Salmonella enterica Typhimurium *fljBA* operon stability: implications regarding the origin of *Salmonella enterica* I 4,[5],12:i:-

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Genet. Mol. Res. 14 (4): 19057-19065 (2015)
Received August 27, 2015
Accepted October 8, 2015
Published December 29, 2015
DOI http://dx.doi.org/10.4238/2015.December.29.13

ABSTRACT. *Salmonella enterica* subsp *enterica* serovar 4,5,12:i:- has been responsible for many recent *Salmonella* outbreaks worldwide. Several studies indicate that this serovar originated from *S. enterica* subsp *enterica* serovar Typhimurium, by the loss of the flagellar phase II gene (*fljB*) and adjacent sequences. However, at least two different clones of *S. enterica* 4,5,12:i:- exist that differs in the molecular events responsible for *fljB* deletion. The aim of this study was to test the stability of the *fljBA* operon responsible for the flagellar phase variation under different growth conditions in order to verify if its deletion is a frequent event that could explain the origin and dissemination of this serovar. In fact, coding sequences for transposons are present near this operon and in some strains, such as *S. enterica* Typhimurium LT2, the Fels-2 prophage gene is inserted near this operon. The presence of mobile DNA could confer instability to this region. In order to examine this, the *cat* (chloramphenicol acetyltransferase) gene was inserted adjacent to the *fljBA* operon so that deletions involving this genomic region could be identified. After growing *S. enterica* chloramphenicol-resistant strains under different conditions, more

than 10^4 colonies were tested for the loss of chloramphenicol resistance. However, none of the colonies were sensitive to chloramphenicol. These data suggest that the origin of *S. enterica* serovar 4,5,12:i:- from Typhimurium by *fljBA* deletion is not a frequent event. The origin and dissemination of 4,5,12:i:- raise several questions about the role of flagellar phase variation in virulence.

Key words: *Salmonella enterica*; *fljBA* operon; Flagellar phase variation; Mutation; λ-red; Serovar 4,5,12:i:-

INTRODUCTION

Salmonella enterica subspecies (subsp) *enterica* serovar 4,[5],12:i:- has been frequently isolated from humans and other animals worldwide (reviewed by Moreno-Switt et al., 2009). Considered an emergent and multi-drug resistant serovar, *S. enterica* 4,5,12:i:- has been responsible for outbreaks in several continents (Echeita and Usera, 1998; Amavist et al., 2005; Mossong et al., 2006; Hauser et al., 2010; Hopkins et al., 2010; CDC, 2011; Raguenaud et al., 2012; Guillier et al., 2013; Mandilara et al., 2013; Gallati et al., 2013; Mulvey et al., 2013; Arguello et al., 2014; Garcia et al., 2014). In Brazil, this emergent serovar was isolated in the 1970s (Tavechio et al., 1996; Tavechio et al., 2004; Fernandes et al., 2006).

A number of studies have demonstrated that *S. enterica* 4,[5],12:i:- is a variant of serovar Typhimurium due to the loss of the *fljBA* operon, which is responsible for flagellar phase variation (Echeita et al., 2001; Garaizar et al., 2002; Soyer et al., 2009; Laorden et al., 2010). In serovar Typhimurium, the two flagellar antigens are encoded by two structural genes, *fliC* (flagellar phase I) and *fljB* (flagellar phase II) (Kutsukake et al., 2006). The genes *fljB, fljA,* and *hin* form the *fljBA* operon, responsible for regulation of phase variation. The *fljA* gene encodes a negative regulator which inhibits *fliC* expression by post-transcriptional control (Bonifield and Hughes, 2003; Yamamoto and Kutsukake, 2006). Flagellar phase variation is determined by the inversion of a 996 bp DNA fragment called the H segment, which contains the promoter sequences of *fljB* and *fljA* (Yamamoto and Kutsukake, 2006). This segment is flanked by two inverted repeat sequences where site-specific recombination occurs, leading to H fragment inversion. The recombination process is mediated by the DNA invertase Hin, which is located in the H segment (Heichman and Johnson, 1990). When *hin* is in the "on" position, both the *fljB* and *fljA* genes are transcribed, resulting in the expression of phase II (*fljB*) flagellin. In this situation, *fljA* also inhibits *fliC* expression. Contrary to this, when *hin* is in the "off" position, *fljB* and *fljA* are not expressed, allowing for the expression of *fliC* (phase I flagellin) (Kutsukake et al., 2006).

There is evidence indicating that the origin of *S. enterica* 4,[5],12:i:- is polyclonal with the existence of at least two clones, the United States (US) and the Spanish (S) clones, that originated independently since they differ in the molecular events involved in *fljBA* deletion as well as in other genomic regions. This suggests that *S. enterica* 4,[5],12:i:- is represented by multiple clones with distinct geographical distribution (Soyer et al., 2009; Laorden et al., 2010). Soyer et al. (2009) suggested that deletion of the *fljBA* operon in the US clone could have resulted from an imprecise excision of a prophage. Therefore, prophages appear to have a role in the emergence of at least one of the *S. enterica* 4,5,12:i:- clones.

Based on this observation, we hypothesized that the *fljBA* operon is located in a genomic

region prone to recombination, leading to the origin of distinct clones of *S. enterica* 4,5,12:i:- in different geographic regions. On the other hand, if the deletion of *fljBA* is not a frequent event, other factors related to the fitness of *S. enterica* 4,5,12:i:- should be considered to explain the origin and transmission of this new serovar. In order to answer this question, this study aimed to verify the stability of the *fljBA* operon in a *S. enterica* Typhimurium strain following growth *in vitro* and under conditions known to induce the lytic cycle in prophages.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Three strains of *S. enterica* serovar Typhimurium (ST) were used: ATCC 14028 (American Type Culture Collection, USA), LT2 (kindly provided by Prof. Roy Curtiss III, Arizona State University, Arizona, USA) and a wild type strain (662STm) that is part of our lab collection. This last strain was previously characterized regarding pathogenic factors and molecular characteristics (Sales and Brocchi, 2007). *S. enterica* Enteritidis strains (678SE and 679SE) were used to detect lytic activity of phages in cultures of *S. enterica* Typhimurium, as explained below. Cultures were grown at 37°C in Luria-Bertani (LB) broth or on LB-agar plates (Sambrook and Russell, 2001). MacConkey agar (Difco, Sparks, MD, USA) and *Salmonella-Shigella* Agar (Difco, Sparks, MD, USA) were also used to check strains phenotypically. When required, ampicillin or chloramphenicol was added to culture media at concentrations of 100 or 25 µg/mL, respectively. All the strains were stored at -80°C in glycerol solution (Sambrook and Russell, 2001).

Plasmids and genomic DNA purification

Plasmids pKD46 and pKD3 belong to the λ-*r*ed recombination system (Datsenko and Warnner, 2000) and were used to obtain chloramphenicol-resistant (Cm^r) *S. enterica* Typhimurium strains. Plasmid and genomic DNA purifications were performed with the Illustra Plasmid Prep Minispin and Illustra Bacterial Genomic MiniSpin, respectively (GE Healthcare, Piscataway, NJ, USA).

Polymerase chain reaction (PCR), agarose gel electrophoresis, and DNA electroporation

Plasmid and PCR fragments were electroporated into *S. enterica* strains using the Gene Pulser Xcell™ Electroporation (Bio-Rad, Hercules) as described previously (Sambrook and Russell, 2001). PCR amplification was performed with a Veriti® 96-Well Thermal Cycler (Applied Biosystem, USA) with 10-15 ng/µL template DNA. DNA fragments were separated on a 0.7 to 1% agarose gel, stained with GelRed™ Biotium (Invitrogen, Hayward, CA, USA) and visualized in a Gel Logic 212 PRO (Carestream, USA).

Construction of Cm^r *S. enterica* Typhimurium strains

In order to monitor the stability of *fljBA* operon, recombinant strains were constructed by inserting the *cat* (chloramphenicol acetyltransferase) gene in the genome of *S. enterica* Typhimurium strains, targeting the intergenic region between the *fljA* and STM2769 coding sequences (CDS)

(Figure 1A). This construction was performed using the λ-red recombination system (Datsenko and Warner, 2000). The primers used for *cat* amplification (STM-FljA-F and STM_FljA-R; Table 1) were designed based on the *S. enterica* Typhimurium LT2 genome (McClelland et al., 2001) in order to generate homologous sequences to the target DNA. PCR amplification was as follows: denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, annealing at 65°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 4 min. The pKD3 plasmid was used as the template.

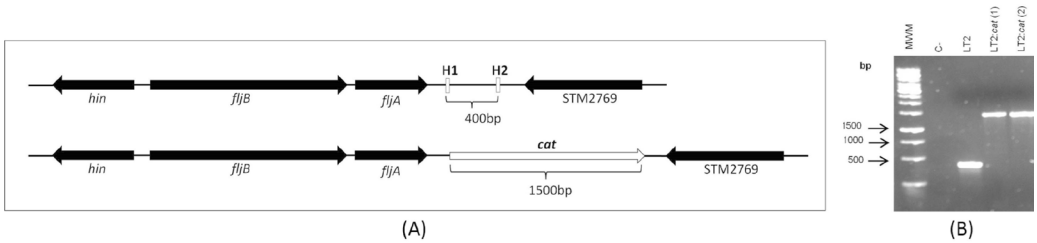

Figure 1. A. Schematic representation of the *cat* gene integration between *fljA* and STM 2769 CDSs. **B.** PCR products amplified using genomic DNA of *Salmonella enterica* Typhimurium LT2 (wild-type) and LT2:*cat* strains (1 and 2) as template with primers STM_FljA_10n-F and -R. *S. enterica* recombinant strains 662STm:*cat* and 14028:*cat* show the same PCR profile (data not shown). As a negative control (C-), the PCR was performed without genomic DNA. Molecular weight marker (MWM) of 1 Kb (Fermentas®) is also shown.

Table 1. Primers used in this study.

Primer	Sequence (5'→3')	Annealing temperature (°C)
STM-FljA-F	GCAAATTGAGAACTTCAGCAAATCGACAAACCAGTTCGAAGTGAGTTGGGGTGTAGGCTGGAGCTGCTTC	65
STM_FljA-R	TACCAGGCCTTTTATGAAGGCTCAAATTTGGCTCAATGGGTACGCCAACGATGGGAATTAGCCATGGTCC	
STM_FljA-10n-F	GGGGGTACTAACACGACCAA	58
STM_FljA-10n-R	CGGAACAGTCCAGGAAAGAG	

The Cm^r cassette was electroporated into pKD46-containing *S. enterica* ATCC 14028 and recombinants were selected in LB + chloramphenicol (25 µg/mL). The *cat* gene was subsequently transferred to ATCC14028, 662STm and LT2 strains by transduction with the bacteriophage P22HT (Maloy, 1990). All insertions were confirmed by PCR with the primers STM_FljA-10n-F and STM_FljA-10n-R (Table 1). PCR conditions are the same as those described above with the exception of the annealing temperature (58°C) with *S. enterica* genomic DNA as a template. Recombinant strains were also checked for P22HT lysogenic activity by phenotypic characteristics in mint green agar plates (Maloy, 1990). The recombinant strains obtained were designated *S. enterica* 662STm:*cat*, 14028:*cat* and LT2:*cat*.

In vitro analyses

The recombinant strains 662STm:*cat*, 14028:*cat* and LT2:*cat* were tested for stability of the *fljBA* operon in two different growth conditions. For the first growth condition, cultures were grown at 37°C for 30 days in LB broth without antibiotics. Aliquots were collected every 5 days and evaluated for the loss of Cm^r. To do this, serial dilutions were performed and spread onto LB-agar plates to obtain isolated colonies that were subsequently transferred to new LB-agar plates

with or without chloramphenicol to pick chloramphenicol-sensitive colonies. This longer period of incubation was used to test the hypothesis that nutritional stress would interfere with any genomic recombination event. The second growth condition was performed only with the LT2:*cat* strain. For this condition, the *S. enterica* Typhimurium LT2:*cat* strain was grown in LB in the same conditions described in the first condition for 10 days. Aliquots of the cells were collected every 2 days. Isolated colonies were transferred to 96 well plates containing 200 µL *LB broth*, incubated at 37°C for 15 min and transferred to LB agar plates with or without chloramphenicol or SS agar plates.

Induction of prophages

Prophages were induced in order to test if imprecise phage excision or recombination could be responsible for the deletion of the *fljBA* operon. Cultures of the LT2:*cat* strain were treated with 2 *µg/mL* mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for prophage induction as described by Frye et al. (2005). After induction, cells were washed three times with phosphate buffered saline (PBS) pH 7.4 (Sambrook and Russell, 2001) and pelleted by centrifugation. After serial dilution and plating, isolated colonies were transferred to 96 well plates containing 200 µL LB broth, incubated at 37°C for 15 min and transferred to LB agar plates with or without chloramphenicol or SS agar plates.

To test for the presence of phage particles in the supernatants of mitomycin C treated cultures, 10 µL the supernatant was transferred to plates inoculated with the 678SE and 679SE strains. These strains were selected because they are lysed by phages induced from LT2 cultures (data not shown).

In vivo analyses

These analyses were conducted in order to verify if the *fljBA* operon remains stable under *in vivo* conditions. Six- to eight-week-old female BALB/c mice were used. The animals obtained from CEMIB (Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório, Brasil) were kept from food and water for 6 and 2 h, respectively, prior to oral and intraperitoneal inoculation. Thirty mice were divided into two groups. Group 1 (15 mice) were inoculated orally with 10^8 UFC (colony forming units) of *S. enterica* Typhimurium LT2:*cat* strain. Group 2 (15 mice) were inoculated intraperitoneally with 10^6 UFC of the same strain.

Mice in group 1 were sacrificed 3, 7 or 14 days after inoculation, and blood, spleen and Peyer's patches were collected. Mice from group 2 were sacrificed at 1, 3 or 7 days after intraperitoneal inoculation and blood and spleen samples were collected. Organs were homogenized in PBS pH 7.4 using a Tissue Master 125 (Omni International, USA) and plated onto LB-agar with or without chloramphenicol. Peyer's patches were plated on MacConkey agar with or without chloramphenicol. Isolated colonies from LB and MacConkey agar without antibiotic were transferred onto new LB agar with or without antibiotic to detect chloramphenicol-sensitive bacteria. These experiments were approved by the Ethics Committee of Animal Experimentation of University of Campinas, Campinas, São Paulo, Brazil.

RESULTS

Construction of Cm^r *S. enterica* Typhimurium strains

Cm^r *S. enterica* Typhimurium strains ATCC 14028, LT2 and 662STm were constructed by

the λ-red recombination system followed by P22HT transduction. As expected, PCR amplification with primers STM_FljA-10n-F and STM_FljA-10n-R revealed a fragment of about 400 bp when genomic DNA of the wild-type strain (LT2) was used as the template. A fragment of about 1500 bp was observed when genomic DNA from recombinant strains was used as the template (Figure 1). The results were identical for the LT2:*cat*, 662STm:*cat* and ATCC 14028:*cat* strains.

Cm^r as an indicator of *fljBA* stability

All bacterial clones isolated from cultures *in vitro* before or after mitomycin C treatment were phenotypically characterized as Cm^r (Table 2). In total, more than 2 x 10^4 colonies were assayed for the loss of antibiotic resistance in the various experiments. Around 10^4 colonies from *in vitro* cultures were evaluated for the Cm^r phenotype and the same number of colonies was evaluated after mitomycin C treatment (Table 2). The supernatant recovered from mitomycin C-treated cultures presented lytic activity to the 678SE and 679SE strains, indicating the presence of phages. As the *cat* gene was inserted adjacent to the *fljBA* operon, deletion events that would affect this operon have a very high probability of also affecting the *cat* gene. Therefore, we can assume that the presence of *cat* indicates *fljBA* integrity. These results suggest that the genomic region of *fljBA* is not highly predisposed to be engaged in recombination or deletion processes.

Table 2. Number of colonies of *Salmonella enterica* Typhimurium strains 662STm:*cat*, 14028:*cat,* and LT2:*cat* assayed *in vitro* to determine the loss of chloramphenicol resistance.

Experiment	Number of colonies assayed	Number of replications	Chloramphenicol-resistant colonies
LT2:*cat*			
9-day protocol	600	1	600
30-day protocol	500	3	1500
662STm:*cat*			
9-day protocol	600	1	600
30-day protocol	500	3	1500
14028:cat			
9-day protocol	600	1	600
30-day protocol	500	3	1500
Total			6300

Bacterial clones isolated from mouse tissues were all phenotyped as Cm^r (Table 3). This experiment was performed in order to verify if growth in mouse tissues could induce a recombination event that would give results for the *fljBA*$^-$/*cat*$^-$ strains. However, the number of clones recovered from the animal tissues was not exhaustive because from the 15 mice tested in intraperitoneal administration, four died before the day of sacrifice. Therefore, the data presented in Table 4 are based on 11 mice.

Table 3. Number of colonies of *Salmonella enterica* Typhimurium strain LT2:*cat* assayed *in vitro* for the loss of chloramphenicol resistance after treatment (MT) or without treatment (NT) with mitomycin C.

Experiment	Number of colonies assayed	Number of replications	Chloramphenicol-resistant colonies
MT*	2500	4	10,000
NT	2500	4	10,000
Total			20,000

*MT = mitomycin C-treated (2 µg/mL); NT = not treated with mitomycin C.

Table 4. Number of colonies of *Salmonella enterica* Typhimurium strain LT2:*cat* isolated from mice and assayed for the loss of chloramphenicol resistance.

Inoculation route	Number of colonies assayed/organ	Chloramphenicol-resistant colonies
Intragastric	400 (Peyer patches)	400
	400 (Spleen)	400
	40 (Blood)	40
Intraperitoneal	500 (Spleen)	500
	250 (Blood)	250
Total (oral and intraperitoneal)	1590 (any organ)	1590

DISCUSSION

S. enterica 4,[5],12:i:- is a serovar that is distributed worldwide. A number of studies indicate that it is a monophasic variant of serovar Typhimurium that lacks *fljB* expression (Garaizar et al., 2002; Moreno-Switt et al., 2009). Soyer et al. (2009) identified the existence of at least two clones of 4,[5],12:i:-, the Spanish (S) and the United States (US), that differ in the type of molecular events associated with the deletion of the *fljBA* operon. In the US clone sequence, 76 CDSs were absent including the *fljBA* operon and Fels-2 gene (Soyer et al., 2009). On the other hand, four different types of genomic deletions were characterized in S strains but it is not clear if they represent variants of a unique bacterial clone or if they arose independently (Laorden et al., 2010).

In addition, other studies have found that 4,[5],12:i:- strains differ regarding the presence or absence of *fljBA* and surrounding genes (Bugarel et al., 2012). The isolation of this serovar worldwide and the existence of at least two different clones that most likely originated independently prompted us to investigate if the genomic region of the *fljBA* operon is prone to recombination or deletion events. This is reinforced by the existence of transposons and the Fels-2 prophage gene in the vicinity of this region and by the fact that some phage-related genes are inserted in the region deleted from the US strains (Soyer et al. 2009). Therefore, our hypothesis was that the *fljBA* operon is in the vicinity of a hot spot of recombination or deletion.

To examine this, a resistance cassette was inserted between *fljA* and STM 2769 and flanking the *fljBA* operon with the goal being to monitor any deletion and/or recombination involving this region. Therefore, the presence of a resistance gene close to this operon made it possible to verify the instability of this region. In other words, if natural deletion of the *fljBA* operon occurred, the resistance cassette would be deleted along with the operon and the bacterial recombinant colonies would become chloramphenicol-sensitive. However, no chloramphenicol-sensitive colonies were detected in all experiments performed, even in prolonged cultures, under bacteriophage induction or after infection of mouse tissues. As shown previously, the Fels-2 prophage gene was absent in monophasic S and US 4,[5],12:i:- strains (Garaizar et al., 2002; Soyer et al., 2009), which has led some groups to hypothesize that imprecise excision of this prophage could delete the *fljBA* operon along with adjacent sequences. Even after the growth of bacteria in a condition described to induce the lytic cycle in prophages, none of the colonies assayed were chloramphenicol-sensitive. The number of bacterial colonies screened for the Cm^r phenotype in this work was not exhaustive. Therefore, we cannot exclude the possibility that if more colonies were screened, some chloramphenicol-sensitive colonies would be identified.

Our data suggest that deletion of *fljBA* occurs at frequencies lower than 10^{-4} cells. It has been suggested that *fljBA* deletion in US clones was likely caused by an abortive or imprecise

phage excision (Soyer et al., 2009). On the other hand, deletion of *fljBA* in S clones was probably mediated by the insertion of a IS26 transposon element. It is tempting to speculate that the spread of *S. enterica* 4,5,12:i:- to different countries and continents is probably due to the characteristics of these clones that confer adaptability to diverse environments and/or host tissues rather than a continued origin of strains carrying *fljB* deletion. However, more studies are necessary in order to better characterize this serovar.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Grants #2009/15956-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Grants #308955/2012-9), Brazil. D.B. Nóbrega, G.P. Milanez, J.P. Pereira and A.P. Calarga were supported by a FAPESP fellowship (FAPESP #2012/10608-3, #2012/05382-6, #2014/11280-7, and #2013/11880-1, respectively). C.H. Werle was supported by fellowships from CNPq (#141629/2012-6). M.P.O. Tomiyama was supported by fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (#01P-04520-2013).

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