

## Anti-flagellin antibody responses elicited in mice orally immunized with attenuated *Salmonella enterica* serovar Typhimurium vaccine strains

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*In the present study we investigated the flagellin-specific serum (IgG) and fecal (IgA) antibody responses elicited in BALB/c mice immunized with isogenic mutant derivatives of the attenuated Salmonella enterica serovar Typhimurium (S. Typhimurium) SL3261 strain expressing phase 1 (FliCi), phase 2 (FljB), or no endogenous flagellin. The data reported here indicate that mice orally immunized with recombinant S. Typhimurium strains do not mount significant systemic or secreted antibody responses to FliCi, FljB or heterologous B-cell epitopes genetically fused to FliCi. These findings are particularly relevant for those interested in the use of flagellins as molecular carriers of heterologous antigens vectored by attenuated S. Typhimurium strains.*

Key words: flagellin - antibody response - attenuated *S. Typhimurium* strains

Attenuated *Salmonella enterica* strains have been studied intensively as live carriers of heterologous vaccine antigens delivered by mucosal or parenteral routes (Brey et al. 1991, Garmory et al. 2002). Orally delivered, attenuated *Salmonella* strains induce both systemic and secretory immune responses against the carrier strain as well as the heterologous passenger antigen (Garmory et al. 2002). Additionally, peptides, particularly those encoding B-cell epitopes, may be expressed by attenuated *Salmonella* strains as hybrid proteins following genetic fusion to bacterial proteins. This approach may improve both the stability and the immunogenicity of target antigens delivered orally in live bivalent vaccines (Dougan et al. 1987, Chatfield et al. 1992).

The fusion of heterologous antigens to *Salmonella* flagellin, the structural subunit of bacterial flagella, has piqued considerable interest as an approach to enhancing the immunogenicity of vaccine peptides (Newton et al. 1989, Stocker 1990, Newton et al. 1991, Stocker & Newton 1994). As originally proposed, the heterologous peptide is fused in-frame to the central hypervariable domain of *Salmonella* FliC<sub>D</sub> flagellin, which is derived from *Salmonella* Muenchen and expressed by an attenuated *Salmonella* Dublin strain. The chimeric flagellins are exported to the bacterial surface where the subunits assemble into the flagellar shaft without a significant impact on bacterial motility and host tissue colonization (Newton et al. 1989, Stocker & Newton 1994). Nonethe-

less, our previous results showed that the genetic fusion of heterologous peptides to flagellins may not enhance antigen-specific antibody responses in mice orally immunized (p.o.) with recombinant *S. Dublin* vaccine strains (De Almeida et al. 1999, Sbrogio-Almeida & Ferreira 2001). Interestingly, the genetic background of both the mouse lineage and the *Salmonella* strain affected the immunogenicity of flagellins delivered orally using recombinant vaccine strains (Sbrogio-Almeida 2004). Indeed, recent evidence indicates that *Salmonella* flagellin administered via the oral route may trigger immunological hyporesponsive responses in healthy mice, although the precise mechanism underlying these responses remains unknown (Sanders et al. 2006).

In contrast to *S. Dublin* strains, wild-type *S. Typhimurium* strains express two flagellar phases: phase 1 (FliC<sub>i</sub>) and phase 2 (FljB), which are alternately produced by a single cell but may be found concurrently in the same bacterial population (Ikeda et al. 2001, Yamamoto & Kutsukake 2006). So far, the oral immunogenicity of the two *S. Typhimurium* flagellins, as well as their role as potential heterologous peptide carriers, has not been investigated. To explore these issues, the present study analyzed the antibody-inducing properties of flagellins expressed by the attenuated *S. Typhimurium* SL3261 strain.

The bacterial strains and plasmids used in this study are described in Table I. Recombinant plasmids were introduced by electroporation into the non-flagellated *S. Typhimurium* strain LDV321. The plasmid pFF400 is a pUC18 derivative carrying the *fliC* gene of SL3261. pFF408 was constructed by replacing a 48 bp region between two natural *EcoRV* sites with a polylinker containing unique *XhoI* and *BglIII* sites at the central region of FliC<sub>i</sub>-encoding gene (Newton et al. 1989). pFF302 is a pFF408 derivative that carries a hybrid *fliCi* gene encoding a 15-amino acid peptide (VDPVIDLLQADGNAL). This peptide defines B and T-cell epitopes of the structural subunit (C<sub>fa</sub>B) of the C<sub>FA</sub>/I fimbriae from entero-

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TABLE I  
*Salmonella* Typhimurium strains and plasmids used in the present study

Strains	Relevant characteristics	Reference
SL3261	<i>aroA, his</i>	Hoiseith & Stocker 1981
LDV321	<i>aroA, his; fliC(i)::FRT<sup>a</sup>, fljBA::FRT</i>	This study
LDV322	<i>aroA, his; fljBA::FRT</i>	This study
LDV323	<i>aroA, his; fliC(i)::FRT</i>	This study
LDV3211 LDV321, pFF408		This study
LDV325 LDV321, pFF411		This study
LDV3212 LDV321, pFF302		This study
TT22971	<i>metA22 metE551 trpD2 ilv-452 leu pro hsdLT6 hsdSA29 hsdB strA120</i>	Court et al. 2002
Plasmids		
pKD46	<i>araC, bla, oriR101, repA101(Ts), lambda red genes (gam<sup>+</sup> bet<sup>+</sup> exo<sup>+</sup>)</i>	Datsenko & Wanner 2000
pCP20	<i>FLP<sup>+</sup>, λ cI857+, λ p<sub>R</sub> Rep<sup>ts</sup>, bla, catF</i>	Cherepanov & Wackernagel 1995
pFF408	pUC18 derivative containing the <i>fliCi</i> gene with a deletion of 48 bp, <i>bla</i>	This study
pFF411	pFF408 derivative with a 45 bp insert encoding the CTP3 epitope	This study
pFF302	pFF408 derivative with a 45 bp insert encoding the CfaB <sub>11-25</sub> epitope	This study

a: FRT- FLP Recognition Target (Cherepanov & Wackernagel 1995).

toxigenic *Escherichia coli* (ETEC) (Luna et al. 1997) at the *FliCi* central hypervariable domain. Similarly, pFF411 delivers a recombinant *fliCi* gene encoding the 15 amino acid-long CTP3 peptide (VEVPGSQHID-SQKKA) derived from the B subunit of the cholera toxin (Newton et al. 1989) at the same flagellin domain.

Flagellin-deficient *S. Typhimurium* strains have been generated following chromosomal deletions of each native flagellin gene (*fliCi* and *fljB*) by allelic replacement (Datsenko & Wanner 2000). Primers were designed based on the flagellin sequences of the *S. Typhimurium* LT2 strain (McClelland et al. 2001). Deletion of *fljB* also included the *fljA* gene, which encodes the *fliC* gene transcriptional repressor. The amplified fragments were introduced by electroporation into the attenuated SL3261 strain transformed with the arabinose-inducible *red* genes-encoding plasmid (pKD46), previously propagated in the restriction minus (*rm<sup>+</sup>*) metilation plus *S. Typhimurium* TT22971 strain. The phage lambda recombinase allows high-frequency recombination between linear DNA fragments generated by polymerase chain reaction (PCR) and homologous regions in the bacterial chromosome (Datsenko & Wanner 2000). To avoid undesirable mutations and to obtain the double-deletion mutant ( $\Delta$ *fliC*,  $\Delta$ *fljBA*) the strains were submitted to transductional crosses using a high frequency-transducing P22 phage (HT105 int) as previously described (Schmieger 1971). The strains were also transformed with pCP20, encoding resistance to ampicillin and chloramphenicol, to remove the resistance cassettes inserted in the target chromosomal sites. As previously reported, this plasmid undergoes temperature-sensitive replication and induction of *flp* (the gene encoding the *Saccharomyces cerevisiae* F1p recombi-

nase) synthesis after a temperature shift (Cherepanov & Wackernagel 1995). The genomic deletions of the resulting *S. Typhimurium* LDV322 ( $\Delta$ *fljBA*), LDV323 ( $\Delta$ *fliC*) and LDV321 ( $\Delta$ *fliC*,  $\Delta$ *fljBA*) strains were confirmed by PCR and immunoblot assays. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to standard procedures (Sbrogio-Almeida & Ferreira 2001). Motility was assessed by applying the bacterial strains to the center of plates containing 0.35% agar, followed by incubation at 37°C for 32 h. Motility agar plates were prepared according to previously established methods (Ikeda et al. 2001).

Flagellins expressed by *S. Typhimurium* LDV322 or LDV323 were harvested from cultures incubated overnight under mild aeration (80 rpm) at 37°C. Cells were collected by centrifugation, suspended in phosphate-buffered saline (pH 7.4) and homogenized for 2 min using a bench mixer. The cells were maintained on ice and the above procedure was repeated three times. The mixture was then centrifuged at 10,000 g for 10 min to remove the bacteria and flagella were collected from the supernatant following acetone precipitation. Contaminating lipopolysaccharide (LPS) was removed using a gel-detoxi column (Pierce). Recombinant His-tagged *FliCi* flagellin was produced in *E. coli* transformed with pET28a carrying the cloned *fliCi* gene. Protein concentration was measured using the BCA quantification method (Pierce).

Isogenic, pathogen-free 8-12 week old female BALB/c mice were supplied by the Isogenic Mouse Breeding Facility of the Departamento de Parasitologia do Instituto de Ciências Biomédicas da Universidade de São Paulo and all procedures were approved by the ethics committee on use of laboratory animals. Immunizations were

carried out using viable bacterial strains harvested during exponential growth (optical density at 600 nm of 0.8). Mice were p.o. with 0.5 ml aliquots of approximately  $10^{10}$  CFU using a stainless steel, round-tip gavage cannule on days 0, 21 and 35. The same immunization schedule was followed for the intraperitoneally (i.p.) immunizations, but the bacterial loads were reduced to  $10^7$  CFU per dose. Serum and fecal wash samples were collected individually, but samples belonging to the same immunization groups (with 5-10 individuals) were pooled for antibody titer determination (Sbrogio-Almeida & Ferreira 2001).

Microtiter plates (Nalge Nunc) were coated (0.1  $\mu$ g/well) with purified FliCi and FljB or the CfaB (Luna et al. 1997) and CTP3 oligopeptides (De Almeida et al. 1999) in 0.05 M sodium bicarbonate buffer (pH 9.6) overnight at RT. Experimental procedures for detecting specific serum IgG and fecal IgA responses have been described previously (Sbrogio-Almeida et al. 2004).

Isogenic flagellin-deficient knockout mutants of the attenuated *S. Typhimurium* SL3261 strain, affected in either *fliCi*, *fljB* or both genes, were selected after homologous recombination mediated by the bacteriophage lambda recombinase encoded by the *red* genes (Fig. 1). In vitro tests carried out with the flagellin-deficient derivatives of the *S. Typhimurium* SL3261 strain showed that deletion of either *fliCi* or *fljB* did not impair the motility of the respective strains. Nonetheless, as expected, deletion of both flagellin-encoding genes rendered the LDV321 strain non-motile (Fig. 1).

Initial attempts to measure the flagellin-specific antibody responses in vaccinated mice indicated that mice immunized with the *S. Typhimurium* SL3261 or

LDV321 strains mounted similar systemic and secreted anti-flagellin antibody responses following oral administration (Fig. 2). As demonstrated in Fig. 3, removal of LPS from flagellin preparations drastically reduced the reactivity of sera in mice immunized with the *Salmonella* strains. Indeed, the recombinant FliCi flagellin, produced in *E. coli*, reacted weakly with the antibodies generated in mice p.o. with the SL3261 strain (Fig. 3). Additionally, western blots carried out with *Salmonella* flagellin preparations and sera from mice vaccinated with the *S. Typhimurium* SL3261 strain showed that, besides the contaminant LPS present in flagellin preparations, a minor protein with an estimated molecular mass of 35 kDa and not detected in Coomassie blue-stained gels reacted with the tested serum sample (Fig. 3).

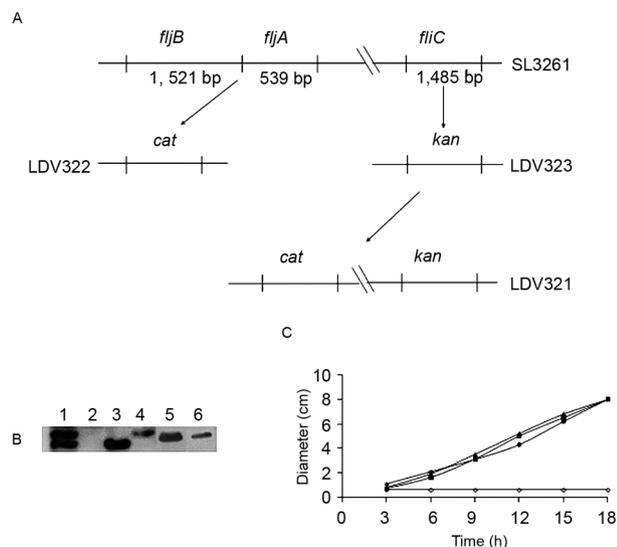


Fig. 1: generation of flagellin-deficient *Salmonella Typhimurium* strains. A: schematic representation of the construction steps leading to *S. Typhimurium* strains expressing only FliCi (LDV322), FljB (LDV323) or none flagellin (LDV321); B: immunological detection of flagellins expressed by different *S. Typhimurium* strains. Lanes 1: SL3261 strain; 2: LDV321 strain; 3: LDV322 strain; 4: LDV323 strain; 5: LDV325 strain; 6: LDV3212 strain; C: in vitro motility of *S. Typhimurium* strains on a semi-solid medium at 37°C; tested strains: SL3261 (●); LDV321 (○); LDV322 (▲); LDV323 (■).

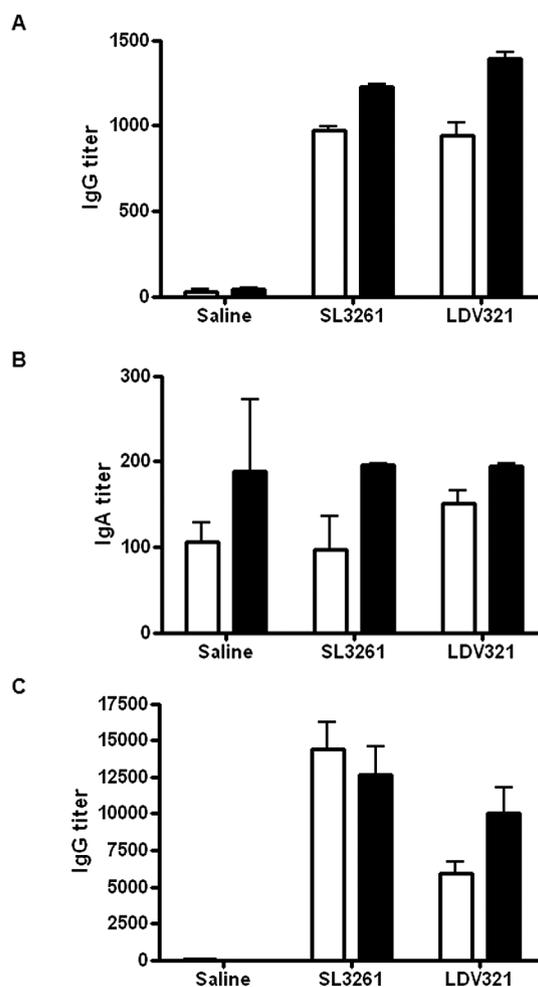


Fig 2: specific antibody responses to flagellins in BALB/c mice immunized with *Salmonella Typhimurium* SL3261 or LDV321 strains. A: serum IgG responses to FliCi (white bars) and FljB (black bars) after oral immunization with the *S. Typhimurium* strains expressing FliCi and FljB (SL3261) or none (LDV321) flagellin; B: fecal IgA responses to flagellins after p.o. with the *S. Typhimurium* SL3261 or LDV321 strains; C: serum IgG responses to flagellins after intraperitoneal immunization route; control groups represented by non immunized mice; values are means of endpoint titers  $\pm$  SEM of serum pools (n = 5) prepared from each mouse group.

The following Enzyme Linked Immuno Sorbent Assay (ELISA) measurements were thus carried out using LPS-free *Salmonella* flagellin preparations. The results obtained after the p.o. trials with the *S. Typhimurium* SL3261, LDV322 or LDV323 strains indicated that no significant flagellin-specific serum IgG responses were induced in mice immunized with the flagellated *S. Typhimurium* strains (Table II). Similar conclusions were drawn following measurement of flagellin-specific IgA in fecal extracts of mice p.o. with flagellated *S. Typhimurium* vaccine strains (data not shown). In contrast, mice immunized via the i.p. route with flagellated *S. Typhimurium* strains developed high FliC*i* or FljB-specific serum IgG responses following vaccination with the LDV322 or LDV323 strain, respectively (Table II). Collectively, these results indicate that mice p.o. with flagellated *S. Typhi-*

murium strains did not develop significant systemic or secreted flagellin-specific antibody responses.

To determine if the lack of antibody responses to *S. Typhimurium* flagellins observed in mice p.o. immunized with the attenuated LDV3212 or LDV325 strain would affect antibody responses to heterologous epitopes genetically fused at the central hypervariable region of FliC*i*, we measured the systemic (serum IgG) and secreted (fecal IgA) responses to peptides derived from the ETEC CFA/I fimbriae structural subunit and the B subunit of cholera toxin. Although the presence of the flagellin inserts could be confirmed by either DNA sequencing or western blot with anti-flagellin serum (Fig. 1B and unpublished observations), BALB/c mice p.o. with the recombinant *S. Typhimurium* strains did not develop significant serum or fecal antibody responses to the he-terologous epitopes (data not shown).

Using the isogenic, flagellin-deficient *S. Typhimurium* strains, it was possible to show that the previously detected flagellin-specific serum antibody responses elicited in mice p.o. with flagellated strains were due to the presence of contaminating LPS, as well as an outer membrane protein in flagellin preparations used as solid-phase bound antigen. Thus, the generation of flagellin preparations with higher purity and the use of a non-flagellated strain helped us to demonstrate that mice p.o. with flagellated *S. Typhimurium* strains failed to mount significant systemic and secreted flagellin-specific antibody responses, similar to results previously reported for *S. Dublin* strains (Sbrogio & Almeida 2001, Sbrogio et al. 2004, Sanders et al. 2006).

Since mice parenterally immunized with flagellated *S. Typhimurium* strains developed strong antibody re-

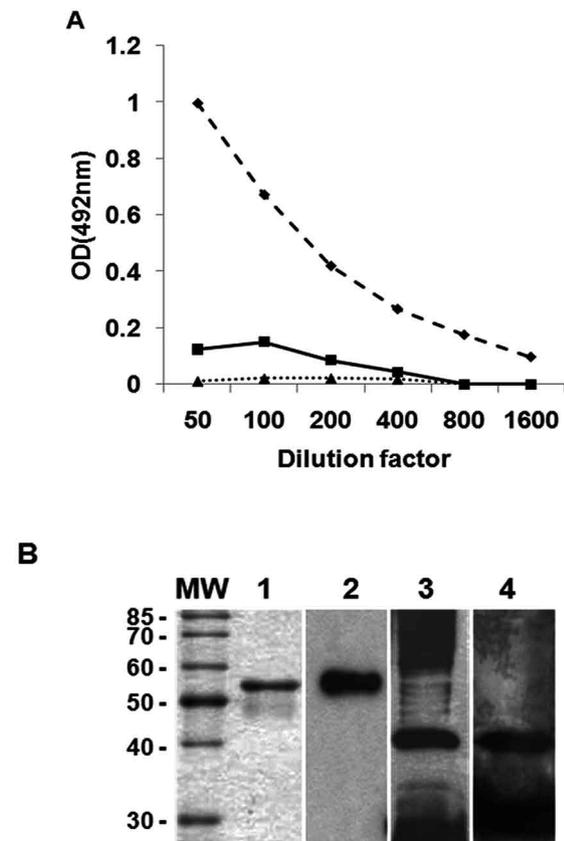


Fig. 3: detection of non-specific reaction of serum samples harvested from mice immunized with the *Salmonella* Typhimurium SL3261 strain. A: ELISA carried out with different solid phase bound antigen preparations: crude FliC*i* preparation extracted from *S. Typhimurium* (◆); lipopolysaccharide (LPS) free FliC*i* preparation (■) and affinity purified *S. FliC*i** produced by a recombinant *Escherichia coli* strain (▲); B: detection of cross-reacting materials in flagellin preparations extracted from *S. Typhimurium* SL3261. Lanes MW: molecular weight markers; 1: Coomassie blue-stained FliC*i* preparation (1 µg); 2: immunoblot developed with anti-FliC*i* serum raised in mice immunized with purified protein; 3: immunoblot developed with serum pool (n = 5) harvested from mice orally immunized with the *S. Typhimurium* SL3261 strain; 4: immunoblot of a LPS-free FliC*i* preparation developed with serum pool (n = 5) harvested from mice orally immunized with the *S. Typhimurium* SL3261 strain.

TABLE II

Flagellin-specific antibody responses (serum IgG and fecal IgA) elicited in BALB/c mice immunized via the oral (p.o.) or intraperitoneally (i.p.) immunization routes with the *Salmonella* Typhimurium SL3261 strain and its flagellin-deficient derivatives

Strains	Immunization routes <sup>a</sup>	Flagellin antibody responses <sup>b</sup>	
		Serum IgG <sup>c</sup>	
<i>S. Typhimurium</i>		AntiFliC <i>i</i>	AntiFljB
SL3261	oral	36 ± 0.10	0
	i.p.	11,626 ± 0.19	5,986 ± 0.25
LDV322	oral	0	ND
	i.p.	5,331 ± 0.2	ND
LDV323	oral	ND	0
	i.p.	ND	10,690 ± 0.25

a: immunizations were carried out with three doses of 10<sup>10</sup> CFU p.o. or 10<sup>7</sup> CFU i.p. on days 0, 21 and 35. Serum and fecal samples were collected one week after the last immunization dose; b: titers were determined in ELISA plates treated with purified FliC*i* or FljB flagellins. Data are based on two independent experiments; c: values are means of endpoint titers ± SEM of serum pools (n = 5) prepared from each mouse group; ND: not done.

sponses to flagellins, the observed flagellin-hyporesponsive state might be restricted to the murine mucosal immune system. Recent findings confirmed that bacterial flagellins do not elicit inflammatory responses in the normal gut epithelium, a behavior explained by the lack of functional TLR5 expression at the apical surface of enterocytes (Gewirtz et al. 2001), the activation of apoptotic responses by flagellin-exposed antigen-presenting cells (Ren et al. 2006) and/or the activation of suppressive CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells (Crellin et al. 2005). Although our findings indicate that the expression of chimeric flagellins by recombinant attenuated *S. Typhimurium* strains has an inherently reduced immunogenicity as an oral bivalent vaccine approach, stimulation of specific immune responses triggered by purified flagellins delivered via the nasal or parenteral routes (Honko et al. 2006) represents more promising alternative for the use of *Salmonella* flagellins in vaccine development.

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