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First Detection of a Fosfomycin Resistance Gene, *fosA7*, in *Salmonella enterica* Serovar Heidelberg Isolated from Broiler Chickens

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ABSTRACT We previously described *Salmonella enterica* serovar Heidelberg isolates harboring a chromosomal gene cluster similar to the glutathione *S*-transferase gene, a putative *fosA* gene conferring resistance to fosfomycin. Here, we show that this new gene, named *fosA7*, confers resistance to fosfomycin. The introduction of *fosA7* into the fosfomycin-susceptible *Salmonella enterica* serovar Enteritidis resulted in a substantial increase in the fosfomycin MIC. This finding increases the awareness of antibiotic resistance in *Salmonella* Heidelberg from broilers as related to the food safety and public health.

KEYWORDS fosfomycin resistance, *fosA7* gene, *Salmonella* Heidelberg, broiler chicken

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Many pathogenic bacteria have shown resistance to fosfomycin. Recently, a high prevalence of plasmid-mediated fosfomycin resistance among CTX-M-producing *Escherichia coli* strains in clinical settings (1–5) and in companion animals (6, 7) has been reported in several countries. Most bacteria are inherently resistant to fosfomycin and carry chromosomal mutations that impair its transport, while others possess fosfomycin-modifying enzymes. Several of these enzymes have been reported in a wide range of Gram-negative bacteria and in some Gram-positive pathogenic bacteria. Fosfomycin resistance was first reported in the early 1980s in a clinical *Serratia marcescens* strain carrying a plasmid-mediated transposable element, Tn*2921*, which harbored the *fosA* gene flanked by two terminal copies of an identical insertion sequence (8). The *fosA* gene product is a glutathione *S*-transferase, a metalloenzyme transferred through plasmids to *Enterobacteriaceae*, with two other variants found in *Pseudomonas aeruginosa* (9). New subtypes of *fosA* with similar structures have been described (*fosA2*, *fosA3*, *fosA4*, *fosA5 fosA6*, *fosB*, *fosC*, *fosC2*, *fosX*, and *fosK*); the mechanism of resistance associated with each of them has been reviewed elsewhere (10–14).

We previously described *Salmonella enterica* serovar Heidelberg isolates harboring a glutathione *S*-transferase gene cluster having 76 to 80% amino acid sequence similarity with *fosA* (15). Since such similarities do not translate into a function for an antimicrobial resistance phenotype, and due to the clinical relevance of fosfomycin, the objective of this study was to investigate the ability of this new gene to confer a fosfomycin resistance phenotype in *S. enterica* serovars. This gene was named *fosA7* to remain consistent with the nomenclature after the latest *fosA6* gene recently described by Guo et al. (14).

The *fosA7* gene was identified on the chromosomes of all four *Salmonella* Heidelberg strains, surrounded by two hypothetical genes of unknown functions (Fig. 1a). The *fosA7* gene has a coding sequence of 423 bp encoding a predicted FosA7 protein of 140 amino acid residues. NCBI-BLAST (16) analysis revealed that the FosA7 protein is highly

FIG 1 (a) Genetic environment of a chromosomal fosfomycin resistance gene, *fosA7*, and its neighboring region (~19.7 kb) in *Salmonella* Heidelberg ABB07-SB3031. The gene cassettes are not drawn to scale. (b) Alignment generated in T-Coffee of amino acid sequences of the newly identified FosA7 (in bold) with 13 known fosfomycin resistance proteins found in different bacterial species. Asterisk, amino acid

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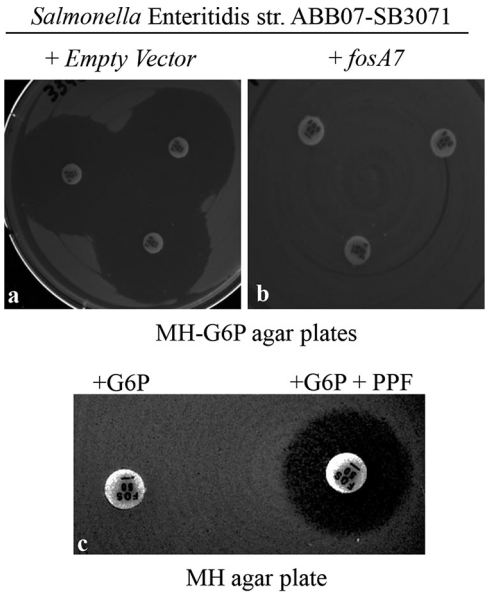

FIG 2 Inhibition zone sizes by fosfomycin (50 μg/disk) in the Kirby-Bauer drug susceptibility test (KBDST) on MH-G6p agar plates (n = 3). (a and b) *Salmonella* Enteritidis ABB07-SB3071 containing the empty vector (a) and containing the *fosA7* gene from *Salmonella* Heidelberg ABB07-SB3031 (b). (c) *Salmonella* Enteritidis ABB07-SB3071 transformed with *fosA7* on Mueller-Hinton (MH) agar plate; the fosfomycin disk on the left contained 50 μg glucose-6-phosphate (G6P), and the disk on the right contained 50 μg of G6P in addition to 1 mg of phosphonoformate (PPF).

conserved among different bacteria. Overall, FosA7 shared 30% to 78% amino acid sequence identity with other reported fosfomycin resistance gene subtype products (Fig. 1b). This suggests phylogenetic relationships and a common ancestry with FosA2, FosA4, FosA5, FosA6, FosA^{TN} (found in *Serratia marcescens*), and FosA^{PA} (found in *Pseudomonas aeruginosa*) (Fig. 1c). FosA7 shares 78.6%, 63.8%, 64.0%, and 62.6% sequence identities with FosA2, FosA4, FosA5, and FosA6, respectively, and 76.4% and 60.3% identities with FosA^{TN} and FosA^{PA}, respectively. Interestingly, no *fosA7* homolog was found in the closely related *Salmonella enterica* Enteritidis serovar.

To assess the fosfomycin resistance phenotype conferred by the chromosomal *fosA7* gene, the corresponding 720-bp region derived from the genomic DNA of donor *Salmonella* Heidelberg strain ABB07-SB3031 was cloned into a high-copy-number vector and transformed into the fosfomycin-susceptible *Salmonella* Enteritidis ABB07-SB3071. This *fosA7*-transformed *S.* Enteritidis strain exhibited complete resistance to fosfomycin (Fig. 2b), in comparison to the recipient transformed with the empty vector, which showed susceptibility to fosfomycin, with an inhibitory zone size greater than 35 mm (Fig. 2a). As shown on Fig. 2c, the contribution of *fosA7* in the resistance to fosfomycin was further confirmed in *Salmonella* Enteritidis ABB07-SB3071 by performing a disk potentiation test, using phosphonoformate (PPF), a specific inhibitor of the *fosA* gene product, as described by Wachino et al. (17).

The susceptibility results obtained from the disk diffusion assay were validated using serial broth and agar dilution methods, according to CLSI guidelines (18, 19). The results were interpreted according to the current European Committee on Antimicrobial

FIG 1 Legend (Continued)
residues conserved among the 14 fosfomycin resistance determinants; colon and dots, amino acid substitutions that result in homologous amino acid residues. The protein GenBank accession numbers are FosA7, KKE03230; FosA2, ACC85616; FosA3, AB522970; FosA4, AB908992; FosA5, AJE60855; FosA6, NG051497; FosA^{PA}, AAT49669; FosA^{TN}, AAA98399; FosB, ABS73480; FosC, AAZ14834; FosC2, AB522969; FosK, AB917040; FosX, CWV56762; and open reading frame 1 (ORF1), AAP50248. (c) Phylogenetic relationships between 14 fosfomycin determinants, including the newly described FosA7 (boxed) calculated at http://www.phylogeny.fr.

TABLE 1 List of *Salmonella* strains used in this study and their fosfomycin MIC values with their interpretations

Inventory no.	Collection date (day-mo-yr)	Organism	Strain or isolate	Zone of inhibition (mm)	Fosfomycin MIC (μg/ml) (resistance)[a]
1768	04-Oct-04	*Salmonella* Heidelberg	SALB-47-2	26	>32 (R)
1770	04-Oct-04	*Salmonella* Heidelberg	SALB-46	26	>32 (R)
1773	25-Oct-04	*Salmonella* Heidelberg	SALB-159-4	26	>32 (R)
3342	28-Jun-05	*Salmonella* Heidelberg	ABB07-SB3031	26	>32 (R)
3346	28-Jun-05	*Salmonella* Enteritidis	ABB07-SB3071	35	<2 (S)
3346-1	28-Jun-05	*Salmonella* Enteritidis	ABB07-SB3071 + empty vector	35	<2 (S)
3346-2	28-Jun-05	*Salmonella* Enteritidis	ABB07-SB3071 + *fosA7*	0	>512 (R)
12[b]	NA[c]	*Escherichia coli*	ATCC 25922	27	<2 (S)

[a]MICs were measured by both the broth and agar dilution method recommended by the CLSI and interpreted by EUCAST standard; the interpretation is designated I (intermediate), S (susceptible), or R (resistant).
[b]Quality control strain.
[c]NA, not applicable.

Susceptibility Testing (26) criteria (susceptible, ≤32 μg/ml; resistant, >32 μg/ml). The MIC values and the Kirby-Bauer test results of four *fosA7*-positive *Salmonella* Heidelberg strains and a *fosA7*-negative *Salmonella* Enteritidis strain used in this study are presented in Table 1.

The recipient *Salmonella* Enteritidis ABB07-SB3071 (containing the *fosA7* gene) showed a >256-fold increase in fosfomycin MIC (≥512 μg/ml), thus demonstrating an increased resistance compared to the parent donor strain (Table 1). These results further suggest that *fosA7* is responsible for fosfomycin resistance, and if transferred on plasmids, it can induce a high level of resistance in the recipient bacterial strain.

Fosfomycin was recently reintroduced in Canada for the treatment of acute uncomplicated urinary tract infections (UTIs) in adult women caused by *E. coli* and *Enterococcus faecalis* (20). Several studies have reported the presence of fosfomycin resistance genes, along with various β-lactamases, including the AmpC-like and extended-spectrum β-lactamases (6, 10). In this study, we characterized a newly identified *fosA7* gene that confers resistance to fosfomycin in *Salmonella* Heidelberg strains isolated from broiler chickens. This *fosA7* gene was expressed on a plasmid in a recipient *S.* Enteritidis strain to induce a >256-fold increase in the fosfomycin MIC value (MIC, ≥512 μg/ml) compared to that of the parental nontransformed strain (MIC, ≥2 μg/ml). The increase in fosfomycin resistance when *fosA7* was transferred from the chromosome onto a plasmid suggests that the genetic background may play a role in the expression of this gene. A similar phenomenon was observed for several well-characterized resistance genes, such as chromosomal β-lactamases (21).

Fosfomycin resistance is typically plasmid mediated in most members of *Enterobacteriaceae* (7). Several published studies have determined the genetic environment of subtypes of this resistance gene, especially *fosA3* by PCR mapping and sequencing (1, 22). Two recent studies conducted by Yao et al. (7) and Lin and Chen (23) identified two genetic environments for *fosA3* harboring two IS*26* transposable elements surrounding the *fosA3* gene. The cooccurrence of the fosfomycin resistance gene has often been detected in bla_{CTX-M}-producing and multidrug-resistant bacteria (5, 23). This could further challenge the use of fosfomycin as an alternative treatment approach against UTIs caused by both *E. coli* and *Salmonella* (24). Contrary to all previously published data, Hou et al. (6) recently reported that the major type of genes conferring resistance to fosfomycin appears to be chromosomal rather than plasmid mediated. Kitanata et al. (25) identified an integron-mediated chromosomal fosfomycin resistance gene, *fosK*, in *Acinetobacter soli* harboring aminoglycoside-modifying enzymes encoded by the *aacA4* gene. In the present study, to further explore the prevalence of *fosA7*, we performed a PCR in 15 *Salmonella* Heidelberg isolates in our collection, and all were found positive for the *fosA7* gene. Additionally, we also performed a BLAST analysis of the FosA7 protein sequence (query length, 140 amino acids) among *Salmonella* serotypes (~2,610 known to date) against the microbial protein database. Only 11 matches were found among *Salmonella enterica* strains. However, in our nucleotide BLAST search (query

length, 423 nucleotides) against both the complete and draft genomes (41,831 total genomes available from NCBI RefSeq on 17 February 2017) of *Salmonella* species available in GenBank, the *fosA7* gene was detected in only 35 *Salmonella* genomes. Of these, 26 genomes (74.3%) were *Salmonella* Heidelberg, with 100% sequence identity to the query sequence. Others include four *Salmonella enterica* serovar Agona genomes, three *S. enterica* serovar Montevideo genomes, and two *S. enterica* serovar Tennessee genomes, with sequence similarities ranging from 94 to 97%. The nucleotide BLAST against the plasmids (2,417 available in total in the GenBank database on 17 February 2017) showed no significant match. These findings reveal a limited prevalence of *fosA7* among the *Salmonella* serotypes, with *Salmonella* Heidelberg being the most common carrier of this gene. Unlike *E. coli* and other pathogens where plasmid-mediated fosfomycin has been detected, the *fosA7* gene is exclusively located on the *Salmonella* chromosome.

In conclusion, we report for the first time a new fosfomycin resistance gene, *fosA7*, in *Salmonella* Heidelberg isolates recovered from broiler chickens in British Columbia, Canada. Currently, the presence of fosfomycin resistance among *Salmonella* species appears to be limited to a few serotypes. Because the *fosA7* gene can confer a high level of resistance and is potentially transferable, it is of great concern that *fosA7* could further spread via horizontal gene transfer within bacterial communities due to the increased use of fosfomycin in both clinical and veterinary settings. Vigilant monitoring for the spread of fosfomycin resistance in bacteria isolated from humans and animals is needed.

Accession number(s). The fosfomycin resistance gene (*fosA7*) sequence is available in GenBank under the accession number KKE03230.1.

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We declare no conflicts of interest.

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