Extensive Genetic Variability Linked to IS26 Insertions in the *fljB* Promoter Region of Atypical Monophasic Variants of *Salmonella enterica* Serovar Typhimurium

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Fifty-nine monophasic *Salmonella enterica* serovar Typhimurium isolates, collected in Belgium during the period from 2008 to 2011, have been serotyped as 4,[5]:i:− and shown to harbor an *fljB* coding sequence. The genetic differences between these strains and phenotypically biphasic *Salmonella* Typhimurium were analyzed through PCR and DNA sequencing. Genetic alterations in the *fljB* promoter region affecting expression of the phase 2 flagellin were observed in 53 isolates. Other genetic events in the invertible region carrying the *fljB* promoter were observed in 2 isolates. For the remaining 4 isolates, no molecular differences with a reference biphasic *Salmonella* Typhimurium strain could be observed. Next-generation sequencing of one representative isolate affected in the *fljB* promoter region revealed a 26-kb IS26 composite transposon insertion along with a local genomic rearrangement. Several other IS26 element-mediated alterations of this genomic region were observed. This group of monophasic *Salmonella* Typhimurium isolates was genetically heterogeneous, as revealed by multilocus variable-number tandem-repeat analysis (MLVA), PCR, and sequencing. Pigs and pork represented a major source of such monophasic isolates in Belgium, as reported in other countries. Three out of 5 isolates of human origin presented genetic profiles identical to those of food isolates, demonstrating the pathogenic potential of the newly characterized variants and potential dissemination along the food chain. This study highlighted the key role played by IS26 insertions in the loss of phase 2 flagellin expression and the subsequent generation of multiple monophasic variant lineages from biphasic *Salmonella* Typhimurium ancestors.

The *Salmonella* genus is currently divided into 2 species, *Salmonella bongori* and *Salmonella enterica*, the latter being classified into 6 subspecies, *S. enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (1). Most salmonellae possess two flagellin genes, *fliC* and *fljB*, encoding the so-called phase 1 and phase 2 flagellins, respectively. Only one flagellin type is expressed at a time in a *Salmonella* cell. Isolates are termed biphasic, monophasic, or nonmotile if they can express both, one, or no flagellar phases, respectively. Expression of the two genes is under the control of a switch mechanism called "phase inversion." The switch mechanism consists of the DNA inversion of a sequence segment, the so-called invertible region, by recombination of the two flanking sites, *hixL* and *hixR*, mediated by an invertase encoded by the *hin* gene included in this segment (2). The promoter of the *fljBA* operon is born on this invertible region. The *fljBA* operon encodes the FljB flagellin and FljA, a repressor of the *fliC* gene. The orientation of the invertible region determines whether the *fljBA* operon or the *fliC* gene will be expressed (2).

Characterization of *Salmonella* spp. below the subspecies level is traditionally obtained by serotyping based on the Kauffmann-White-Le Minor scheme (1). Serovars are determined following agglutination of entire bacteria with specific sera to identify variants of the somatic (O) and flagellar (H) antigens. Serotyping results in an antigenic formula, indicating the O, H1, and H2 antigens separated by colons. The different O and H antigens are named by numbers, alphabetical characters, or both. Some O antigens can be present or absent without interfering with serovar

identification. These antigens are interesting only as epidemiological markers within a given serovar. These accessory O antigens are underlined when their presence is linked to the integration of a prophage. O or H antigens are written within square brackets if they may be present or absent without relation to phage conversion. The 1,4,[5],12:i:1,2 antigenic formula, corresponding to *S. enterica* serovar Typhimurium, therefore indicates that (i) the O4 and O12 antigens are always present in this serovar, (ii) the O1 antigen can be present or absent due to phage conversion, (iii) the O5 antigen can be present or absent without relation to phage conversion, and (iv) the H1 antigen "i" and the H2 antigens "1" and "2" are always present. If no H1 or H2 antigen can be detected,

a minus sign (−) is written at the corresponding place in the antigenic formula and the strain is termed monophasic.

Monophasic *Salmonella* Typhimurium strains serotyped as 1,4,[5],12:i:− have emerged in the mid-1990s and account currently for one of the most common serotypes observed in many countries worldwide (3). Since the public health risk posed by *Salmonella* 1,4,[5],12:i:− strains is considered comparable to that of *Salmonella* Typhimurium strains (4), epidemiological surveillance and molecular characterization of this serovar should be part of the *Salmonella* control programs. It has been suggested that the success of *Salmonella* 1,4,[5],12:i:− variants might be related to the selective advantage offered by multidrug-resistant profiles associated with stable genetic elements, which also carry virulence features. Such variants might be well adapted to the animal production context and capable of causing human infections (5). However, other studies have shown that not all *Salmonella* 1,4,[5],12:i:− variants are systematically associated with multidrug-resistant profiles (3, 4). The explanation may therefore be more complex and certainly deserves further investigation.

Isolates serotyped as *S. enterica* subsp. *enterica* 1,4,[5],12:i:− express only the phase 1 flagellin. They can be monophasic variants of several *S. enterica* serovars: *S.* Typhimurium, *S.* Lagos, *S.* Agama, *S.* Farsta, *S.* Tsevie, *S.* Gloucester, and *S.* Tumodi. A PCR assay was developed by Tennant et al. (6) to determine whether an isolate serotyped as 1,4,[5],12:i:− was related to *Salmonella* Typhimurium or to the rarely observed *S.* Farsta. This PCR assay targets (i) the *fliB-fliA* intergenic region hosting an IS*200* element found only in *S.* Typhimurium or *S.* Farsta or their variants (among serovars expressing the O:4 and H:i antigens) and (ii) *fljB*, the gene coding for the phase 2 flagellin. *Salmonella* 1,4,[5],12:i:− isolates related to *Salmonella* Typhimurium and lacking the *fljB* coding sequence, as demonstrated by this PCR assay, were recognized as *Salmonella* Typhimurium monophasic variants by the European Food Safety Authority (EFSA) (4). Beside these isolates, here referred to as *fljB*-negative *Salmonella* Typhimurium monophasic variants (STMV Δ*fljB* strains), *Salmonella* 1,4,[5],12:i:− strains characterized by a typical *Salmonella* Typhimurium IS*200* signature and the presence of the *fljB* coding sequence (STMV *fljB*+ strains) were also reported (7–9). These STMV *fljB*+ strains are neither phenotypically biphasic nor typical *Salmonella* Typhimurium monophasic variants like the one recognized by EFSA (4) and the Spanish and U.S. clones described by Soyer et al. (10). Theoretically, the presence of an intact *fljB* coding sequence is not the unique genetic requirement for phase 2 flagellin expression. A fully operational invertible region, also called the H segment, carrying the *hin* gene (encoding the main invertase involved in phase inversion), a functional *fljB* promoter, and the two recombination sites *hixL* and *hixR*, required for the inversion mechanism, is also needed (11). In-depth molecular characterization of the entire H segment of such STMV *fljB*+ isolates has, to our knowledge, not been reported.

In a previous study, 59 *Salmonella* isolates serotyped as 1,4,[5],12:i:− and collected during the period from 2008 to 2011 at the Belgian *Salmonella* National Reference Laboratories for Public Health, Animal Health, and Food were reported (9). These isolates were routinely identified by classical serotyping, following at least one phase inversion assay, and were characterized by PCR analysis as STMV *fljB*+ strains. Such STMV *fljB*+ variants were also reported elsewhere (7–9). The objectives of the present study were (i) to increase awareness that not just *fljB*-negative strains are

monophasic, (ii) to provide nucleotide sequence material for the development of improved molecular assays targeting most signatures of such strains, and (iii) to stress the need to provide a regulatory context for *fljB*-positive monophasic *S.* Typhimurium strains, given the large proportion (23.3% of Belgian monophasic strains) and the public health risk (comparable to biphasic *S.* Typhimurium) they represent.

MATERIALS AND METHODS

Bacterial isolates. A collection of 59 *Salmonella* 4,[5]:i:− isolates, 37 from pig, 2 from poultry, 14 from pork (i.e., pig meat), 1 from unspecified meat, and 5 from human origins, was investigated. They were previously assayed with the PCR test developed by Tenant et al. (6) and identified as *fljB*-positive *Salmonella* Typhimurium (9). This STMV *fljB*+ strain collection includes all strains isolated during the period from 2008 to 2011 at the Belgian *Salmonella* National Reference Laboratories for Public Health, Animal Health, and Food. They were phenotypically confirmed after at least one phase inversion assay. Strains used for nucleotide sequencing are stored in the Belgian Co-ordinated Collections of Microorganisms (BCCM).

DNA extraction. The genomic DNA used as a template for PCR or sequencing assays was extracted from bacteria that were grown on brilliant green agar plates (Oxoid, Aalst, Belgium) by using the DNeasy blood and tissue kit according to the manufacturer's instructions for Gram-negative bacteria (Qiagen, Valencia, CA).

PCR. (i) Invertible H segment. PCR targeting the entire or a portion of the invertible H segment was performed with the FastStart PCR master mix (Roche Diagnostics, Mannheim, Germany) by using different primer pairs. PCRs were conducted in a 25-μl final volume consisting of FastStart PCR master mix, 0.5 μM each primer, 10 ng of template DNA, and Milli-Q water. The amplification procedure consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C, followed by a final extension of 10 min at 72°C.

If the entire invertible H segment of a given *fljB*-positive STMV isolate was not PCR amplifiable (Table 1, first primer mix) or yielded a PCR amplicon whose size differed from that of the reference *Salmonella* Typhimurium LT2 strain, two PCRs (Table 1, second and third primer mixes) targeting different portions of the H segment (in phase 2 OFF orientation) were assayed. All PCR products were analyzed by gel electrophoresis in 1% agarose gel (Tris-acetate-EDTA [TAE] buffer) using SYBRSafe (Life Technologies Europe, Ghent, Belgium) as the intercalating dye and a 200-bp to 10-kb DNA ladder (Eurogentec, Seraing, Belgium).

(ii) Junction PCRs. As shown in Fig. 1, the insertion of a transposon was observed in the invertible region of a representative STMV *fljB*+ isolate. Two 3-primer PCR assays were designed to assess the occurrence of this transposon in the other STMV *fljB*+ strains (Table 1, fourth and fifth primer mixes). These two PCR assays targeted either the left (LJ) or right (RJ) junction of the inserted transposon or the intact *fljB* promoter region found in the *Salmonella* Typhimurium LT2 reference (biphasic) strain. The left junction PCR amplifies either the transposon left junction (primers BG1215 and BG1216) in the target *fljB*+ STMV isolates or the conserved *hin-iroB* intergenic region in phase 2 OFF orientation (control primers BG1215 and BG1218). The right junction PCR amplifies either the transposon right junction (primers BG1218 and BG1232) or the conserved *hin-iroB* intergenic region (control primers BG1215 and BG1218). Both PCRs were conducted in a 25-μl final volume consisting of FastStart PCR master mix, 0.5 μM each primer, 10 ng of template DNA, and Milli-Q water. The amplification procedure consisted of an initial denaturation at 95°C for 10 min followed by 15 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, followed by another 15 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C (for the last step, with 5-s increment at each cycle), followed by a final extension of 10 min at 72°C. PCR products were analyzed by gel electrophoresis in 1% agarose gel (TAE buffer) by using SYBRSafe (Life Technologies Europe, Ghent, Belgium) as the intercalating dye and a 200-bp to 10-kb DNA ladder (Eurogentec, Seraing, Bel-

TABLE 1 Summary of PCRs and primers used to map the 26-kb transposon insertion and other genetic alterations of the invertible H segment

PCR target	Primer mix (name, nucleotide sequence from 5′ to 3′)	Amplicon size in *Salmonella* Typhimurium LT2 (bp)	Amplicon size in *Salmonella* 4,5:i:− VAR-2009/08643/1 (bp)
Entire invertible H segment	BG1132, CAGTGCGGACTGGGATTTGTTCA BG1133, AATGTTGCGAACGAACAGCTCCT	1,200	No amplicon
hixR-hin[a]	BG1132, CAGTGCGGACTGGGATTTGTTCA BG1137, AGCCGACTAATCTGTTCCTGT	600	600
hin-fljB promoter-*hixL*[a]	BG1136, CAGAGCGCAAGGACGA BG1133, AATGTTGCGAACGAACAGCTCCT	600	No amplicon
Left junction of inserted transposon or intact *hin-iroB* region[a,b]	BG1215, ATACAGGCGTGTGGCATAAATAAACCGA BG1216, GCAATCTGTGCGGCCAGT BG1218, CAAGGTAACGGGAAACGTAGGGAGT	600 (with BG1215 and BG1218)	1,000 (with BG1215 and BG1216)
Right junction of inserted transposon or intact *hin-iroB* region[a,b]	BG1218, CAAGGTAACGGGAAACGTAGGGAGT BG1232, TGGCGGGAAATGCGTGTTTACA BG1215, ATACAGGCGTGTGGCATAAATAAACCGA	600 (with BG1215 and BG1218)	1,200 (with BG1232 and BG1218)
Left IS*26* element of inserted transposon[a]	BG1215, ATACAGGCGTGTGGCATAAATAAACCGA BG1225, TGAATCGCGGGATCTGCCACTTCT	No amplicon	600
Right IS*26* element of inserted transposon	BG1218, CAAGGTAACGGGAAACGTAGGGAGT BG1225, TGAATCGCGGGATCTGCCACTTCT	No amplicon	500

[a] A PCR product will be observed with this primer mix only if the H segment is in phase 2 OFF orientation.

[b] Three-primer PCR yielding two possible PCR products.

gium). Where no amplification was observed with either the left or the right junction PCR nor with the control primers BG1215 and BG1218, PCRs targeting just the IS*26* element either at the left (BG1215 and BG1225) or right (BG1218 and BG1225) junction were performed in the same conditions as for the transposon junction PCRs. Amplification of the conserved *hin-iroB* intergenic region was designed to occur only if the tested strain was in phase 2 OFF orientation (Fig. 1, Table 1).

PCR amplicon sequencing. Sanger sequencing was completed on PCR fragments amplified from strains displaying atypical genetic profiles (listed in Table S1 in the supplemental material) by using the primers listed in Table 1. Sequencing reaction mixtures were obtained using the BigDye Terminator v3.1 cycle sequencing kit and the BigDye XTerminator purification kit. Analyses were conducted on an ABI 3130 genetic analyzer according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA).
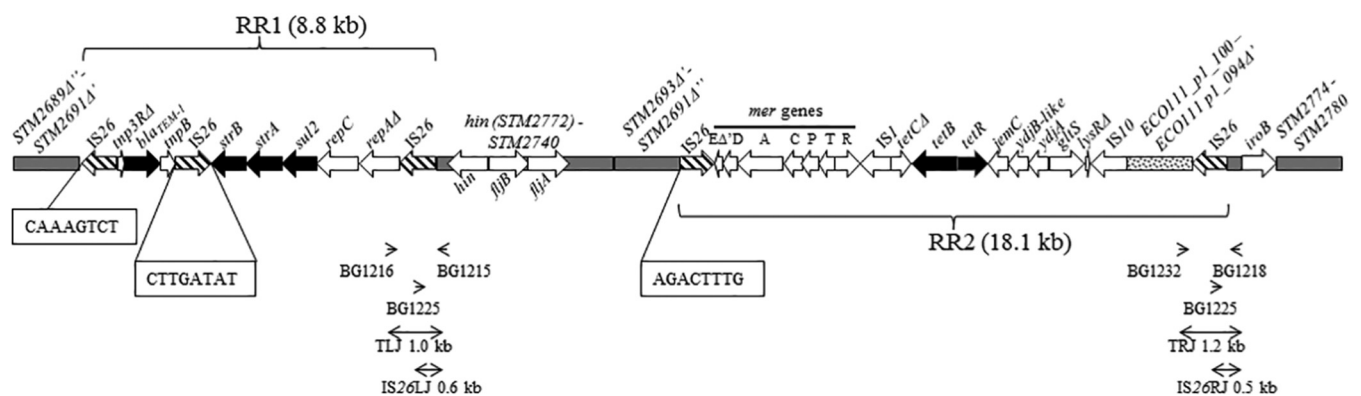

FIG 1 Map of the *fljBA* operon region of the monophasic variant strain VAR-2009/08643/1 (83,173 bp). IS*26* copies are drawn with hatched arrows. Black arrows delineate antibiotic resistance genes. Gray boxes indicate regions homologous to *Salmonella* Typhimurium LT2 (GenBank accession number AE006468). The dotted box is homologous to a region found on the *E. coli* plasmid pO111_1 (GenBank accession number AP010961). Antibiotic resistance regions 1 (RR1) and 2 (RR2) are similar to a *Salmonella* 4,[5],12:i:− chromosomal sequence (GenBank accession number HQ331538) except for (i) a deletion covering partial *merE*, *urf2*, and *tniA*Δ*1*, (ii) an inverted IS*26* element, (iii) a complete IS*10* copy, and (iv) an additional region (dotted box) in RR2. Δ′ and Δ″ refer to deletions noticed in the 3′ or 5′ part of some genes, respectively. Boxed nucleotides refer to IS*26*-associated repeats. The insertion of a 26-kb transposon consisting of the RR1 and RR2 elements occurred at an intermediate stage and was followed by a genomic rearrangement between the first and fourth IS*26* copies, leading to an inverted DNA sequence in between. This is supported by the finding of inverted repeats bordering the first and fourth IS elements (boxed nucleotides) and the orientation of the genes within the inverted segment (gray boxes). The arrowheads at the bottom point to primers used to demonstrate the presence of IS*26* elements (possibly as part of the 26-kb transposon). Double-headed arrows show the corresponding amplicons with their sizes (TLJ, transposon left junction; IS*26*LJ, IS*26* left junction; TRJ, transposon right junction; IS*26*RJ, IS*26* right junction).

Next-generation sequencing. Genomic DNA of the *Salmonella* 4,5:i:− VAR-2009/08643/1 isolate was extracted as explained above. A sequencing library was prepared from the extracted DNA according to the manufacturer's instructions for the Titanium Series rapid library preparation method (Roche, Mannheim, Germany). Sequencing was performed with a GS Junior instrument (Roche, Mannheim, Germany) with Titanium Series reagents and run protocol (200 cycles), generating 97,082 reads with an average read length of 480 bases. Three hundred ninety-seven contigs were generated by *de novo* assembly by using GS *De Novo* Assembler software v2.7 (Roche, Mannheim, Germany) with the default parameters. This analysis also produced a contig graph indicating potential contig links. Since repeated elements were found in the genomic region of interest (surrounding the *fljB* promoter region), contigs were joined by PCRs to assemble an 83,173-bp sequence (GenBank accession number KJ999732) (primers are specified in Table S2 in the supplemental material). This sequence was annotated according to the published sequences of *Salmonella* Typhimurium LT2 (GenBank accession number AE006468) (12), the pO111_1 plasmid of *Escherichia coli* O111:H− strain 11128 (GenBank accession number AP010961.1) (13), and *Salmonella* 1,4,[5],12:i:− strain 105/7/03 (GenBank accession number HQ331538) (14). Due to the inherent limits of the technology used for whole-genome sequencing, four loci with homopolymers apparently different from the *Salmonella* Typhimurium LT2 and pO111_1 plasmid sequences were corrected by Sanger sequencing (nucleotides 6996 to 6999, 18916 to 18921, 19376 to 19381, and 24165 to 24171 of GenBank accession number KJ999732).

MLVA. Multilocus variable-number tandem-repeat analyses (MLVA) were conducted as described by Lindstedt et al. (15), following the nomenclature proposed by Larsson et al. (16). Allele assignment was performed using a correction table created with 33 calibration strains provided by the European Centre of Disease Prevention and Control (17). MLVA profiles are reported as a string of 5 numbers (STTR9-STTR5-STTR6-STTR10-STTR3), representing the number of repeats at the corresponding locus, or as "NA" when no amplification product was obtained.

Nucleotide sequence accession numbers. All sequences used in this study were sent to GenBank, and accession numbers KJ999729 to KJ999733 were assigned.

RESULTS

Fifty-nine *Salmonella* isolates serotyped as 4,[5]:i:− previously reported as STMV *fljB⁺* strains (9) and isolated in Belgium during the period from 2008 to 2011 were investigated to decipher the molecular differences between phenotypically biphasic *Salmonella* Typhimurium and these monophasic variants. They were isolated at different stages of the food chain: pigs (37 isolates), poultry (2 isolates), pork (14 isolates), unspecified meat (1 isolate), and humans (5 isolates). Thirty-five isolates were serotyped as *Salmonella* 4:i:− isolates and 24 as *Salmonella* 4,5:i:− isolates (only the O:4 and O:5 antigens were tested).

At the first stage, the invertible H segment carrying the *fljB* promoter was targeted by PCR with different primer pairs, allowing the amplification of various parts of this region in the 39 STMV *fljB⁺* animal isolates to map possible insertions or deletions compared to the *Salmonella* Typhimurium LT2 reference genome. Part of the H segment, carrying the *fljB* promoter and *hixL*, could not be amplified in 33/39 STMV *fljB⁺* animal isolates (84.6%). In two isolates (5.1%), a genetic event had occurred at the other side of the invertible H segment carrying *hixR* and the 5′ part of *hin*, while in two other isolates (5.1%), PCRs indicated genetic alteration encompassing the entire invertible H segment. No sequence length alteration could be identified in the PCR pro-

files generated from the last two animal isolates (5.1%) compared to that of *Salmonella* Typhimurium LT2.

One representative isolate (VAR-2009/08643/1) of the largest variant population displaying a profound alteration in the DNA sequence around the *fljB* promoter was fully sequenced (GenBank accession number KJ999732). Comparison with the *Salmonella* Typhimurium LT2 genome (GenBank accession number AE006468) identified a 26-kb IS*26* composite transposon insertion in this region, along with a 451-bp deletion encompassing the *fljB* promoter and *hixL*. This transposon consists of two resistance regions (Fig. 1) carrying several antibiotic resistance genes involved in resistance to ampicillin (*bla*$_{TEM-1}$), streptomycin (*strA*, *strB*), sulfonamides (*sul2*) (region RR1), and tetracycline (*tetB* and *tetR*) (region RR2). Phenotypical resistance to these four antibiotics (known as the ASSuT profile) was confirmed using the Kirby-Bauer disc diffusion method by following recommendations of the Clinical and Laboratory Standards Institute. In addition to this IS*26* composite transposon insertion, the *fljB* promoter-surrounding region of strain VAR-2009/08643/1 still differs from the reference genome of *Salmonella* Typhimurium LT2 by a 40-kb sequence inversion involving the whole *fljBA* operon, with only part of the invertible H segment. More precisely, after insertion of the transposon, which is supposed to have occurred without further rearrangement in an ancestor lineage not formally identified in our study, inversion of a segment bordered by the first and fourth IS*26* copies subsequently occurred, as suggested by the presence of inverted repeats bordering the IS*26* elements found at the edges of the inverted sequence and by the orientation of the genes born on the inverted sequence element (Fig. 1). This genomic rearrangement resulted in the dissociation of the two resistance regions. Eventually, a 451-bp deletion encompassing the *fljB* promoter and *hixL* occurred which irreversibly canceled FljB flagellin expression.

Two PCR assays were designed to assess the occurrence of this "new" genetic profile in STMV *fljB⁺* strains isolated from animals, food, and humans. These PCRs targeted the left or right transposon junctions, i.e., segments encompassing distal parts of the composite transposon, and the *fljB* promoter region from the original (intact) genome. A control amplification primer was added to each PCR assay to amplify the intact *fljB* promoter region in strains where no mobile element had inserted at that position. Amplification of both left and right transposon junctions (PCR profile "TLJ, TRJ" in Table S1 in the supplemental material) was observed for 31 isolates, 1 of human, 21 of pig, 1 of poultry, 7 of pork, and 1 of unspecified meat origin (Table 2; see also Table S1). In 12 isolates (9 from pigs and 3 from pork), an IS*26* element, alone or as part of the composite transposon, was detected at the left junction along with different profiles at the right junction (Table 2; see also Table S1). One IS*26* copy, combined with either a 482-bp or 536-bp deletion encompassing the *fljB* promoter and *hixL*, was observed in 1 isolate of pig origin and 2 isolates of human and pork origins (GenBank accession numbers KJ999731, KJ999730, and KJ999729) (Fig. 2 and Table 2; see also Table S1). Thus, in 78.0% (46/59) of the isolates, an IS*26* element either found alone or as part of a composite transposon was inserted between the *hin* gene and the *fljB* promoter in phase 2 OFF orientation, affecting expression of the phase 2 flagellin gene. In 7 other isolates, a genetic alteration in the *fljB* promoter region was observed by PCRs targeting the invertible H

TABLE 2 Summary of genetic alterations supporting the monophasic phenotype found in the invertible H segment of the 59 *fljB*-positive *Salmonella* 1,4,[5],12:i:− isolates collected during the period from 2008 to 2011 in Belgium

Genetic alteration	Transposable element found in the invertible H segment	PCR profile[a] associated with the genotype (amplicon size[s] in kb)	No. of isolates
451-bp deletion encompassing the *fljB* promoter and *hixL*	26-kb IS*26* composite transposon[b] inserted between *hin* and *iroB*	Amplification of both left and right transposon junctions (1.0 and 1.2)	31
Disruption of the invertible H segment between *hin* and the *fljB* promoter	IS*26* element associated or not with a transposon inserted between *hin* and the *fljB* promoter	Amplification of the left IS*26* element of the inserted transposon (0.6)	12
536-bp deletion encompassing the *fljB* promoter and *hixL*	One single IS*26* copy inserted between *hin* and *iroB*	Atypical fragment observed with the two 3-primer junction PCR assays (0.9)	2
482-bp deletion encompassing the *fljB* promoter and *hixL*	One single IS*26* copy inserted between *hin* and *iroB*	Atypical fragment observed with the two 3-primer junction PCR assays (1.0)	1
Other genetic alteration in the *fljB* promoter region	Not found	No part of the invertible H segment amplified	6
		Only the *hixR-hin* portion of the invertible H segment amplified (0.6)	1
Genetic alteration in the H segment part containing *hixR* and a portion of *hin*	Not found	Only the *hin-hixL* portion of the invertible H segment amplified (0.6)	2
Not found	Not found	Entire invertible H segment amplified (1.2)	4

[a] Profiles obtained when performing the invertible H segment and junction PCRs described in Materials and Methods.
[b] This is the 26-kb composite transposon identified in this study and to which GenBank entry KJ999732 refers.

segment, but no IS*26* or transposon was identified at this place with the junction PCRs (Table 2). In total, genetic alterations in the *fljB* promoter region affecting the expression of the phase 2 flagellin were observed in 53 isolates. For 2 isolates, a genetic alteration was observed in the other part of the H segment (Table 2). For the remaining 4 isolates, no molecular differences with *Salmonella* Typhimurium LT2 could be observed in the invertible H segment. Overall, 17 different genetic profiles were identified by different PCR and sequencing reactions among the 59 STMV *fljB*+ isolates (see Table S1).

MLVA was then performed to complete the molecular characterization of these isolates. Twenty-one different MLVA subtypes were observed among the 59 isolates. The STTR10 locus could not be detected in any of the strains under study, and the STTR9 locus showed no variability (see Table S1 in the supplemental material). A Simpson diversity index of 0.900 was associated with STMV *fljB*+ isolates and was slightly lower than the one associated with STMV Δ*fljB* isolates (0.942) recovered during the same period in Belgium (9). Thirteen out of all STMV *fljB*+ isolates (22.0%), 12 of pig and 1 of pork origin, displayed the same MLVA profile (3-13-
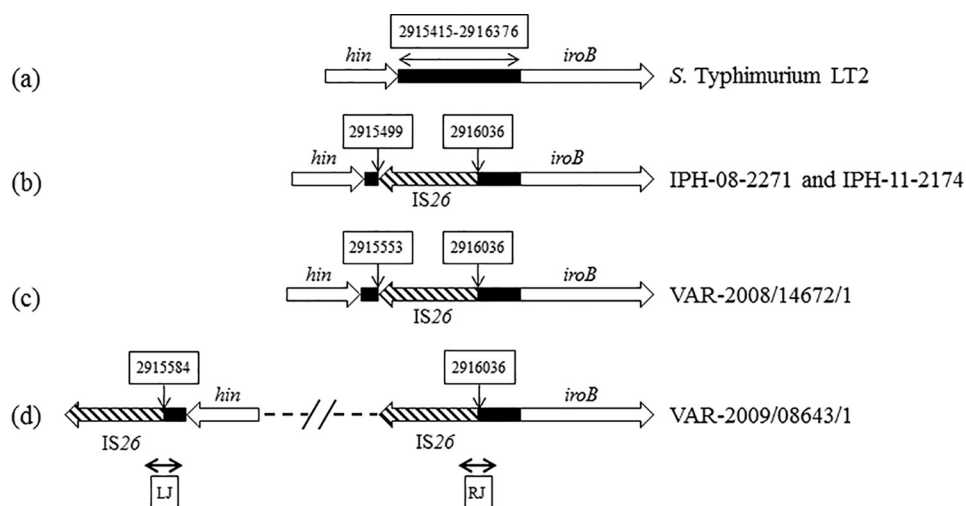

FIG 2 Map of the *hin-iroB* intergenic sequence of various monophasic variants. The intergenic sequence lying between the *hin* and *iroB* genes and observed in the *Salmonella* Typhimurium LT2 reference strain as well as in some representative isolates of the monophasic variants described in this work is shown as a black box. IS*26* elements are indicated by hatched arrows. Nucleotide positions in the intergenic sequence bordering IS*26* elements are given in white boxes above vertical arrows and follow the nucleotide sequence numbering of *Salmonella* Typhimurium LT2 (AE006468). Compared to *S.* Typhimurium LT2 (panel a), the *hin-iroB* intergenic sequence was deleted by 536 (panel b), 482 (panel c), or 451 (panel d) bp. LJ and RJ indicate the left and right junctions, respectively, amplified by the junction PCRs described in Materials and Methods. The dotted line in panel d delineates the large (56.6-kb) intervening region detailed in Fig. 1.

10-NA-0211), junction PCR profile ("TLJ, TRJ"), and antigenic formula (4:i:−) (see Table S1). Other small clusters of strains, including up to 4 isolates and displaying the same antigenic and molecular typing characteristics, were also observed.

DISCUSSION

This study revealed several genotypes supporting the monophasic phenotype of STMV *fljB*+ isolates. In 78.0% (46/59) of the isolates, the insertion of an IS*26* element, either alone or as part of a composite transposon, between the *hin* gene and the *fljB* promoter affected expression of the phase 2 flagellin gene. Nonexpression of *fljB* in 9 isolates could be explained by other genetic alterations in the *fljB* promoter or in the other part of the invertible region. Future whole-genome sequencing studies could help to decipher the nature of the genetic events associated with the lack of phase 2 flagellin expression in the 4 remaining isolates.

The insertion of one IS*26* element at nucleotide position 2916036 (*Salmonella* Typhimurium LT2 reference genome) in the *hin-iroB* intergenic region illustrated in Fig. 2b, c, and d was observed in 47 isolates (PCR profiles detailed in Table S1 in the supplemental material) and was reminiscent of previously characterized *Salmonella* Typhimurium monophasic strains (14) (C. Lucarelli, personal communication) (GenBank accession number GU226187). However, the genomic sequence at the other side of the inserted IS*26* element in these 47 isolates was characterized by various deletions, yielding genetically distinct groups within the above-described general category. This suggests the existence of a common ancestor harboring a single IS*26* copy at position 2916036 with no further genomic deletion or rearrangement event. This ancestor would be a phenotypically biphasic strain which would have generated different STMV lineages after the insertion of another IS*26* copy (possibly as part of a composite transposon) at several positions, followed by genetic recombination between both IS*26* copies and deletion of the sequence in between. These deletions suppressed the invertible character of the *fljB* promoter region and rendered the strains phenotypically monophasic. The diverse STMV *fljB*+ population investigated in this study and characterized by numerous PCRs and MLVA profiles would therefore not represent a single lineage but rather a diversity of lineages originating from a few common ancestors.

Our study thus highlighted the key role played by IS*26* insertions in the loss of genetic functionality required for phase 2 flagellin expression. In addition, IS*26* composite transposon insertion in some monophasic variants caused acquisition of antibiotic resistance genes in the bacterial chromosome, illustrating again the importance of IS*26* in the dissemination of antibiotic resistance genes, as reported by Tremblay (18). The two resistance regions, RR1 and RR2 (Fig. 1), born on an IS*26* composite transposon, are 100% identical at the nucleotide level to two regions of the *Escherichia coli* pO111_1 plasmid (GenBank accession number AP010961.1). This perfect conservation suggests that the IS*26* composite transposon is easily transferable and may not be restricted to the *Salmonella* genus, as proposed earlier (19). This is supported by the occurrence of the RR1 resistance element on the *E. coli* pO111_1 plasmid and on Tn*6029*, a transposon inserted in an IncHI2 plasmid isolated from *Salmonella* Typhimurium. (GenBank accession number GQ150541) (20, 21). The IS*26* composite transposon could have been inserted through homologous recombination with IS*26* elements already present in the *Salmonella* strain, as suggested by the absence of paired direct repeats

flanking the third and fifth IS*26* elements (22) (Fig. 1). Chromosomal integration of closely related RR1 and RR2 resistance elements was also reported at other positions in *Salmonella* 1,4,[5],12:i:− strain 105/7/03 (GenBank accession number HQ331538) (14) (Fig. 1). The ASSuT resistance profile associated with these resistance genes cannot therefore be used as a phenotypical discrimination marker, since it is frequently observed in both monophasic and biphasic *Salmonella* Typhimurium isolates (4, 23).

This study also broadens our knowledge on the genetic variability associated with the monophasic character of *Salmonella* 1,4,[5],12:i:− strains and the genomic plasticity of the region carrying the genetic elements needed for expression of the phase 2 flagellin. Interestingly, the *hin-fljB* region would have been acquired itself by horizontal gene transfer, as indicated by its low G+C content compared to that of the *Salmonella* core genome (24). These findings highlight the diversity of STMV isolates and the challenging identification of these isolates in routine diagnosis. Although important for epidemiological traceability, the distinction between phenotypically biphasic and monophasic *Salmonella* Typhimurium may not be that critical from a public health perspective. Indeed, similar pathogenicity gene repertoires have been reported in monophasic and biphasic *Salmonella* Typhimurium isolates (25). Our results show that PCR assays targeting the *fljB* coding sequence are not sufficient to rule in the monophasic character of *Salmonella* isolates displaying the 1,4,[5],12:i:− antigenic formula. Molecular identification of the genetic alterations in STMV *fljB*+ isolates supporting/explaining the monophasic phenotype is needed to confirm the classification of these isolates as *S.* Typhimurium variants and thereby fit to sanitary regulations targeting *S.* Typhimurium. PCR assays developed in this study targeting portions of the invertible H segment are now available for the identification of such variants. A recommended laboratory scenario may be to first confirm the phylogenetic relatedness of monophasic 1,4,[5],12:i:− isolates to *Salmonella* Typhimurium (6) and to pinpoint the *fljB*-positive or -negative genotype of the STMV isolates (4). Eventually, the monophasic character of Δ*fljB* STMV strains can be validated with the PCR assays reported in the present article, which target the genetic signatures associated with the impaired functionality of the *fljB* promoter. Given the large number of genetic modifications associated with the monophasic character of *Salmonella* 1,4,[5],12:i:− strains, multiplexed molecular tests may rapidly become a strong requirement for routine or reference laboratories. Future studies with similar PCRs and sequencing approaches could help to describe currently uncharacterized variants.

Pigs and pork represent major sources of STMV *fljB*+ isolates collected in Belgium, as also reported in other countries (4, 25). The proportion of isolates originating from pigs and pork among the nonhuman isolates was even higher in STMV *fljB*+ isolates than in STMV Δ*fljB* isolates (94.4% [this study] compared to 66.0% [9], respectively). Among the 5 characterized human isolates, 3 displayed PCR and MLVA types identical to those of food isolates (pork or unspecified meat), demonstrating the pathogenic potential of the new variants and their possible dissemination along the food chain. Thus, losing a flagellar phase would not be detrimental for the success of *Salmonella* Typhimurium as a mammalian pathogen, as discussed elsewhere (26).

In conclusion, this study revealed new *Salmonella* 1,4,[5],12:i:− genotypes, explaining the lack of phase 2 flagellin expression

in isolates harboring an *fljB* coding sequence. IS*26* insertions played a key role in the loss of genetic elements needed for phase 2 flagellin expression, which would have generated several STMV lineages from *Salmonella* Typhimurium biphasic strains. Genetic diversity observed by PCR highlighted the nonclonality of STMV isolates circulating in Belgium, as observed in other countries (8, 27, 28). New STMV will probably be observed in the future due to the genomic plasticity of the region carrying elements needed for expression of phase 2 flagellin, especially IS*26* element-mediated genomic rearrangements. As these new variants were also observed in humans, molecular epidemiological follow-up is essential for tracing such contaminations throughout the food chain, particularly in the pig and pork sector.

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