rounds of screening, plasmid inserts were amplified from the surviving bacteria by PCR and sequenced by using an ABI310 sequencer and a Big-dye sequencing kit (Applied Biosystems, Foster City, Calif.).

Sequence analysis of *arcA* loci of SE2472 and SE8743. To compare the *arcA* locus of SE2472 and the *arcA* locus of SE8743, we amplified the genomic DNA of each isolate with primers PK2F3 (5′-AGGTAGCAAACATGCAGACC) and PK2R2 (5′-CAGGAGGGAAAGCGAGGC). This pair of primers was designed to amplify a DNA sequence from 11 bp upstream to 100 bp downstream of *arcA*. The PCR products were then sequenced with the ABI310 sequencer and a Big-dye sequencing kit (Applied Biosystems).

Mutagenesis of *S. enterica* serovar Enteritidis. Mutagenesis of the *arcA* gene of *S. enterica* serovar Enteritidis SE2472 was carried out by using the gene disruption method described by Datsenko and Wanner (9), except that 10 mM arabinose was used to induce expression of the Red recombinase instead of 1 mM arabinose as described by Datsenko and Wanner. Oligonucleotide primers 5′-TCTTATCGTTGAAGACGAGTTGGTAACACGCAACACGTTGAAAA GTATTTTCGAAGCGGAGTGTAGGCTGGAGCTGCTTC and 5′-CTTATC GTTGAAGACGAGTTGGTAACACGCAACACGTTGAAAAGTATTTTCG AAGCGGAGTGTAGGCTGGAGCTGCTTC were used to amplify the kanamycin resistance gene, and pKD4 was used as the template. These oligonucleotide primers contain a sequence of the kanamycin resistance gene and nucleotides 17 to 76 and 667 to 726 of the *arcA* gene, respectively. The *arcA* sequence from nucleotide 77 to nucleotide 666 was replaced with the kanamycin resistance gene by homologous recombination between the genomic DNA and the PCR product. Individual colonies obtained after electroporation were cultured in 1 ml of superbroth (1) overnight. The cultures were pelleted and resuspended in 100 μl of water. The resuspended bacteria were then boiled for 10 min and centrifuged for 10 min, and the supernatant was used to characterize the targeted region by performing PCR with flanking primers PK2F3 and PK2R2 and internal primers K1 and K2 (9). To confirm the interruption of the *arcA* locus, the junctions of the recombination sites were sequenced. Once homologous recombination was confirmed, the *arcA* mutation was transduced into a fresh culture of SE2472 by using phage P22, and individual phage-free transductants were selected for further analysis.

Assays of bacterial survival after exposure to nitrosative, oxidative and other stresses. Freshly transformed bacteria were cultured in 2 ml of LB medium at 37°C overnight with shaking. Antibiotics were added as appropriate. Portions (20 μl) of the overnight cultures were added to 2-ml portions of LB medium containing one of the following chemicals: sodium nitrite, hydrogen peroxide, sodium chloride, or sodium dodecyl sulfate (SDS). Survival in the presence of GSNO was assayed in M9 minimal medium with 1:1,000 dilutions of overnight

cultures. Survival in the presence of sodium nitrite and GSNO was assayed at pH 5.0, while other stress assays were performed at pH 7.0. In all assays the cultures were grown aerobically with shaking at 225 rpm. After exposure to RNI, ROI, or other stresses, aliquots of cultures were diluted and plated in triplicate. Bacterial colonies were enumerated by determining the number of CFU after overnight incubation in order to determine the bacterial concentrations.

Mouse oral infection studies. Mice were infected with wild-type SE2472 and its derivatives as previously described (29). The bacteria were diluted in phosphate-buffered saline and used for infection. Groups of eight 6- to 8-week-old BALB/c and C3H/HeN mice (Charles River Laboratories, Kingston, N.Y.) were used for intragastric infection experiments. Each mouse received approximately 3×10^5 to 5×10^5 organisms in 0.25 ml of phosphate-buffered saline through a feeding needle. The mice were monitored for 2 weeks after infection, and mortality during this period was recorded.

RESULTS

Construction of the library and screening clones for ASN resistance. We showed previously that the clinical isolate *S. enterica* serovar Enteritidis SE2472 is more resistant to killing than other clinical *S. enterica* serovar Enteritidis isolates after exposure for 6 h to medium containing 20 mM sodium nitrite at pH 5 (29). To identify genes associated with resistance to ASN, we constructed a genomic DNA library of SE2472 using plasmid pRB3-273C. We obtained approximately 5×10^3 independent clones with an average DNA insert size of 1.5 kb, representing approximately 85% of the genome. The library was transformed into SE8743, an *S. enterica* serovar Enteritidis isolate susceptible to ASN (29). The SE8743 transformants were subjected to selection in ASN medium, and resistant clones were recovered and characterized. We found one plasmid clone, pK2, that consistently conferred a survival advantage upon transformed SE8743 exposed to ASN. SE8743 transformed with pK2 exhibited more than 10-fold-greater survival after 6 h of exposure to 15 mM sodium nitrite at pH 5.0 than untransformed or vector pRB3-273C-transformed SE8743. However, SE8743 containing pK2 still exhibited a lower level of survival than wild-type resistant isolate SE2472 (Fig. 1). The growth kinetics of SE8743 transformed with pK2 and the growth kinetics of SE8743 transformed with the vector pRB3-273C were identical in LB broth (data not shown).

Characterization of plasmid pK2. Plasmid pK2 contained an approximately 1.7-kb insert. The insert was sequenced and was found to contain a complete 714-bp open reading frame that was highly homologous to genes encoding the global regulator ArcA of *E. coli* and *S. enterica* serovar Typhimurium. In addition to the *arcA* coding sequence, the insert also contained 423 bases upstream and 543 bases downstream of the *arcA* sequence. The *S. enterica* serovar Enteritidis *arcA* gene exhibited 91 and 99% nucleotide sequence homology to the *E. coli* and *S. enterica* serovar Typhimurium *arcA* genes, respectively. The amino acid sequence of the *S. enterica* serovar Enteritidis ArcA protein differed from that of *S. enterica* serovar Typhimurium ArcA at position 127 (alanine → valine) and from that of *E. coli* ArcA at positions 127 (alanine → valine) and 237 (glutamine → glutamic acid). We renamed plasmid pK2 pRB3arcA to reflect the nature of the insert.

pRB3arcA provides GSNO and H$_2$O$_2$ resistance to SE8743. Since plasmid pRB3arcA provided ASN resistance to SE8743, we determined whether this plasmid also mediated resistance to other RNI/ROI products. Survival of SE8743 transformed with pRB3arcA was assayed in the presence of 4 mM GSNO at pH 5.0 and in the presence of 5 mM hydrogen peroxide at
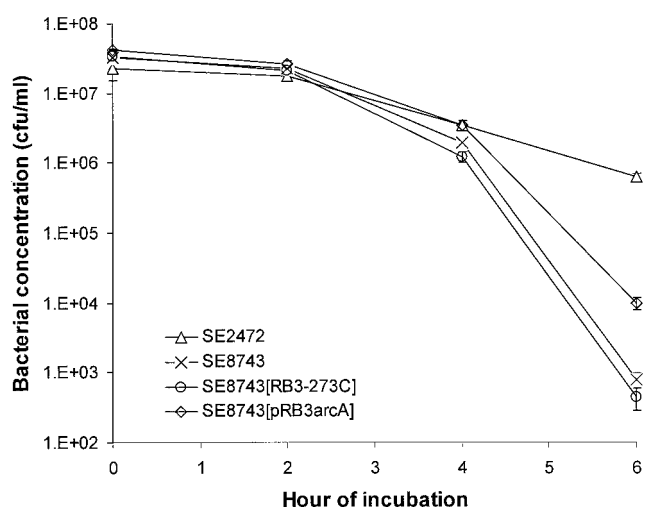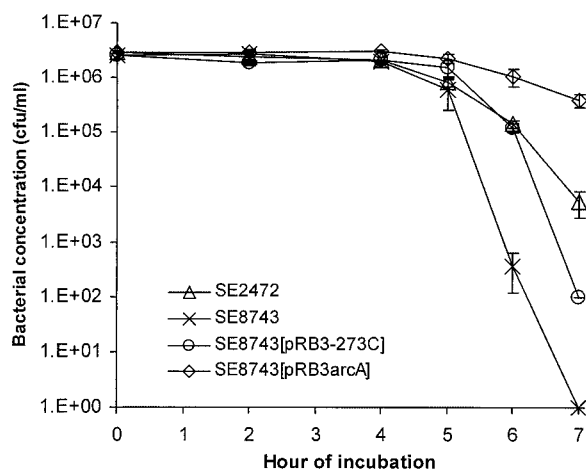

FIG. 1. ArcA increases resistance of *S. enterica* serovar Enteritidis to ASN. Cultures of SE2472, SE8743, and SE8743 transformed with vector pRB3-273C or plasmid pRB3arcA were grown in the presence of 15 mM sodium nitrite in pH 5.0 LB medium, and bacterial concentrations were determined by plating. The values are the concentrations of surviving bacteria after exposure to ASN. At least three experiments were performed, and the results of a representative experiment performed in triplicate are shown. The error bars indicate standard deviations.

neutral pH. Compared to untransformed SE8743 and SE8743 transformed with vector pRB3-273C alone, SE8743 transformed with pRB3arcA exhibited enhanced survival under both conditions (Fig. 2). After 7 h of exposure to 4 mM GSNO at pH 5.0, the concentration of viable SE8743 transformed with pRB3arcA decreased less than 1 $\log_{10}$. In contrast, the concentration of SE8743 transformed with pRB3-273C decreased from more than 10^6 CFU/ml to approximately 10^2 CFU/ml over the same period, and untransformed SE8743 was completely killed. This indicated that pRB3arcA conferred at least a 3-$\log_{10}$-fold survival advantage on SE8743 in the presence of the stress induced by 4 mM GSNO (Fig. 2A). Interestingly, pRB3arcA-transformed SE8743 exhibited even better survival than resistant isolate SE2472, further demonstrating that overexpression of ArcA effectively increased the resistance of *S. enterica* serovar Enteritidis to GSNO (Fig. 2A). Exposure to 5 mM hydrogen peroxide for 2 h at pH 7.0 resulted in about a 1-$\log_{10}$ reduction in the concentration of viable SE8743 organisms transformed with pRB3arcA, and the bacterial count returned to the original level at the end of the assay (6 h). Exposure of untransformed SE8743 or SE8743 transformed with pRB3-273C to the same conditions resulted in sharp decreases, about 3 $\log_{10}$ after 2 h and more than 4 $\log_{10}$ after 6 h. Therefore, by the end of the assay SE8743 transformed with pRB3arcA had a survival advantage of 4-$\log_{10}$-fold compared to bacteria transformed with vector pRB3-273C (Fig. 2B). In contrast to the effect of plasmid pRB3arcA on GSNO resistance, pRB3arcA did not increase the level of resistance of transformed SE8743 to hydrogen peroxide to the level of resistance of SE2472 (Fig. 2B). This result suggests that overexpression of ArcA might be more effective against RNI than against ROI.
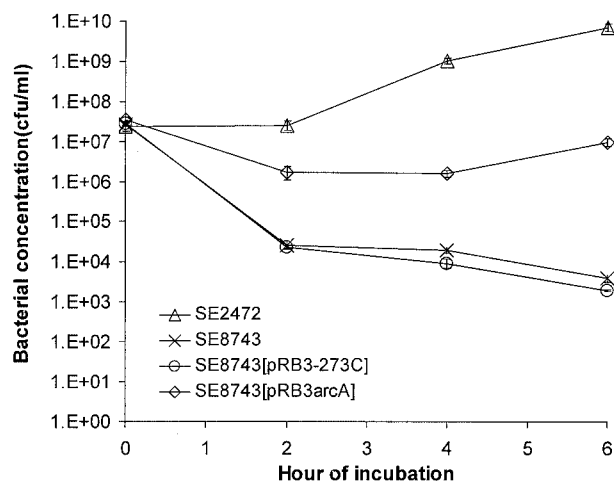
A. GSNO

B. H2O2

FIG. 2. ArcA increases the resistance of *S. enterica* serovar Enteritidis to GSNO and hydrogen peroxide. Cultures of SE2472, SE8743, and SE8743 transformed with vector pRB3-273C or plasmid pRB3arcA were grown in the presence of 4 mM GSNO in pH 5.0 M9 minimal medium (A) or in the presence of 5 mM hydrogen peroxide in pH 7.0 LB medium (B). Bacterial concentrations were determined by plating. The values are the concentrations of surviving bacteria after exposure to GSNO (A) or H_2O_2 (B). At least three experiments were performed, and the results of a representative experiment performed in triplicate are shown. The error bars indicate standard deviations.

pRB3arcA does not provide protection against general stress. We determined whether the protective effect of pRB3arcA was specific for RNI/ROI stresses or provided protection against stresses in general. Standard assays for general stress conditions used by other investigators were used in this study (16, 35). SE8743 transformed with pRB3arcA was exposed to heat (52°C), salt (1 M NaCl), acid (pH 4.0), or detergent (5% SDS), and the survival of this organism was compared to the survival of SE8743 transformed with the pRB3-273C vector. No differences in survival and growth were observed between *S. enterica* serovar Enteritidis transformed with pRB3arcA and

S. enterica serovar Enteritidis transformed with pRB3-273C under any of these conditions (Fig. 3). Of all the stress conditions tested, incubation at 52°C was the most detrimental to the bacteria. Approximately 3 to 4 $\log_{10}$ of killing occurred in the first 3 h, and there was no further change between 3 and 6 h. *S. enterica* serovar Enteritidis transformed with pRB3arcA was slightly more susceptible to thermal stress than the strain containing the vector alone. Salt or acid stress had little or no killing effect on *S. enterica* serovar Enteritidis, although these stresses inhibited the growth of the bacteria. These results demonstrate that plasmid pRB3arcA does not provide a survival and growth advantage to *S. enterica* serovar Enteritidis under the nonoxidative stress conditions which we evaluated. This finding supports the hypothesis that the protection of SE8743 associated with pRB3arcA is specifically directed against RNI/ROI stresses.

Comparison of *arcA* sequences in isolates SE2472 and SE8743. Since isolate SE2472 exhibited greater resistance against RNI/ROI stress than SE8743 and since this resistance could be transferred to susceptible isolate SE8743 by plasmid pRB3arcA, we compared the sequence of the *arcA* locus of SE2472 and the sequence of the *arcA* locus of SE8743. The coding sequences of the *arcA* locus were the same in the two isolates.

Construction of an *arcA* mutant. We demonstrated that overexpression of *arcA* on a multicopy plasmid protects *S. enterica* serovar Enteritidis against RNI and ROI stresses. It was therefore of interest to determine whether *arcA* is essential for bacterial defense against these stresses at the physiological level of expression. An *arcA* mutant of *S. enterica* serovar Enteritidis was generated by using the Red recombinase system described by Datsenko and Wanner (9). The DNA sequence encoding ArcA amino acids 28 to 223 (of a total of 238 amino acids) was successfully replaced with a kanamycin resistance cassette. Four candidate mutant colonies were screened by PCR performed with both flanking primers and internal kanamycin resistance cassette primers. The primers and expected size of a PCR product obtained following the gene replacement are shown in Fig. 4A. All four colonies displayed the expected pattern after PCR, and two of the patterns are shown in Fig. 4B. To confirm that the expected homologous recombination event occurred, we sequenced the junction regions of the kanamycin resistance and *arcA* genes. The sequences observed at both the upstream and downstream junction regions were exactly as predicted.

Morphology and growth characteristics of the *arcA* mutant. The *S. enterica* serovar Enteritidis *arcA* mutant exhibited the same smooth colony morphology that wild-type bacteria exhibited. However, the colonies were smaller than those of the wild type. Since the growth kinetics of an *arcA* mutant of *Salmonella* have not been reported previously, we compared the growth curve of the *arcA* mutant to that of wild-type strain SE2472. The wild type and the *arcA* mutant of SE2472 showed similar growth kinetics when they were grown either aerobically or anaerobically (Fig. 5). When the organisms were grown in minimal or LB medium, the optical density of the log-phase culture of the *arcA* mutant was less than the optical density of the wild-type culture. However, the bacterial concentrations were similar when the organisms were enumerated by plating (data not shown). This observation showed that an *arcA* mu-

A

52 degree C

B

5% SDS

C

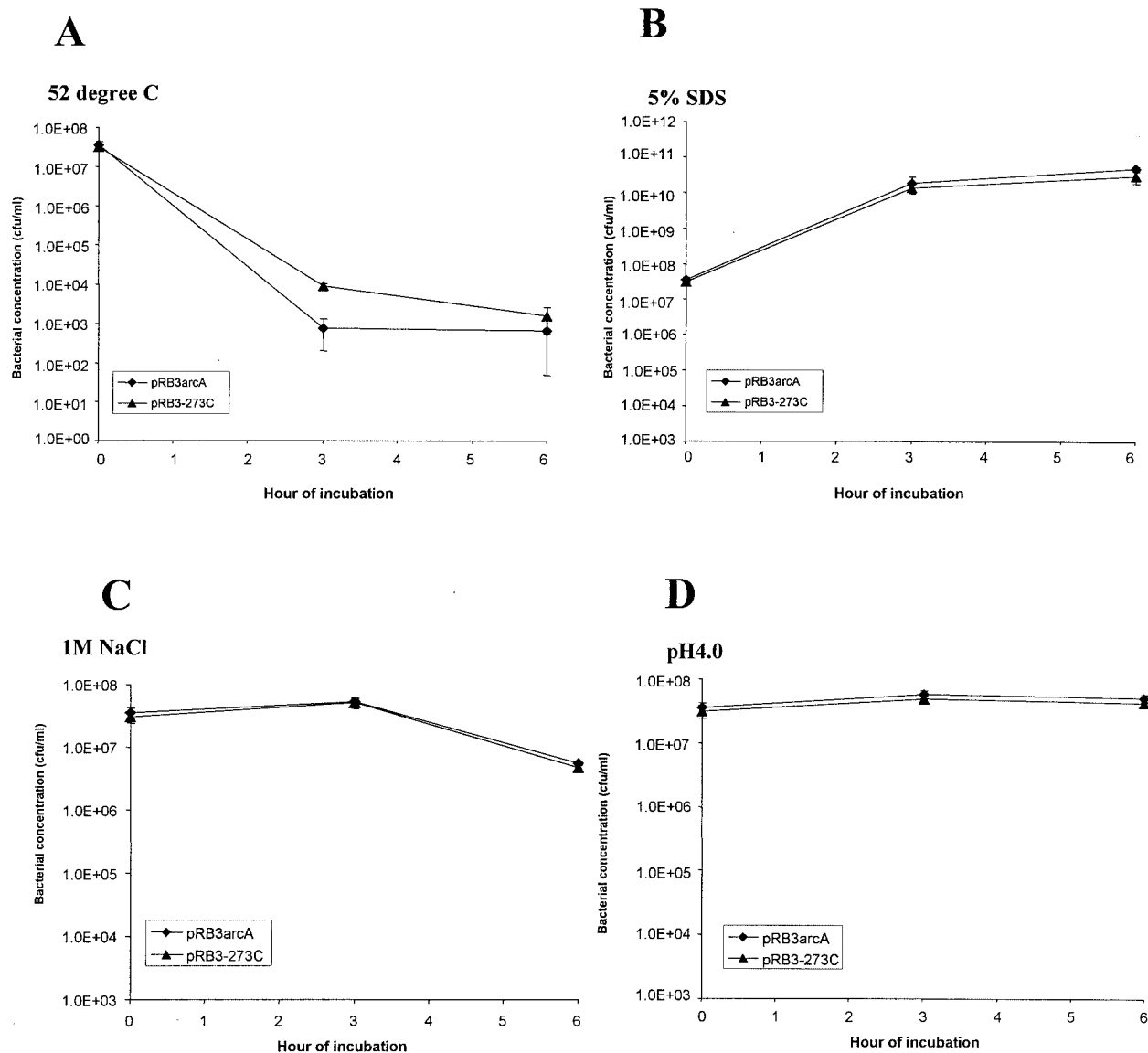### 1M NaCl

D

pH4.0

FIG. 3. ArcA does not protect *S. enterica* serovar Enteritidis from heat, detergent, salt, or low pH. Cultures of SE8743 transformed with vector pRB3-273C or plasmid pRB3arcA were subjected to 52°C (A), 5% SDS (B), 1 M NaCl (C), or pH 4.0 in LB medium (D). Bacterial concentrations were determined by plating. The values are the concentrations of surviving bacteria after exposure to the stress conditions. At least two experiments were performed, and the results of a representative experiment performed in triplicate are shown. The error bars indicate standard deviations.

tant of SE2472 grows normally in culture and provided the basis for the analysis of resistance of the *arcA* mutant to RNI and ROI.

Resistance of *arcA* mutant of *S. enterica* serovar Enteritidis to RNI and ROI. Since *S. enterica* serovar Enteritidis transformed with an *arcA* plasmid exhibited enhanced resistance to RNI and ROI, we sought to determine if endogenous *arcA* is essential for the bacterial defense against nitrosative and oxidative stresses. The *arcA* mutant was exposed to GSNO, hydrogen peroxide, or acidified nitrite, and its survival was compared to that of the wild-type bacteria. All assays were performed under aerobic conditions with vigorous shaking since ArcA is known to be the global regulator induced under

anaerobic conditions and anaerobic conditions may affect bacterial resistance to other stresses.

After 4 h of exposure to 4 mM GSNO at pH 5.0 in M9 medium, the *arcA* mutant decreased over 4 logs, while the wild-type strain decreased minimally (Fig. 6A). To demonstrate that the observed phenotype was specifically attributable to the *arcA* mutation, we transformed pRB3arcA into the *arcA* mutant and tested the transformants in the same assay. While the pRB3-273C vector-transformed *arcA* mutant exhibited the same level of survival as the untransformed mutant, the pRB3arcA plasmid fully compensated for the deficit, indicating that the increased susceptibility to GSNO was indeed due to the *arcA* mutation (Fig. 6A).
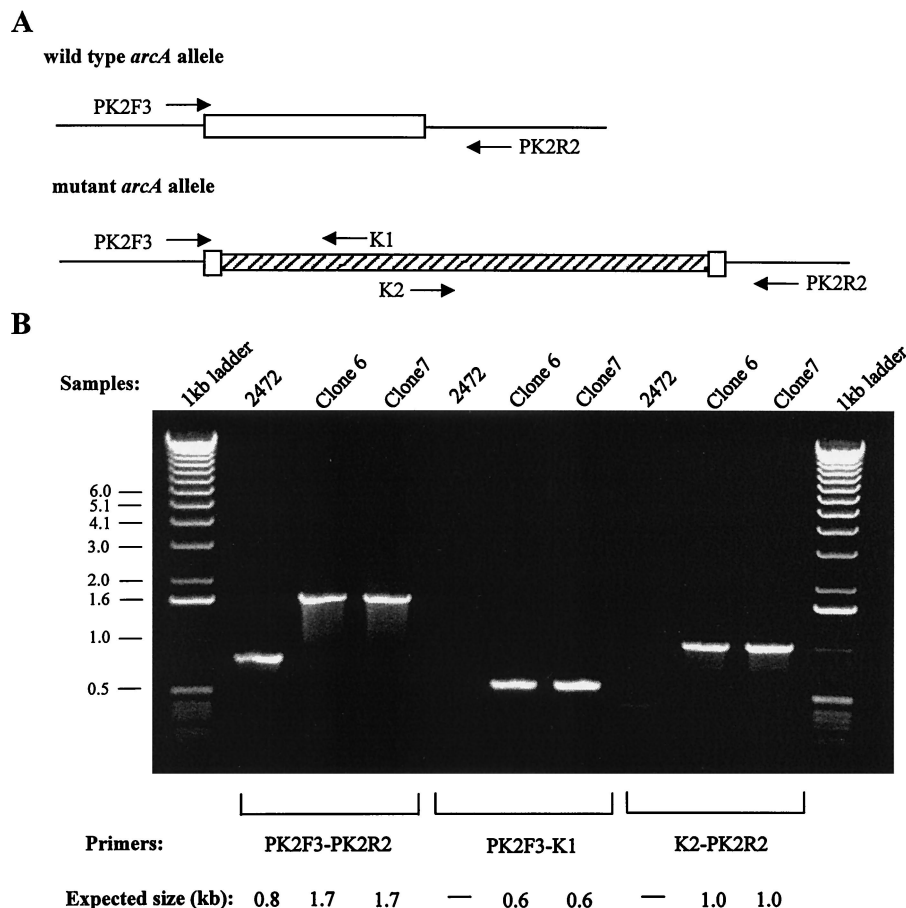
A

FIG. 4. Generation of an *arcA* mutation in *S. enterica* serovar Enteritidis SE2472. (A) Structures of the wild-type and mutant *arcA* alleles. The coding region of *arcA* is represented by an open box, and Kan^r is represented by a cross-hatched box. The positions and designations of the primers used to characterize the mutants are shown next to the alleles. (B) Characterization of two *arcA* mutant clones by PCR. The genomic DNA of wild-type strain SE2472 and mutant clones 6 and 7 were PCR amplified by using the primers shown in panel A. Numbers on the left are molecular sizes in kilobases.

We also tested whether the *arcA* mutant was more susceptible to ROI. The *arcA* mutant was exposed to 5 mM hydrogen peroxide at pH 7.0. The concentration of mutant bacteria decreased about 1 $\log_{10}$ after the initial 2 h of exposure, remained constant after 4 h, and eventually returned to the level in the starting culture. In contrast, the concentration of wild-type bacteria slightly decreased initially, and then growth of the bacteria resumed. By 6 h after exposure, there was a more-than-2-$\log_{10}$ difference in the concentrations of wild-type and *arcA* mutant bacteria. The survival and growth disadvantage of the *arcA* mutant bacteria in the presence of hydrogen peroxide were fully compensated for by transformation with pRB3arcA (Fig. 6B).

The resistance of the *arcA* mutant to ASN was also analyzed. However, no difference between the wild type and the *arcA* mutant could be consistently demonstrated in the ASN assay with sodium nitrite concentrations of 10 to 35 mM at pH 5.0.

***arcA* mutant of *S. enterica* serovar Enteritidis is not deficient in resistance against heat, salt, detergent, and acid.** To determine the specificity of the increased susceptibility of the SE2473 *arcA* mutant to RNI/ROI, we determined the susceptibility of the *S. enterica* serovar Enteritidis *arcA* mutant to heat

(52°C), salt (1 M NaCl), detergent (5% SDS), or acid (pH 4.0). No difference in survival or growth was detected between the *arcA* mutant and wild-type strain SE2472 under any of these conditions (Fig. 7).

***arcA* mutant of *S. enterica* serovar Enteritidis is not significantly attenuated in virulence during oral infection of mice.** To evaluate the effect of *arcA* on the virulence of *S. enterica* serovar Enteritidis in vivo, we orally infected mice with the SE2472 *arcA* mutant and compared the ability of this organism to cause death in mice with that of wild-type strain SE2472. Both BALB/c and C3H/HeN mice were used. The *arcA* mutant of SE2472 was not attenuated in orally infected BALB/c mice (data not shown). The *arcA* mutant bacteria were slightly attenuated in orally infected C3H/HeN mice compared to wild-type strain SE2472 and an pRB3arcA-complemented *arcA* mutant. However, the difference was not statistically significant (data not shown).

DISCUSSION

In a previous report, we described an analysis of a collection of clinical isolates of *S. enterica* serovar Enteritidis that exhib-

A.   aerobic growth
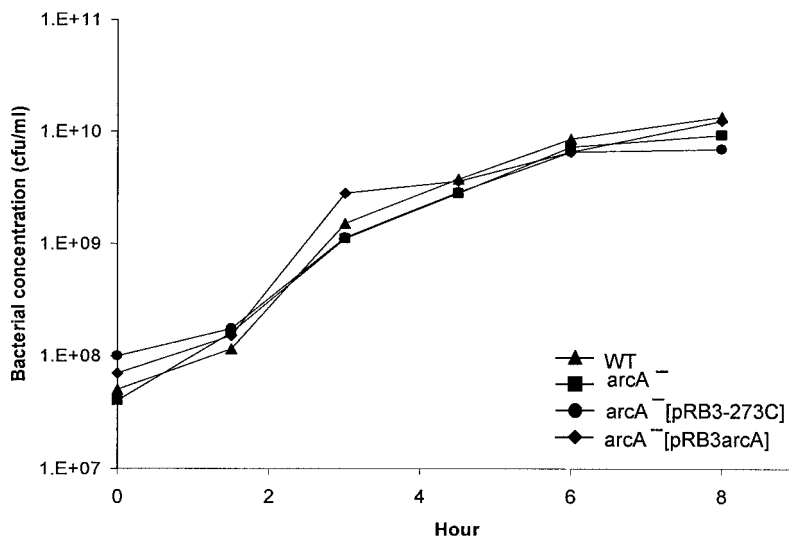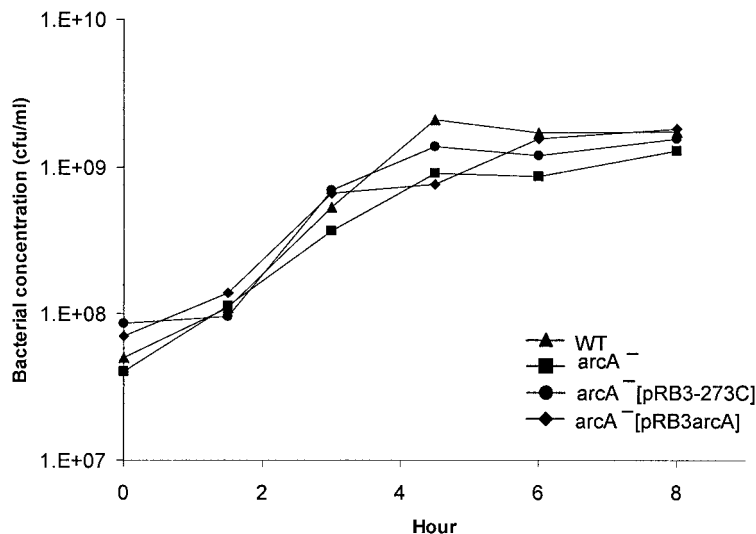

B.   anaerobic growth

FIG. 5. ArcA mutant of *S. enterica* serovar Enteritidis exhibited normal growth under both aerobic and anaerobic conditions. Wild-type strain SE2472 (WT), the SE2472 *arcA* mutant (arcA⁻), and vector pRB3-273C- and plasmid pRB3arcA-transformed *arcA* mutant SE2472 (arcA⁻[pRB3-273C] and arcA⁻[pRB3arcA], respectively) were grown in peptone-yeast extract medium either aerobically (A) or anaerobically (B). The concentrations of bacteria were determined by sampling and plating after incubation.

ited a wide range of LD_{50} for oral infection of BALB/c mice (29). The results of in vitro virulence assays showed little correlation with these oral LD_{50}. However, isolate SE2472, which had the lowest LD_{50} (16 organisms), was observed to be most resistant to ASN. In the present study, SE2472 was found to be more resistant than a less virulent isolate (SE8743) to GSNO and hydrogen peroxide in liquid culture assays. We initiated a study to search for the molecular mechanisms of RNI/ROI resistance of SE2472. In this study, the global regulator ArcA was found to be associated with the oxidative and nitrosative
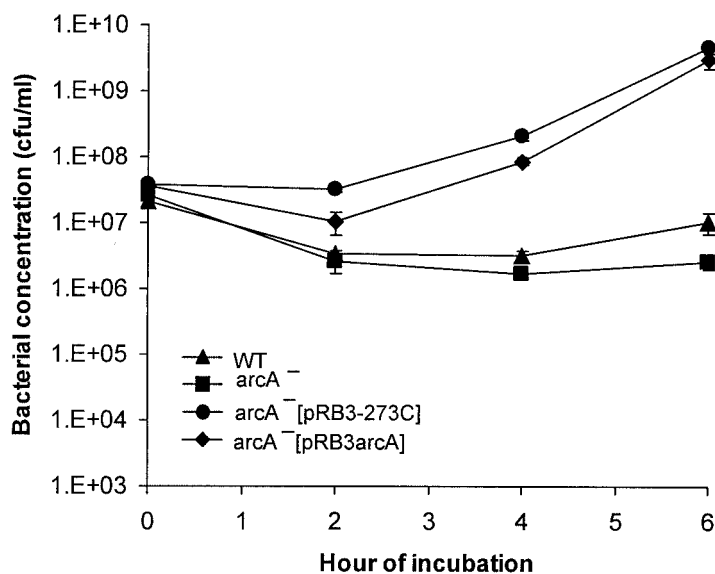
A. GSNO
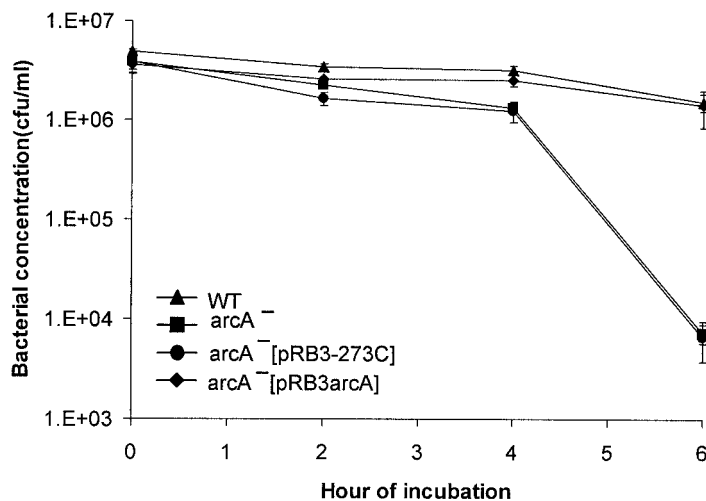
B. H2O2

FIG. 6. ArcA mutant of *S. enterica* serovar Enteritidis is more susceptible to GSNO and hydrogen peroxide. Cultures of SE2472 (WT), the SE2472 *arcA* mutant (arcA⁻), and vector pRB3-273C- and plasmid pRB3arcA-transformed *arcA* mutant SE2472 (arcA⁻[pRB3-273C] and arcA⁻[pRB3arcA], respectively) were grown in the presence of 4 mM GSNO in pH 5.0 M9 minimal medium (A) or in the presence of 5 mM hydorgen peroxide in pH 7.0 LB medium (B). Bacterial concentrations were determined by plating. The values are the concentrations of surviving bacteria after exposure to GSNO (A) or H_2O_2 (B). At least three experiments were performed, and the results of a representative experiment performed in triplicate are shown. The error bars indicate standard deviations.

stress response. The evidence that *arcA* is essential for resistance of *S. enterica* serovar Enteritidis to RNI and ROI is as follows: (i) an RNI/ROI-susceptible clinical isolate of *S. enterica* serovar Enteritidis (SE8743) became more resistant when it was transformed with a multicopy plasmid containing *arcA*, (ii) disruption of *arcA* by a kanamycin resistance cassette in an RNI/ROI-resistant isolate of *S. enterica* serovar Enterit-

idis (SE2472) made the isolate susceptible to GSNO and H_2O_2, and (iii) transformation of an *arcA* mutant of SE2472 with the *arcA* gene carried on a multicopy plasmid restored the isolate's resistance to both GSNO and H_2O_2.

We observed, however, that, while transformation of RNI-susceptible isolate SE8743 with a plasmid expressing *arcA* made it resistant to ASN, disruption of *arcA* in relatively RNI-

A

Heat (52 degree C)

B

5% SDS

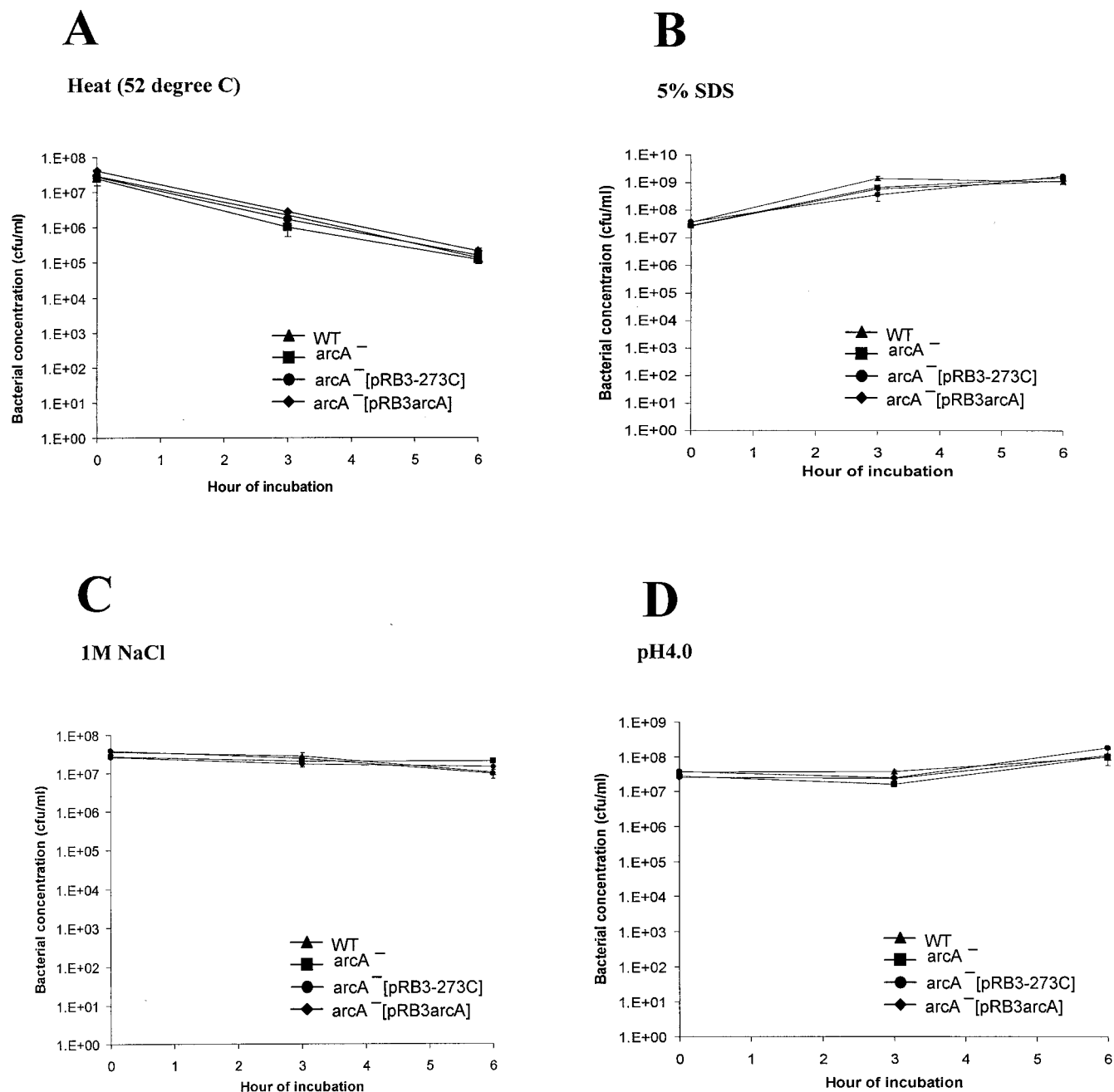
C

1M NaCl

D

pH4.0

FIG. 7. ArcA mutation does not make *S. enterica* serovar Enteritidis susceptible to heat, detergent, salt, or low pH. Cultures of SE2472 (WT), the SE2472 *arcA* mutant (arcA$^-$), and vector pRB3-273C- and plasmid pRB3arcA-transformed *arcA* mutant SE2472 (arcA$^-$[pRB3-273C] and arcA$^-$[pRB3arcA], respectively) were subjected to 52°C (A), 5% SDS (B), 1 M NaCl (C), or pH 4.0 in LB medium (D). Bacterial concentrations were determined by plating. The values are the concentrations of surviving bacteria after exposure to the stress conditions. At least two experiments were performed, and the results of a representative experiment performed in triplicate are shown. The error bars indicate standard deviations.

resistant isolate SE2472 did not significantly affect its susceptibility to ASN, suggesting that *arcA* is not required for resistance of SE2472 to ASN. Instead, the *arcA* mutant of SE2472 became susceptible to GSNO, a donor of RNI products. Other workers have shown that GSNO mediates oxygen-independent cytostasis of *S. enterica* serovar Typhimurium and that another NO redox form, NO·, has no antimicrobial activity against this organism (11). Thus, *Salmonella* appears to exhibit different

patterns of susceptibility to different redox forms of NO. The disruption of *arcA* in SE2472 may have affected a bacterial product that mediates resistance to the bacteriostatic redox form of NO generated by GSNO (presumably nitrosonium [NO$^+$] [11]), whereas the susceptibility of SE8743 to ASN may be mediated by different NO redox forms that are overcome by the multicopy effect of *arcA*. It is also possible that the difference in the genetic backgrounds of SE8743 and SE2472 is

responsible for the difference in susceptibility to ASN of SE8743 transformed with multicopy *arcA* and SE2472 disrupted in a singe copy of gene *arcA*.

ArcA is one component of the *E. coli* and *Salmonella* global control systems that respond to molecular oxygen, including the OxyR, SoxRS, Fnr, and ArcAB systems (2, 28, 31). Together with Fnr, ArcAB regulates anaerobic growth of *E. coli*. ArcA is the transcriptional regulator, and ArcB is a transmembrane protein that contains receiver and transmitter domains. Signals recognized by ArcB, thought to be membrane potential or metabolites generated during anaerobic growth, may activate the system (4, 20, 21, 26–28). Recently, Georgellis et al. reported that the redox signals for the ArcAB system are quinones which are membrane-associated electron carriers (21). Anaerobic induction of ArcAB appears to be dependent on Fnr, which also regulates many of the ArcAB-regulated genes involved in cellular adaptation to anaerobic growth (31). Together, ArcAB and Fnr repress genes involved in aerobic metabolism, but ArcAB may also induce positive regulation of the cytochrome *d* oxidase operon (8, 31). The function of ArcA in aerobic growth has not been reported. Here we provide evidence for the first time that ArcA is important for RNI/ROI resistance under aerobic conditions.

In addition to ArcAB and Fnr, OxyR and SoxRS are the other major global control systems that regulate genes in response to oxidative stress. Both OxyR and SoxRS systems are also involved in resistance to RNI (14, 24, 34). SoxRS appears to be important for RNI resistance in *E. coli* but not in *S. enterica* serovar Typhimurium (19). Both OxyR and SoxR can be directly modified by S nitrosylation (14, 24). This report provides evidence for the first time that *arcA* of the ArcAB regulon is important for *Salmonella* resistance to nitrosative stress. In an anaerobic environment, nitrogen oxides can serve as electron acceptors. It would be interesting to determine whether expression or activity of the ArcAB system is induced by RNI and whether the sensor ArcB is modified by nitrosylation, as OxyR and SoxR are.

Although we found that *arcA* is necessary for resistance of *S. enterica* serovar Enteritidis to both nitrosative stress and oxidative stress in vitro, we did not detect significant attenuation of an *arcA* mutant *S. enterica* serovar Enteritidis strain in mice. It is possible that an *arcA* mutation is compensated for by other factors in vivo. As mentioned above, many of the ArcA-regulated genes are coregulated by the Fnr modulon. Since the RNI/ROI-susceptible *arcA* mutant strain of *S. enterica* serovar Enteritidis was able to kill mice in most of the experiments, we concluded that nitrosative and oxidative stresses in vivo are distinct from the stresses examined in vitro in this study or that other factors induced in vivo compensate for the susceptibility to stresses observed in vitro. The observation that an *arcA* mutant is not clearly attenuated in vivo is not entirely surprising. Two other global transcriptional regulators, OxyR and SoxS, have been shown to be nonessential for virulence of *S. enterica* serovar Typhimurium in vivo, even though both are essential for resistance to RNI or ROI in vitro (19, 39).

Since all *S. enterica* serovar Enteritidis isolates carry *arcA* and the sequence of this gene is the same in SE2472 (RNI/ROI resistant) and SE8743 (RNI/ROI susceptible), the relative RNI/ROI resistance of SE2472 compared to that of other clinical isolates cannot be attributed to this gene alone. Intro-

duction of *arcA* on a multicopy plasmid was not sufficient for isolate SE8743 to become as resistant to ASN and hydrogen peroxide as SE2472, further indicating that other unidentified loci play a role. Nevertheless, our analyses showed that *arcA* is necessary for SE2472 to resist some redox forms of RNI, as well as ROI. In the future, it would be of interest to identify ArcA-regulated genes involved in RNI/ROI resistance. The *arcA* mutant which we constructed provides an opportunity to study such genes. It is becoming clear that the study of the role of global regulators in stress response and *Salmonella* pathogenesis requires detailed analyses of the interactions of all of these systems in vivo and of the overlapping regulation of the genes that they control.

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