

## Atypical, *fljB*-Negative *Salmonella enterica* subsp. *enterica* Strain of Serovar 4,5,12:i:– Appears To Be a Monophasic Variant of Serovar Typhimurium

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**An *fljB*-negative, multidrug-resistant *Salmonella enterica* serovar 4,5,12:i:– phage type DT U302 strain (resistant to ampicillin, chloramphenicol, sulfonamide, gentamicin, streptomycin, tetracycline, and sulfamethoxazole-trimethoprim) emerged and spread in Spain in 1997. Sequences specific for *Salmonella* serovar Typhimurium and phage type DT 104 and U302 were present in this atypical *Salmonella* strain, suggesting that it is a monophasic *Salmonella* serovar Typhimurium variant.**

*Salmonella enterica* is responsible for the majority of food-borne cases of disease worldwide. The epidemiological marker generally used for the identification of *S. enterica* is serotyping. Knowledge of serovar distributions allows the detection of new serovars, an increase in the frequency of already known serovars, and the geographical and temporal distributions of serovars.

An atypical *S. enterica* subsp. *enterica* strain of serovar 4,5,12:i:– emerged and spread in Spain in 1997 (5). The main strain characteristics are lysis by phage 10, recently incorporated into the *Salmonella* serovar Typhimurium phage typing scheme (phage type DT U302) (1); resistance to ampicillin, chloramphenicol, sulfonamide, gentamicin, streptomycin, tetracycline, and sulfamethoxazole-trimethoprim; and the absence of second-phase flagellar antigen by detection by either slide agglutination or specific PCR amplification (5).

According to the Kauffman-White scheme, the strain could be either a *Salmonella* serovar Typhimurium strain (serovar 4,5,12:i:1,2), a serovar Lagos strain (serovar 4,5,12:i:1,5), a monophasic variant, or a new serovar (9). Monophasic *Salmonella* strains could represent ancestral forms which did not acquire a second flagellar antigen or the necessary switching mechanism during evolution. Alternatively, they could originate as mutants of biphasic strains which have lost the switching mechanism, either the *fliC* or the *fljB* flagellar gene, or the ability to express one of these genes (4).

Until 1981, *Salmonella* serovar Typhimurium was the most frequently isolated serovar in Spain. Today, it is the second most frequently isolated serovar (12). *Salmonella* serovar Lagos has never been detected in Spain. If *Salmonella* serovar 4,5,12:i:– is a monophasic variant, it is most likely a *Salmonella* serovar Typhimurium variant.

The selective advantage of multidrug resistance can probably be one of the factors that has influenced the extension of

this strain in Spain, which became the fourth most common serovar during the period from 1998 to 2000 (12, 13).

These *Salmonella* serovar 4,5,12:i:– isolates possess two or three small cryptic plasmids together with a large 140-kb *spvC* (*Salmonella* plasmid virulence gene)-positive or 120-kb *spvC*-negative plasmid (2). The 140- or 120-kb plasmid could be derived from the *Salmonella* serovar Typhimurium-associated 90-kb virulence plasmid by the acquisition of class I integrons (7).

To determine the reason why second-phase flagellar antigens were not expressed in these isolates, PCR amplification of a selection of these monophasic strains was carried out to selectively amplify the total or partial second-phase (*fljB*) flagellar gene. PCR was performed as described previously with primers sense-56, sense-60, antisense-58, and antisense-83 (14) and sense-F1, antisense-R5, antisense-R6, antisense-R7, and antisense-R1 (6). No amplification of the 18 different possible fragments of the *fljB* gene was obtained. This result indicates the absence of the *fljB* gene.

To determine if *Salmonella* serovar 4,5,12:i:– is a *Salmonella* serovar Typhimurium monophasic variant, two type-specific sequences of *Salmonella* serovar Typhimurium and phage type DT 104 and U302, respectively, were investigated.

Thirteen multidrug-resistant (resistant to ampicillin, chloramphenicol, sulfonamide, gentamicin, streptomycin, tetracycline, and sulfamethoxazole-trimethoprim) *Salmonella* serovar 4,5,12:i:– (*fljB*-negative) strains were selected. One *Salmonella* serovar Typhimurium phage type LT2, two *Salmonella* serovar Typhimurium phage type DT 104, and two *Salmonella* serovar Typhimurium phage type DT U302 strains were selected as control strains. Nineteen different *Salmonella* serovars were selected as negative controls (Table 1).

*Salmonella* serovar Typhimurium strains harbor a specific IS200 fragment within the flagellin gene cluster. The IS200 fragment is located downstream of the *fliB* gene and upstream of the noncoding *fliA* gene region. This location is *Salmonella* serovar Typhimurium specific (3).

The *fliB*-*fliA* intergenic regions of all *Salmonella* strains tested were amplified with two primers, primers FFLIB (5'-C TGGCGACGATCTGTCGATG-3') and RFLIA (5'-GCGGT

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TABLE 1. Serogroup, serovar, and PCR results for 37 isolates of *S. enterica* used in the present study

Serogroup	Serovar	No. of strains	Size (by) of <i>fliB-fliA</i> intergenic region	Presence of 162-bp amplicon <sup>a</sup>
B	4, 5, 12:i:-	13	1,000	+
	Typhimurium LT2	1	1,000	-
	Typhimurium DT 104	2	1,000	+
	Typhimurium DT U302	2	1,000	+
	Lagos	1	250	-
	Agona	1	250	-
	Brandenburg	1	250	-
	Derby	1	250	-
	4, 12:b:- subsp. II	1	250	-
C1	Infantis	1	250	-
	Mbandaka	1	250	-
	Mikawasima	1	250	-
	Montevideo	1	250	-
	Ohio	1	250	-
	Rissen	1	250	-
	Virchow	1	250	-
C2	Goldcoast	1	250	-
	Hadar	1	250	-
	Muenchen	1	250	-
D	Enteritidis	1	250	-
E1	Anatum	1	250	-
E4	Senftenberg	1	250	-
O:61	61:lv:z subsp. IIIb	1	250	-

<sup>a</sup> +, present; -, absent.

ATACAGTGAATTCAC-3'), that anneal with the last *fliB* gene nucleotides and the first *fliA* gene nucleotides, respectively. The primers were designed by using already published sequences (3, 8). PCR amplification was performed with a Ready-to-Go system (Amersham Pharmacia Biotech Inc.). Five microliters of a strain suspension boiled for 10 min was used as a template. PCR amplification was as follows: denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and 1 final extension cycle at 72°C for 7 min. Fragments were separated in a 3% agarose gel by unidirectional electrophoresis. The fragments were visualized by staining with ethidium bromide.

PCR amplification of the *fliB-fliA* intergenic region of *Salmonella* serovar 4,5,12:i:- generated a 1,000-bp fragment, similar to the fragments from *Salmonella* serovar Typhimurium phage types LT2, DT 104, and DT U302. All 19 different *Salmonella* serovars tested, including *Salmonella* serovar Lagos, generated a 250-bp fragment, similar as those described previously (3) (Fig. 1).

The amplification products from four *Salmonella* serovar 4,5,12:i:- strains and *Salmonella* serovar Typhimurium phage type LT2 were purified and sequenced by using primers FFLIB and RFLIA with an Applied Biosystems ABI PRISM377 DNA Sequenator and the *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems/Perkin-Elmer). Sequence analysis was performed with PC-Gen software (Intelligenics Inc.). All five sequences were identical and harbored the *Salmonella* serovar Typhimurium IS200-specific fragment already de-

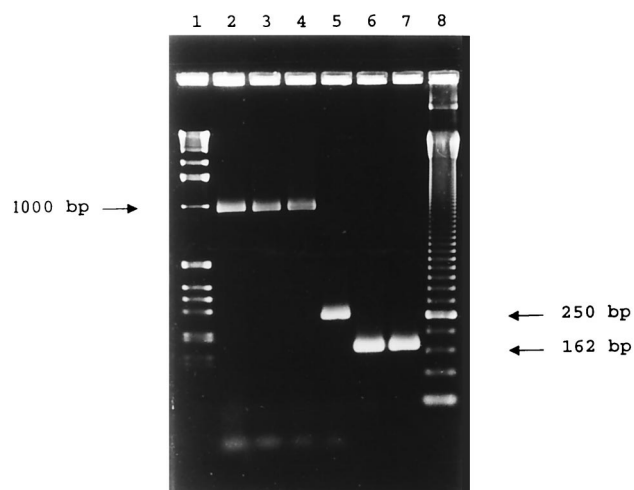


FIG. 1. PCR amplification products of *Salmonella* isolates analyzed. Lanes 2 through 5, *fliB-fliA* intergenic region of *Salmonella* serovar 4,5,12:i:-, serovar Typhimurium phage type LT2, serovar Typhimurium phage type DT U302, and serovar Brandenburg (3), respectively; lanes 6 and 7, phage type DT 104 and U302 162-bp specific amplicon of *Salmonella* serotype 4,5,12:i:- and serotype Typhimurium phage type DT U302, respectively (10); lane 1, molecular weight marker X (Roche Molecular Biochemicals); lane 8, 50-bp ladder (Amersham Pharmacia Biotech Inc.).

scribed (3). The IS200 element obtained (i) started 38 nucleotides downstream of the stop codon of the *fliB* gene, (ii) lacked a terminal inverted repeat, (iii) had two additional base pairs at the 5' end, and (iv) had an additional C at nucleotide 1542.

PCR amplification of a 162-bp *Salmonella* serovar Typhimurium phage type DT 104- and U302-specific region has been used as a rapid technique for the identification of both phage types. This fragment was also obtained from 15 *Salmonella* strains belonging to 6 different serovars among the 36 *Salmonella* serovars studied. This fragment can be considered specific for *Salmonella* serovar Typhimurium phage types DT 104 and U302 (10).

Because bacteriophages are serovar specific and because *Salmonella* serovar Typhimurium phage 10 was able to lyse *Salmonella* serovar 4,5,12:i:-, the strain would probably be a monophasic variant of *Salmonella* serovar Typhimurium phage type DT U302.

All *Salmonella* 4,5,12:i:- and *Salmonella* serovar Typhimurium phage type DT 104 and U302 strains tested amplified a 162-bp fragment (Fig. 1). *Salmonella* serovar Typhimurium phage type LT2, *Salmonella* serovar Lagos, and 18 other *Salmonella* serovars tested did not express any fragment, as expected. Fragments of 162 bp from four *Salmonella* 4,5,12:i:- strains were sequenced, with sequences identical to those of *Salmonella* serovar Typhimurium phage types DT 104 and U302 published previously being obtained (10).

The present study demonstrates the presence of the IS200-IV fragment (11) in the intergenic *fliB-fliA* flagellin cluster region in all *Salmonella* serovar 4,5,12:i:- strains tested. This fragment is located at the same position at which it is located in *Salmonella* serovar Typhimurium and has the same sequence as *Salmonella* serovar Typhimurium (3). It was also

possible to amplify a 162-bp fragment that was similar in size and sequence to those from *Salmonella* serovar Typhimurium phage types DT 104 and U302.

We conclude that the emerging *fliB*-negative *Salmonella* serovar 4,5,12:i:– phage type DT U302 strain resistant to ampicillin, chloramphenicol, sulfonamide, gentamicin, streptomycin, tetracycline, and sulfamethoxazole-trimethoprim should be considered a *Salmonella* serovar Typhimurium 5+ monophasic variant.

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