# Influence of SOS-inducing agents on the expression of ArtAB toxin gene in *Salmonella enterica* and *Salmonella bongori*

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**Abstract**

*Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) definitive phage type 104 (DT104), *S. enterica* subspecies *enterica* serovar Worthington (*S.* Worthington), and *S. bongori* produce ArtA and ArtB (ArtAB) toxin homologs, which catalyze ADP-ribosylation of pertussis toxin-sensitive G protein. ArtAB gene (*artAB*) is encoded on prophage in DT104 and its expression is induced by mitomycin C (MTC) and hydrogen peroxide ($H_2O_2$) that trigger the bacterial SOS response. Although the genetic regulatory mechanism associated with *artAB* expression is not characterized, it is thought to be associated with prophage induction, which occurs when the RecA-mediated SOS response is triggered. Here we show that subinhibitory concentration of quinolone antibiotics that are SOS-inducing agents, also induce ArtAB production in these *Salmonella* strains. Both MTC and fluoroquinolone antibiotics such as enrofloxacin induced *artA* and *recA* transcription and *artAB*-encoding prophage (ArtAB-prophage) in DT104 and *S.* Worthington. However, in *S. bongori*, which harbors *artAB* genes on incomplete prophage,

*artA* transcription was induced by MTC and enrofloxacin, but prophage induction was not observed. Taken together, these results suggest that SOS-response followed by induction of *artAB* transcription is essential for ArtAB production. $H_2O_2$-mediated induction of ArtAB prophage and efficient production of ArtAB was observed in DT104 but not in *S.* Worthington and *S. bongori*. Therefore, induction of *artAB* expression with $H_2O_2$ is strain-specific, and the mode of action of $H_2O_2$ as SOS-inducing agent might be different from those of MTC and quinolone antibiotics.

## Introduction

*Salmonellae* cause infectious diseases associated with enteric fever, gastroenteritis, bacteremia, and systemic infection in both humans and animals. The *Salmonella* genus includes the two species *Salmonella enterica* and *S. bongori*. *S. enterica* is classified into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*, which include over 2,500 serotypes. The subspecies *enterica* is the most closely associated with disease [1]. *Salmonella* serotypes can be divided into two main groups: typhoidal and non-typhoidal. Typhoid fever is a systemic disease with diarrhea, caused by serotypes Typhi and Paratyphi A, which are host-adapted to humans. Non-typhoid *Salmonella* is the most common bacterial pathogen causing gastrointestinal infection worldwide but rarely develops into an invasive infection. *Salmonella enterica* subspecies *enterica* serotype Typhimurium (*S.* Typhimurium) is a type of non-typhoid *Salmonella*, with a broad host range. Multidrug-resistant *S.* Typhimurium definitive phage type 104 (DT104) has emerged

since 1990s as a common cause of salmonellosis in humans and animals [2-6]. The severity of clinical illness in *S.* Typhimurium DT104 outbreaks suggests that this strain possesses enhanced virulence [4, 7]. Recently, the genes *artA* and *artB* (*artAB*) were identified within the prophage of *S.* Typhimurim DT104 (8). These genes encode polypeptides with amino acid sequence similarity to the pertussis toxin (Ptx), ADP-ribosyltransferase A subunit (S1 unit), and one of five components of the heteropentameric B subunits (S2 unit) [8-10]. Additionally, the serotypes *S.* Worthington and *S.* Agoueve and the species *S. bongori* were shown to harbor *artAB* homologs [10]. While the A subunit shows sequence similarity with other ArtABs, the B subunit sequence of *S. bongori* ArtAB (ArtAB-Sb) is divergent [10]. Expression of *artAB* in these bacteria is induced by mitomycin C (MTC). Purified ArtABs showed potent ADP-ribosyltransferase activity for Ptx-sensitive G proteins. Intraperitoneal injection of purified ArtABs was fatal in mice; the 50% lethal dose of ArtAB produced by DT104 (ArtAB-DT104) was the lowest, suggesting that ArtAB-DT104 is the most toxic to mice [10].

Shiga toxin production in *Escherichia coli* is coregulated through induction of the prophage that encodes toxin gene *stx2* [11-18]. Activation of the SOS response by MTC and $H_2O_2$, which cause DNA damage, or antimicrobial agents such as quinolones, which inhibit DNA replication, triggers substantial Stx-phage induction and results in high Stx2 production [11-17, 19]. Since the effect of $H_2O_2$ or antibiotics on ArtAB production in *Salmonella* spp. is unknown, we evaluated the effect of different agents on ArtAB production, ArtAB prophage induction, and SOS response in *Salmonella* spp. that harbor

*artAB* homologs.

## Methods

### Bacterial strains and culture

*S.* Typhimurium DT104 strain U1, *S.* Worthington stain 182, and *S. bongori* strain ATCC43975 used in this study have been described previously [10]. *Salmonella* cultures were grown in syncase broth [20] supplemented with $FeCl_3$ at 10 µg $ml^{-1}$ at 37°C on a shaker at 120 r.p.m.

### Antibiotics and antimicrobial susceptibility testing

Nalidixic acid, enrofloxacin, cefazolin, and trimethoprim were purchased from Sigma Aldrich (St. Louis, MO, USA) and rifampicin from Wako Junnyaku, Co., Ltd. (Japan). The MICs of antibiotics were determined according to the NCCLS guidelines [21].

### Detection of ArtA production by western blotting

Western blotting analysis of ArtA was carried out as described previously [9]. Overnight cultures of *Salmonella* grown in syncase broth were diluted with syncase broth at a ratio of 1:50 and grown for 3 h at 37°C in a shaker at 120 rpm. Cultures were further incubated for 16 h at 37°C in the presence of the following tested agents (added to indicated final concentrations): 0.5 µg $ml^{-1}$ MTC, 1/2 MIC (Table 1) antibiotics, 3 mM $H_2O_2$. Cultures were centrifuged to separate the cells, and the supernatant was passed through a 0.22-µm pore filter and concentrated 20-fold using a Vivaspin 10 K (GE Healthcare, UK). Ten

106 microliters of concentrated supernatants was resolved by 15% SDS-PAGE and proteins in
107 the gel were transferred onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The
108 filters were first probed with an antibody against the 14-amino acid peptide corresponding
109 to the $Arg^{10}$–$His^{23}$ sequence of *S.* Typhimurium DT104 ArtA [9] (1:5,000 dilution in
110 Tris-buffered saline, 0.05% Tween (TBS-T)). This was followed by incubation with
111 anti-rabbit immunoglobulin G horseradish peroxidase-labeled conjugate diluted 1:100,000
112 in TBS-T. Bands were visualized using ECL Prime Western Blotting Detection Reagent
113 (GE Healthcare) and Ez-Capture II (ATTO Corporation, Japan) with ImageSaver5 software
114 (ATTO).
115
116 **Design of primer and standards for quantitative PCR (qPCR) and quantitative**
117 **reverse transcription PCR (qRT-PCR).**
118 Table 2 lists the primers used in this study. Targets for qPCR and qRT-PCR were genes
119 coding for *artA*, *artB*, intergenic region spanning both *artA* and *artB*, and *recA*, as well as
120 corresponding RNAs. To enable an accurate quantification of the RNA targets, RNA
121 standards for each gene were generated as described by Fey et al [22] using T7 RNA
122 polymerase and PCR product. Primer sets (Table 1) for generation of RNA standards were
123 designated for each target, in which the primers were located upstream and downstream of
124 the sequences recognized by the primer sets for each target for qPCR described above, and
125 the forward primer contained the sequences of T7 promoter. PCR products carrying T7
126 promoter sequences were purified with Wizad SV Gel and PCR Clean-Up System

(Promega, Madison, WI). Standard RNA was generated by *in vitro* transcription from PCR templates carrying T7 promoter sequences using ScriptMAX Thermo T7 Transcription Kit (TOYOBO, Japan). This was followed by a digestion with TURBO DNase enzyme (TURBO DNA-free Kit; Ambion, Inc., Applied Biosystem Business, CA) for 15 min at 37°C and a subsequent purification by RNA cleanup protocol of the RNeasy Mini kit (QIAGEN, Germany), which included a second DNase I digestion on the column for 15 min at room temperature during purification.

**Total RNA extraction and quantification of gene expression of by quantitative reverse transcription PCR (qRT-PCR).**

After incubation with each tested agent for 3 h at 37°C as described above, total RNA was extracted from the culture using the RNeasy Protect Bacteria Mini kit (QIAGEN), following manufacturer's recommended procedure. RNA concentration was measured by Eppendorf BioPhotometer D30 (Eppendorf, Germany). Two step qRT-PCR and data analysis were carried out as described by Fey et al [22]. RNA samples (100 ng) were reverse-transcribed using a ReverTra Ace® qPCR RT Master Mix with gDNA Remove (TOYOBO), which included a DNase I digestion at 37°C for 15 min before reverse transcription reaction. In each reverse transcription reaction, some RNA samples were not supplemented with reverse transcriptase to rule out DNA contamination. First strand of cDNA synthesized in this reaction was amplified by qPCR using the THUNDERBIRD SYBR qPCR Mix (TOYOBO) and LightCycler480 System II (Roche Applied Sciences,

Indianapolis, IN, USA). For qPCR, 1 µl of diluted sample was added to 19 µl of a PCR mixture prepared from THUNDERBIRD SYBR qPCR Mix, containing 300 nM of each primer, which was designed to target sequences of standards. The cycle parameters were as follows: 1 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C. For the determination of the melting curve, the temperature was increased of 1°C every 20 s, from 65 to 94°C. To generate a calibration curve, the serially diluted RNA standards were similarly treated and quantified in each qRT-PCR run. The copy numbers of the standards were calculated by assuming average molecular masses of 340 Da for 1-nucleotide single-stranded RNA. Using calibration curve, LightCycler480 System II calculated the number of target copies in the measured samples.

**Quantification of phage particles**

Filtered supernatants obtained after treatment with each agent for 3 h as described above were treated with DNase using a TURBO DNA-free kit (Ambion) for 30 min at 37°C. Phage DNA was released by heat treatment for 10 min for 100°C as described by Bonanno et al [23]. Copy numbers were determined by qPCR using the same primes for qRT-PCR of *artA* as described above. The *artA* gene copy numbers were calculated from the standard curve generated using diluted purified PCR-product of *artA* by assuming average molecular masses of 660 Da for 1 bp of double-stranded DNA.

**Statistical analysis.** Statistical comparisons were made using Student's *t*-test, and the

results with P values of <0.05 were considered to be significant.

## Results

### Effects of MTC, enrofloxacin, and $H_2O_2$ on *artA* expression

In order to examine the increase in extracellular ArtA protein in response to antibiotics, the culture supernatant was analyzed by immunoblotting using a polyclonal antibody raised against a synthetic peptide corresponding to residues 10-23 of mature ArtA [9]. In the western blotting analysis, *S.* Typhimurium DT104 strain U1 showed extracellular ArtA production after exposure to the quinolone antibiotics nalidixic acid and enrofloxacin, at subinhibitory concentrations and MTC; however, ArtA was not detected in the untreated cultures in syncase broth (Fig. 1a). Furthermore, induction of ArtA production by other antibiotics exerting their activity through different mechanism was examined. It was found that ArtA production was not induced by cefazolin, trimethoprim, and rifampicin (Fig. 1a). Consistently, extracellular ArtA production was also induced in *S.* Worthington strain 182 and *S. bongori* strain ATCC43975 after exposure to MTC and enrofloxacin (Fig. 1b). A faint band of ArtA was also detected in the western blot of *S.* Typhimurium DT104 strain U1 and *S. bongori* strain ATCC43975 cultures upon exposure to $H_2O_2$, but not that of *S.* Worthington strain 182 (Fig. 1b).

Next, the effect of MTC, $H_2O_2$, and quinolone antibiotic on *artA* transcription was tested (Fig. 3). MTC and enrofloxacin significantly increased *artA* transcription compared with

untreated culture of *S.* Typhimurium DT104 strain U1 (174- and 479-fold, respectively), *S.* Worthington strain 182 (7079- and 550-fold, respectively), and *S. bongori* strain ATCC43975 (19- and 8-fold, respectively) ($p<0.05$) (Fig. 3). $H_2O_2$ also significantly increased *artA* transcription in *S.* Typhimurim DT104 strain U1 (200-fold) ($p<0.01$); however, *artA* transcription was not significantly upregulated by $H_2O_2$ in either *S.* Worthington strain 182 or *S. bongori* strain ATCC43975 ($p=0.40$, $p=0.25$) (Fig. 3).

**Transcriptional analysis of *artAB***

To examine whether *artA* and *artB* are cotranscribed, we assessed their transcription in *S.* Typhimurium DT104 strain U1 by qRT-PCR using primer pairs that direct amplification of 84- to 225-bp fragments within *artA*, within *artB*, or spanning *artA* and *artB* (Fig. 2A). Copy numbers of each mRNA from *S.* Typhimurium DT104 strain U1 treated with MTC were then compared. As shown in Fig. 2, a 297-bp fragment spanning *artA* and *artB* was detected by qRT-PCR (Fig. 2B) and the target mRNA copy number determined using each primer sets was similar, suggesting that *artA* and *artB* are likely co-transcribed (Fig. 2C). Identical results were obtained using mRNA from *S.* Worthington strain 182 and *S. bongori* ATCC43975 (data not shown).

**Evaluation of ArtAB prophage induction and expression of *recA***

We subsequently examined whether each agent that promotes *artAB* expression also causes prophage induction. Since indicator stain for *artAB*-encoding phage is not yet available at

this stage, we investigated the number of *artA* gene copies in the supernatant of each strain to estimate phage enumeration. As shown in Fig. 4, MTC and enrofloxacin significantly induced prophage in *S.* Typhimurium DT104 strain U1 and *S.* Worthington strain 182 to a greater degree (9- to 219-fold, respectively) than the control ($p<0.05$). $H_2O_2$ induced a 37-fold phage increase in *S.* Typhimurium DT104 strain U1 but had no significant effect in *S.* Worthington strain 182 (Fig. 4). Moreover, no significant increase in phage number was observed in *S. bongori* strain ATCC43975 with any agent (Fig. 4).

Based on the experimental results obtained with *S.* Typhimurium strain U1 and *S.* Worthington strain 187, we assumed that the activation of the SOS response triggers a significant ArtAB prophage induction and results in high ArtAB production. To confirm the link between *artAB* expression and SOS response following exposure to MTC, antibiotics, and $H_2O_2$, we evaluated the expression of *recA*. Both MTC and enrofloxacin increased *recA* expression significantly (10- to 69-fold, respectively) ($p<0.05$) in all three strains, including *S. bongori* strain ATCC43975, which did not show phage induction; however, $H_2O_2$ did not significantly induce *recA* expression (Fig. 5).

## Discussion

We demonstrated that subinhibitory concentration of both quinolone (nalidixic acid) and fluoroquinolone (enrofloxacin) antibiotics induced ArtA production in *S.* Typhimurium DT104 strain U1, while other antibiotics which exert their activity through different

mechanism, antibiotics that target the cell wall (cefazolin), transcription (rifampicin), and folate metabolism inhibitors (trimethoprim) did not influence ArtA production. Enrofloxacin as well as MTC induced transcription of *artA* gene and production of ArtA in *S.* Typhimurium DT104, *S.* Worthington, and *S. bongrori*. Transcriptional analysis indicated that *artA* and *artB* are cotranscribed and form an operon. Therefore, quinolone antibiotics are potent inducers of *artAB* expression, as reported for Shiga toxin gene in *E.col*i [11, 14, 17].

A previous report showed that *artAB* in *S.* Typhimurium DT104 is encoded on prophage (8). Recently, whole-genome sequencing analysis showed that *artAB* homologs in *S.* Worthington strain 182 and *S. bongori* strain ATCC43975 are also encoded on prophage (Y. Tamamura, I. Uchida, M. Akiba, M. Kusumoto, unpublished data). Furthermore, PHASTER prophage analysis [24] showed that *artAB* is encoded on intact prophage (Similar to Gifsy-1) in both *S.* Typhimurium DT104 strain U1 and *S.* Worthington strain 182, but on incomplete prophage in *S. bongori* strain ATCC43975. The number of *artA* copies in the supernatant of *S.* Typhimurium and *S.* Worthington treated with MTC or enrofloxacin was significantly increased compared with the untreated cultures, suggesting that ArtAB-prophage is induced by both agents that are known to be potent SOS inducers. Agents that provoke the bacterial SOS response, damage DNA, or interfere with DNA synthesis trigger activation of the bacterial RecA protein [25, 26]. The first step in prophage induction is a RecA-dependent autocleavage of the cI repressor that is required for

253 lysogenic establishment to repress the prophage replication and subsequent activation of
254 lytic promoters [27]. Expression of *stx2* and induction of Stx-prophage also result from
255 proteolytic cleavage of the cI repressor, allowing expression of early and middle phage
256 genes [19, 28, 29]. Absence of cI leads to the expression of the anti-terminators Q. The
257 protein Q allows read-through of terminators that inhibit transcription of late phage genes,
258 including *stxAB* which is located between Q and Holin genes [15, 28]. The ArtAB-encoding
259 genes of *S.* Typhimurium DT104 strain U1 and *S.* Worthington strain 182 share the location
260 of *stx2* within a functional prophage (Y. Tamamura, I. Uchida, M. Akiba, and M.
261 Kusumoto, unpublished data). As far as the mechanism of prophage induction considered,
262 the large family of lambdoid prophages including Gifsy-1 found in *Salmonella* genomes
263 employs an alternative induction strategy [30]. The repressors of these phages are not
264 cleaved upon induction; instead, they are inactivated by the binding of small antirepressor
265 proteins. Formation of this complex causes the repressor to dissociate from DNA. The
266 antirepressor genes lie outside the immunity region and are under direct control of the
267 LexA repressor, thus plugging prophage induction directly into the SOS response. At this
268 stage, we have not yet identified any such antirepressor gene in the *artAB* encoding phage
269 genome; similar mechanism might confer both prophage induction and *artAB* expression.
270 Furthermore, in our experiment, MTC, enrofloxacin, and $H_2O_2$ induced *recA* expression in
271 all tested *Salmonella* strains. Similar upregulation of *recA* by SOS inducer is reported in
272 Stx-producing *E. coli* [12, 18]. Therefore, induction of ArtAB production in *Salmonella* is
273 suggested to occur via triggering the SOS response and inducing *recA* transcription,

274 followed by increased expression of *artAB* and induction of ArtAB prophage. The proposed
275 mechanism thus matches that of Stx production in *E. coli*. However, in *S. bongori* strain
276 ATCC43975 phage induction might not occur even following SOS response, since *artAB* in
277 this strain is located on incomplete phage. Nonetheless, expression of *artAB* in this strain
278 was increased upon treatment with enrofloxacin or MTC, suggesting that SOS response
279 followed by induction of *artAB* transcription, but not phage induction, might be essential
280 for ArtAB production.
281
282 Previously, we reported that $H_2O_2$ also induced ArtAB production in *S.* Typhimurium
283 DT104 [9]. In our experiments, $H_2O_2$ at a concentration of 3 mM causes increase of *artA*
284 transcription in all three stains examined, the highest in *S.* Typhimurium DT104 strain U1
285 ($p<0.01$). Under this condition, we also observed prophage induction in *S.* Typhimurium
286 DT104 strain U1, but not in both *S.* Worthington strain 182 and *S. bongori* strain
287 ATCC43975. Moreover, $H_2O_2$ did not increase mRNA level of *recA* in *S.* Worthington
288 strain 182. By western blotting, an intense ArtA signal was observed only in supernatant of
289 *S.* Typhimurium DT104 strain U1 by exposure to $H_2O_2$. Thus, although *artAB* expression
290 was induced by both MTC and enrofloxacin in all tested *Salmonella* strains, $H_2O_2$-mediated
291 induction of the ArtAB phage and efficient production of ArtAB was observed only in *S.*
292 Typhimurium DT104 strain U1. Treatment with $H_2O_2$ was reported to cause induction of
293 Stx-converting prophage in *E.coli*, though with efficiency significantly lower than MTC [15,
294 16, 31-33]. In *E. coli* treated with $H_2O_2$, only a very small fraction of cells is induced for

prophage excision and subsequent lytic development, compared to cells treated with MTC [15, 31]. It has been proposed that OxyR protein, a transcription factor acting as a major regulator of the oxidative stress induced by $H_2O_2$, is responsible for low efficiency of prophage induction, since prophage induction is significantly enhanced in *oxyR* mutant host [34]. Furthermore, different patterns of *stx2* gene expression were reported in *E. coli* O157:H7 and non-O157 serotype treated with $H_2O_2$. Shiga toxin gene *stx2* was upregulated in O157:H7, but not in O104:H4 [35]. Thus, similar to *stx2* expression in *E. coli*, the difference in results obtained with the induction of *artAB* expression in *Salmonella* by MTC, antibiotics, and $H_2O_2$ might reflect the mode of action of these agents, and $H_2O_2$-induced *artAB* expression in *Salmonella* appeared to be strain specific. Reactive oxygen species generated and released by host cells, such as macrophages or leukocytes, may induce *artAB* expression by intracellular bacteria. Since evidence of ArtAB expression *in vivo* is currently missing, further studies on the regulation and expression of the toxin are required to clarify the importance of ArtAB in the virulence of *Salmonella*, especially *S.* Typhimurium DT104.

Importantly, we have shown that quinolone antibiotics, used for treatment of diarrheal disease, induced ArtAB production in *Salmonella*. Although the role of this toxin in virulence of *Salmonella* remains poorly understood, potential clinical complications may arise after therapy with fluoroquinolone antibiotics in animals infected with *artAB*-harboring *Salmonella*.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

No experimental work with humans or animals was performed**.**

**References**

1. **Guibourdenche M, Roggentin P, Mikoleit M, Fields PI, Bockemuhl J et al.** Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. *Res Microbiol* 2010; 161:26-29.

2. **Threlfall EJ, Frost JA, Ward LR, Rowe B.** Epidemic in cattle and humans of

*Salmonella* Typhimurium DT 104 with chromosomally integrated multiple drug resistance. *Vet Rec* 1994; 134:577.

3. **Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M et al.** Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *N Engl J Med* 1998; 338:1333-1338.

4. **Villar RG, Macek MD, Simons S, Hayes PS, Goldoft MJ et al.** Investigation of multidrug-resistant *Salmonella* serotype Typhimurium DT104 infections linked to raw-milk cheese in Washington State. *Jama* 1999; 281:1811-1816.

5. **Sameshima T, Akiba M, Izumiya H, Terajima J, Tamura K et al.** *Salmonella* Typhimurium DT104 from livestock in Japan. *Jpn J Infect Dis* 2000; 53:15-16.

6. **Leekitcharoenphon P, Hendriksen RS, Le Hello S, Weill FX, Baggesen DL et al.** Global genomic epidemiology of *Salmonella enterica* serovar Typhimurium DT104. *Appl Environ Microbiol* 2016; 82:2516-2526.

7. **Allen CA, Fedorka-Cray PJ, Vazquez-Torres A, Suyemoto M, Altier C et al.** In vitro and in vivo assessment of *Salmonella enterica* serovar Typhimurium DT104 virulence. *Infect Immun* 2001; 69:4673-4677.

8. **Saitoh M, Tanaka K, Nishimori K, Makino S, Kanno T et al.** The *artAB* genes encode a putative ADP-ribosyltransferase toxin homologue associated with *Salmonella enterica* serovar Typhimurium DT104. *Microbiology* 2005; 151:3089-3096.

9. **Uchida I, Ishihara R, Tanaka K, Hata E, Makino S et al.** *Salmonella enterica* serotype Typhimurium DT104 ArtA-dependent modification of pertussis toxin-sensitive

G proteins in the presence of [$^{32}$P]NAD. *Microbiology* 2009; 155:3710-3718.

10. **Tamamura Y, Tanaka K, Uchida I.** Characterization of pertussis-like toxin from *Salmonella* spp. that catalyzes ADP-ribosylation of G proteins. *Sci Rep* 2017; 7:2653.

11. **Bielaszewska M, Idelevich EA, Zhang W, Bauwens A, Schaumburg F et al.** Effects of antibiotics on Shiga toxin 2 production and bacteriophage induction by epidemic *Escherichia coli* O104:H4 strain. *Antimicrob Agents Chemother* 2012; 56:3277-3282.

12. **Fang Y, Mercer RG, McMullen LM, Ganzle MG.** Induction of Shiga toxin-encoding prophage by abiotic environmental stress in Food. *Appl Environ Microbiol* 2017; 83: e01378-17. .

13. **Fogg PC, Saunders JR, McCarthy AJ, Allison HE.** Cumulative effect of prophage burden on Shiga toxin production in *Escherichia coli*. *Microbiology* 2012; 158:488-497.

14. **Kimmitt PT, Harwood CR, Barer MR.** Toxin gene expression by Shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. *Emerg Infect Dis* 2000; 6:458-465.

15. **Licznerska K, Nejman-Falenczyk B, Bloch S, Dydecka A, Topka G et al.** Oxidative stress in shiga toxin production by enterohemorrhagic *Escherichia coli*. *Oxid Med Cell Longev* 2016; 2016:3578368.

16. **Los JM, Los M, Wegrzyn A, Wegrzyn G.** Hydrogen peroxide-mediated induction of the Shiga toxin-converting lambdoid prophage ST2-8624 in *Escherichia coli* O157:H7. *FEMS Immunol Med Microbiol* 2010; 58:322-329.

17. **Matsushiro A, Sato K, Miyamoto H, Yamamura T, Honda T.** Induction of prophages of enterohemorrhagic *Escherichia coli* O157:H7 with norfloxacin. *J Bacteriol* 1999; 181:2257-2260.

18. **Toshima H, Yoshimura A, Arikawa K, Hidaka A, Ogasawara J et al.** Enhancement of shiga toxin production in enterohemorrhagic *Escherichia coli* serotype O157:H7 by DNase colicins. *Appl Environ Microbiol* 2007; 73:7582-7588.

19. **Chakraborty D, Clark E, Mauro SA, Koudelka GB.** Molecular mechanisms governing "Hair-Trigger" induction of shiga toxin-encoding prophages. *Viruses* 2018; 10: doi: 10.3390/v10050228.

20. **Finkelstein RA, Atthasampunna P, Chulasamaya M, Charunmethee P.** Pathogenesis of experimental cholera: biologic ativities of purified procholeragen A. *J Immunol* 1966; 96:440-449.

21. **Clinical and Laboratory Standards Institute (CLSI).** Performance Standards for Antimicrobial susceptibility Testing. 27th ed 2017.

22. **Fey A, Eichler S, Flavier S, Christen R, Hofle MG et al.** Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. *Appl Environ Microbiol 2004*; 70:3618-3623.

23. **Bonanno L, Petit MA, Loukiadis E, Michel V, Auvray F.** Heterogeneity in induction level, infection ability, and morphology of Shiga toxin-encoding phages (*stx* phages) from dairy and human shiga toxin-producing *Escherichia coli* O26:H11 Isolates.

*Appl Environ Microbiol* 2016; 82:2177-2186.

24. **Arndt D, Grant JR, Marcu A, Sajed T, Pon A et al.** PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* 2016; 44(W1):W16-21.

25. **Patel M, Jiang Q, Woodgate R, Cox MM, Goodman MF.** A new model for SOS-induced mutagenesis: how RecA protein activates DNA polymerase V. *Crit Rev Biochem Mol Biol* 2010; 45:171-184.

26. **Baharoglu Z, Mazel D.** SOS, the formidable strategy of bacteria against aggressions. *FEMS Microbiol Rev* 2014; 38:1126-1145.

27. **Mustard JA, Little JW.** Analysis of *Escherichia coli* RecA interactions with LexA, lambda CI, and UmuD by site-directed mutagenesis of *recA*. *J Bacteriol* 2000; 182:1659-1670.

28. **Tyler JS, Mills MJ, Friedman DI.** The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. *J Bacteriol* 2004; 186:7670-7679.

29. **Wagner PL, Neely MN, Zhang X, Acheson DW, Waldor MK et al.** Role for a phage promoter in Shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. *J Bacteriol* 2001; 183:2081-2085.

30. **Lemire S, Figueroa-Bossi N, Bossi L.** Bacteriophage crosstalk: coordination of prophage induction by trans-acting antirepressors. *PLoS Genet* 2011; 7:e1002149.

31. **Los JM, Los M, Wegrzyn A, Wegrzyn G.** Altruism of Shiga toxin-producing *Escherichia coli*: recent hypothesis versus experimental results. *Front Cell Infect*

*Microbiol* 2012; 2:166.

32. **Los JM, Los M, Wegrzyn G, Wegrzyn A.** Differential efficiency of induction of various lambdoid prophages responsible for production of Shiga toxins in response to different induction agents. *Microb Pathog* 2009; 47:289-298.

33. **Los JM, Los M, Wegrzyn G.** Bacteriophages carrying Shiga toxin genes: genomic variations, detection and potential treatment of pathogenic bacteria. *Future Microbiol* 2011; 6:909-924.

34. **Glinkowska M, Los JM, Szambowska A, Czyz A, Calkiewicz J et al.** Influence of the *Escherichia coli oxyR* gene function on lambda prophage maintenance. *Arch Microbiol* 2010; 192:673-683.

35. **Mei GY, Tang J, Carey C, Bach S, Kostrzynska M.** The effect of oxidative stress on gene expression of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and non-O157 serotypes. *Int J Food Microbiol* 2015; 215:7-15.

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Table 1. MICs of five antibiotics for *Salmonella* strains investigated for antibiotics effects on *artAB* expression

| | MIC ($\mu g\ ml^{-1}$) | | |
|---|---|---|---|
| Antibiotic | *S.* Typhimurium DT104 strain U1 | *S.* Worthington strain 182 | *S. bongori* strain ATCC43975 |
| enrofloxacin | 0.08 | 0.04 | 0.07 |
| nalidixic acid | 20 | — | — |
| cefazolin | 28 | — | — |
| trimethoprim | 3 | — | — |
| rifampicin | 30 | — | — |

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Table 2. Primers used to generate DNA and RNA standards and PCR primers for qPCR

| Target gene[a] | Primer name | Sequence (5' → 3')[b] | Product size | Accession no. |
|---|---|---|---|---|
| Generation of Starndard | | | | |
| *artA* (ST DT104) | ST ArtA T7F | TAATACGACTCACTATAGGGTTGACTCGAGACCTCCGGAT | 601 | AB104436 |
| | ST ArtA T7R | TGCATCATCCCTGCGTACTC | | |
| *artA* (SW) | ST ArtA T7F | TAATACGACTCACTATAGGGTTGACTCGAGACCTCCGGAT | 601 | LC127363 |
| | SW ArtA T7R | TGCATCATCCCTGCGCACTC | | |
| *artA* (Sb) | Sb artA T7F | TAATACGACTCACTATAGGGTGGACTCGAGACCTCCGGAT | 605 | LC127367 |
| | Sb artA R2R | TACGTGTATCATCCCTGCGC | | |
| *artAB* (ST DT104) | ST artAB T7F | TAATACGACTCACTATAGGGAGGAGTACGCAGGGATGATG | 384 | AB104436 |
| | ST artAB T7R | CCCTGACCTATACACGCCATA | | |
| *artB* (ST DT104) | ST artB T7F | TAATACGACTCACTATAGGGGCGTGTATAGGTCAGGGGATA | 318 | AB104436 |
| | ST artB T7R | GGCAACGTAGGTCCCATACA | | |
| *recA* (ST DT104) | ST recA T7F | TAATACGACTCACTATAGGGATTGTGGTCGACTCCGTAGC | 603 | NC_003197 |
| | ST recA T7R | GGCGTGGCATTCTGATTACT | | |
| *recA* (SW) | ST recA T7F | TAATACGACTCACTATAGGGATTGTGGTCGACTCCGTAGC | 603 | JYYI01000003 |
| | ST recA T7R | GGCGTGGCATTCTGATTACT | | |
| *recA* (Sb) | Sb recA T7F | TAATACGACTCACTATAGGGATTGTCGTCGACTCCGTCG | 603 | NC_015761 |
| | Sb recA T7R | GGAGTAGAATCCTGATTGCT | | |
| PCR primers | | | | |
| *artA* (ST DT104) | ST ArtA F1 | AGTTTCTACAGCCTTCCGCC | 84 | AB104436 |
| | ST ArtA R1 | CCGCATCATCACTCGCTCAA | | |
| *artA* (SW) | ST ArtA F1 | AGTTTCTACAGCCTTCCGCC | 84 | LC127363 |
| | ST ArtA R1 | CCGCATCATCACTCGCTCAA | | |
| *artA* (Sb) | Sb artA F1 | AGTTTCTATAGCCTTCCGCC | 84 | LC127367 |
| | ST artA R1 | CCGCATCATCACTCGCTCAA | | |
| *artAB* (ST DT104) | ST artAB F1 | TTATGACGCACGGGGAGTTT | 225 | AB104436 |
| | ST artAB R1 | CACTGGATAACGACGCAAGA | | |
| *artAB* (SW) | SW artAB F1 | TTATGACGCACGGGGAGTTT | 224 | LC127363 |
| | SW artAB R1 | CACTGGATAACGACGCAAGA | | |
| *artAB* (Sb) | Sb artAB F1 | AAATCGTGGCTATCGGGACC | 119 | LC127367 |
| | Sb artAB R1 | ACCCCCAGCCTTAAGAAACG | | |
| *artB* (STDT104) | ST artB F1 | AAGAGGGGGAGTCAAGTTCC | 297 | AB104436 |
| | ST artB R1 | ACATCCTCCCCTGTTGCATA | | |
| *artB* (SW) | SW artAB F1 | AATAATGAGAGTCAAATACC | 126 | LC127363 |
| | SW artAB R1 | ACTTTCTCCCCTGTTGTATA | | |
| *artB* (Sb) | SW artAB F1 | CATCATCAGGTGGTTCAAATGT | 97 | LC127367 |
| | SW artAB R1 | ATTTTCTCACCTGTTGTATA | | |
| *recA* (ST DT104) | ST recA F1 | GGCGAAATCGGCGACTCT | 123 | NC_003197 |
| | ST recA R1 | CATACGGATCTGGTTGATGAAAATC | | |
| *recA* (SW) | ST recA F1 | GGCGAAATCGGCGACTCT | 125 | JYYI01000003 |
| | ST recA R1 | CATACGGATCTGGTTGATGAAAATC | | |
| *recA* (Sb) | Sb recA F1 | GGCGAGATTGGCGACTCTC | 123 | NC_015761 |
| | Sb recA R1 | CATACGGATCTGGTTGATAAAAATC | | |

[a] ST DT104, *S*. Typhimurium DT104; SW, *S*. Worthington; S.b, *S. bongori*.

[b] Sequences corresponding to the T7 promoter are underlined.

**Figure legends**

**Fig. 1.** Western blotting analysis of ArtA. (a) Effects of enrofloxacin (EFX), nalidixic acid (NA), cefazolin (CFZ), trimethoprim (TMP), and rifampicin (RFP) at 1/2 MIC on ArtA production in *S.* Typhimurium DT104 strain U1. Effect of MTC (0.5 μg $ml^{-1}$) was also examined. (b) Induction of ArtA expression in *Salmonella* strains by treatment with MTC (0.5 μg $ml^{-1}$), EFX (1/2 MIC) and $H_2O_2$ (3 mM). Each sample was assessed at least twice using the western blotting assay.

**Fig. 2.** Analysis of transcriptional of *artA* and *artB* by Q-RT-PCR. (a) Map of part of ArtAB-prophage in *S.* Typhimurium DT104 strain U1. Target regions by Q-RT-PCR are indicated below the map. (b) Transcriptional analysis using primers designed to amplify the region within *artA*, *artB* and intergenic region spanning both genes. For each region, two PCR analysis were carried out; +, Q-RT-PCR on cDNA, -, negative control with no reverse transcriptase. M, 100 bp DNA ladder size marker (TOYOBO) was used as size marker (C) Number of copies of mRNA corresponding target regions, as determined by Q-RT-PCR analysis. Error bars indicate the standard errors of the means. Data are from three separate experiments.

**Fig. 3.** Effect of mitomycin, enrofloxacin, $H_2O_2$ on *artA* transcription.

Overnight cultures of *Salmonella* strains were diluted 1:50 in 10 ml syncase broth and cultured with shaking at 37°C for 3 hr. Then, the culture was treated with MTC (0.5 μg

478 $ml^{-1}$), enrofloxacin (1/2 MIC) or $H_2O_2$ (3 mM) as an induction agent. After 3 h of
479 incubation at 37°C with shaking, RNA was extracted and used as a template for qRT-PCR.
480 Number of copies of *artA* mRNA per 100 ng total RNA, was determined by qRT-PCR
481 analysis. Data represent mean ± standard errors of the means from three independent
482 experiments. *, $P<0.05$, **, $P<0.01$ (unpaired Student's t test).
483
484 **Fig. 4.** Effect of MTC, enrofloxacin, and $H_2O_2$ on ArtAB-prophage induction. *Salmonella*
485 strains were grown in a syncase broth at 37°C with shaking with and without an induction
486 agent. The relative *artA* DNA abundance in the culture supernatant was determined by
487 qPCR. Data show fold increase in *artA* copy number compared to that in agent-free cultures.
488 Data represent mean ± standard errors of the means from three independent experiments.
489
490 **Fig. 5.** Expression of *recA* in *Salmonella* strains after treatment with MTC, enrofloxacin,
491 and $H_2O_2$. The culture was treated with MTC (0.5 μg $ml^{-1}$), enrofloxacin (1/2 MIC) or $H_2O_2$
492 (3 mM) as an induction agent. After 3 h of incubation at 37°C with shaking, RNA was
493 extracted and used as a template for qRT-PCR. Number of copies of *recA* mRNA per 100
494 ng total RNA, was determined by qRT-PCR analysis. Data represent mean ± standard
495 errors of the means from three independent experiments. *, $P<0.05$, **, $P<0.01$ (unpaired
496 Student's t test).
497

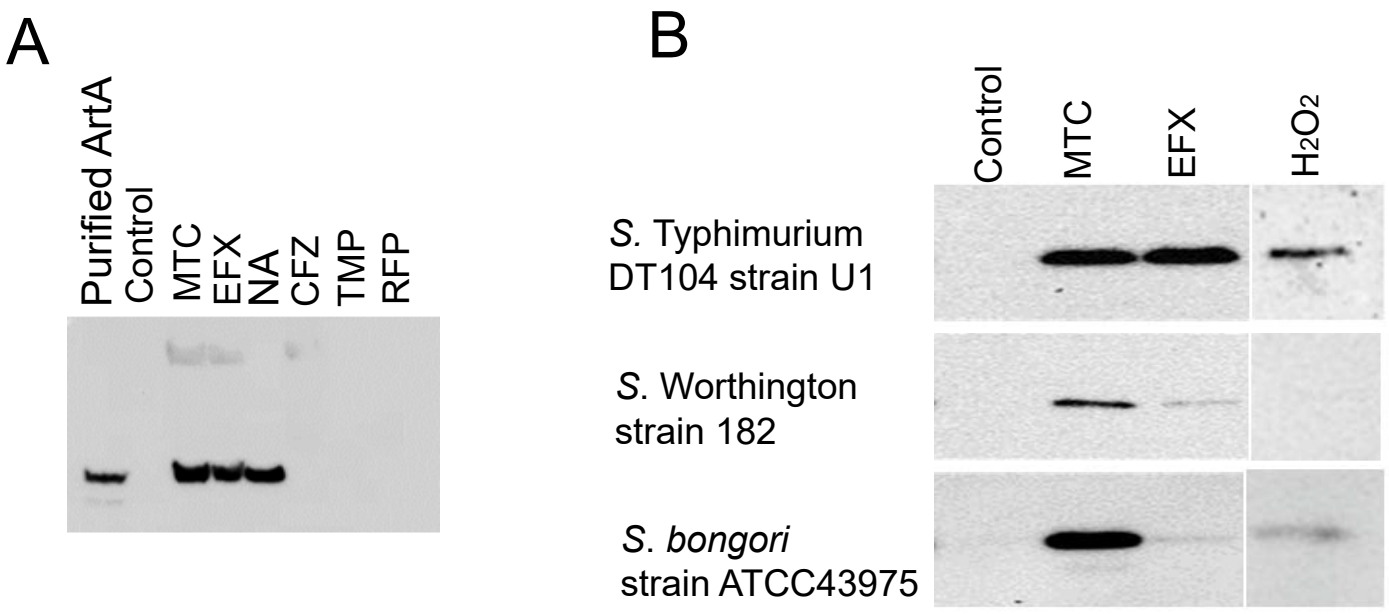


Figure 1. Western blotting analysis of ArtA. (A) Effects of enrofloxacin (EFX), nalidixic acid (NA), cefazolin (CFZ), trimethoprim (TMP), and rifampicin (RFP) at 1/2 MIC on ArtA production in *S.* Typhimurium DT104 strain U1. Effect of MTC (0.5 μg/ml) was also examined. (B) Induction of ArtA expression in *Salmonella* strains by treatment with MTC (0.5 μg/ml), EFX (1/2 MIC) and $H_2O_2$ (3 mM).

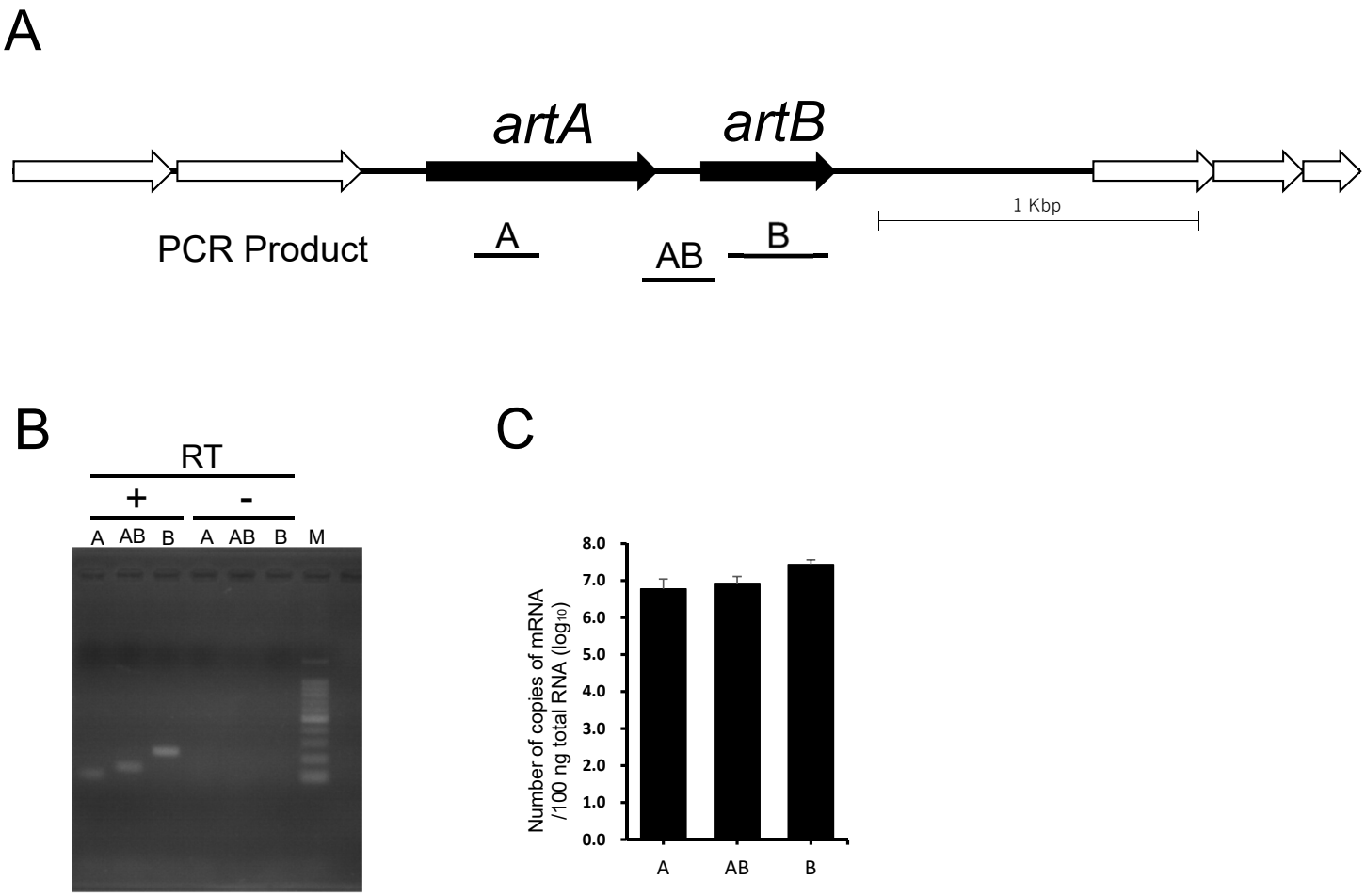


Figure 2. Analysis of transcriptional of *artA* and *artB* by Q-RT-PCR. (A) Map of part of ArtAB-prophage in *S.* Typhimurium DT104 strain U1. Target regions by Q-RT-PCR are indicated below the map. (B) Transcriptional analysis using primers designed to amplify the region within *artA*, *artB* and intergenic region spanning both genes. For each region, two PCR analysis were carried out; +, Q-RT-PCR on cDNA, -, negative control with no reverse transcriptase. M, 100 bp DNA ladder size marker (TOYOBO) was used as size marker (C) Number of copies of mRNA corresponding target regions, as determined by Q-RT-PCR analysis. Error bars indicate the standard errors of the means. Data are from three separate experiments.

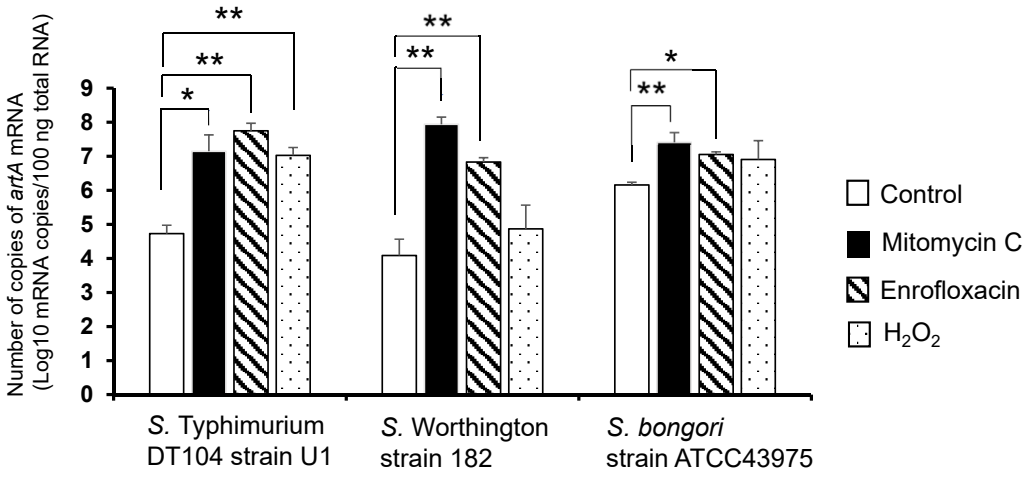


Figure 3. Effect of mitomycin, enrofloxacin, $H_2O_2$ on *artA* transcription.
Overnight cultures of *Salmonella* strains were diluted 1:50 in 10 ml syncase broth and cultured with shaking at 37°C for 3 hr. Then, the culture was treated with MTC (0.5 μg/ml), enrofloxacin (1/2 MIC) or $H_2O_2$ (3 mM) as an induction agent. After 3 h of incubation at 37°C with shaking, RNA was extracted and used as a template for qRT-PCR. Number of copies of artA mRNA per 100 ng total RNA, was determined by qRT-PCR analysis.Data represent mean ± standard errors of the means from three independent experiments. *, $P<0.05$, **, $P<0.01$ (unpaired Student's t test).

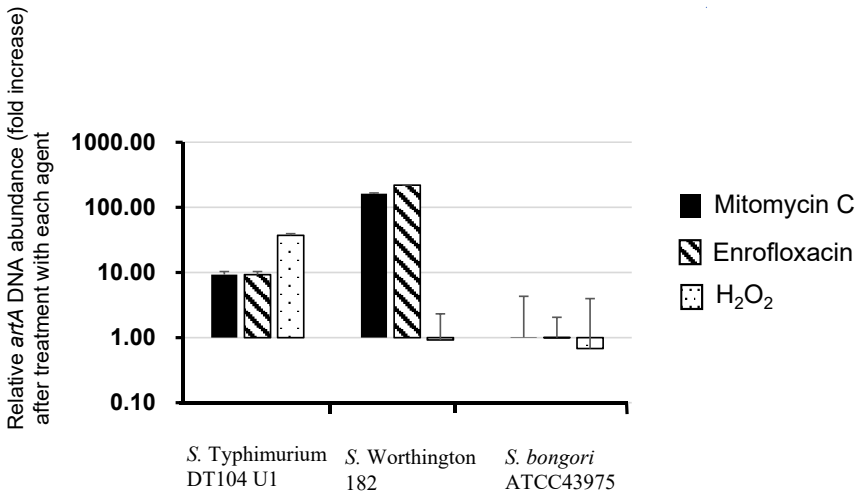


Figure 4. Effect of MTC, enrofloxacin, and hydrogen peroxidase on ArtAB-prophage induction. *Salmonella* strains were grown in a syncase broth at 37°C with shaking with and without an induction agent. The relative *artA* DNA abundance in the culture supernatant was determined by qPCR. Data show fold increase in *artA* copy number compared to that in agent-free cultures. Data represent mean ± standard errors of the means from three independent experiments.

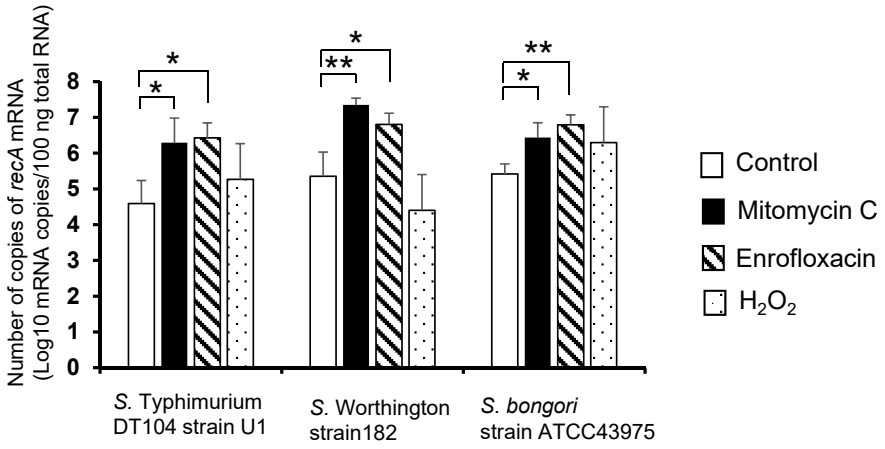


Figure 5. Expression of *recA* in *Salmonella* strains after treatment with MTC, enrofloxacin, and $H_2O_2$. The culture was treated with MTC (0.5 μg/ml), enrofloxacin (1/2 MIC) or $H_2O_2$ (3 mM) as an induction agent. After 3 h of incubation at 37°C with shaking, RNA was extracted and used as a template for qRT-PCR. Number of copies of recA mRNA per 100 ng total RNA, was determined by qRT-PCR analysis. Data represent mean ± standard errors of the means from three independent experiments. *, $P<0.05$, **, $P<0.01$ (unpaired Student's t test).