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Repression of Flagella Is a Common Trait in Field Isolates of *Salmonella enterica* Serovar Dublin and Is Associated with Invasive Human Infections

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The nontyphoidal *Salmonella enterica* serovar Dublin is adapted to cattle but infrequently infects humans, very often resulting in invasive infections with high levels of morbidity and mortality. A *Salmonella*-induced intestinal acute inflammatory response is postulated as a mechanism to prevent bacterial dissemination to systemic sites. In *S. enterica* serovar Typhimurium, flagella contribute to this response by providing motility and FliC-mediated activation of pattern recognition receptors. In this study, we found 4 *Salmonella enterica* isolates, with the antigenic formula 9,12:−:−, that, based on *fliC* sequence and multilocus sequence type (MLST) analyses, are aflagellate *S.* Dublin isolates. Interestingly, all were obtained from human bloodstream infections. Thus, we investigated the potential role of flagella in the unusual invasiveness exhibited by *S.* Dublin in humans by analyzing flagellation and proinflammatory properties of a collection of 10 *S.* Dublin human clinical isolates. We found that 4 of 7 blood isolates were aflagellate due to significantly reduced levels of *fliC* expression, whereas all 3 isolates from other sources were flagellated. Lack of flagella correlated with a reduced ability of triggering interleukin-8 (IL-8) and CCL20 chemokine expression in human intestinal Caco-2 cells and with reduced early inflammation in the ceca of streptomycin-pretreated C57/BL6 mice. These results indicate that flagella contribute to the host intestinal inflammatory response to *Salmonella* serovar Dublin and suggest that their absence may contribute to its systemic dissemination through dampening of the gut immune response. Analysis of FliC production in a collection of cattle isolates indicated that the aflagellate phenotype is widely distributed in field isolates of *S.* Dublin.

Salmonellosis by nontyphoidal salmonellae (NTS) is among the most common food-borne infections worldwide, with an estimated global incidence of 93.8 million cases of gastroenteritis and 155,000 deaths per year (1). Among the >2,500 serotypes assigned to the species *Salmonella enterica*, those able to cause disease in humans are divided into typhoidal serotypes (mainly Typhi and Paratyphi A) and hundreds of NTS serotypes, among which the most prevalent worldwide are Enteritidis and Typhimurium (2, 3). The typhoidal serotypes are restricted to the human host and cause invasive disease in immunocompetent individuals, while NTS typically have a broad vertebrate host range and cause predominantly a self-limiting gastroenteritis, characterized by acute intestinal inflammation and diarrhea, in humans. However, depending on the immune status of the host and the serovar and particular strain involved, NTS can enter the bloodstream and cause systemic disease. In addition, NTS are consistently the most common bacteria isolated from the bloodstream in both adults and children presenting with fever in sub-Saharan Africa, and this is associated with HIV, malaria, and malnutrition (4). In Uruguay, from a total of 495 NTS clinical isolates received at the National *Salmonella* Centre (NSC) from 2008 to 2012, 96 (19.4%) were obtained from bloodstream infections (NSC, unpublished data).

When the invasive index is considered, i.e., the percentage of blood isolates relative to the total number of isolates for each *Salmonella* serovar in a defined geographical region, it becomes evident that certain NTS serovars are more invasive than others (2, 5, 6). This is the case for *Salmonella* serovars Dublin and Choleraesuis, which are consistently reported worldwide, with invasive indexes of >40%, while the invasive indexes of the more ubiquitous *Salmonella* serovars Enteritidis and Typhimurium rarely exceed 5% (2, 5, 7, 8).

S. enterica serovar Dublin (formula 1,9,12 [Vi]:g,p:−) is strongly adapted to cattle, where it causes systemic infections and abortion (9). However, human infections with *S.* Dublin occur at low frequency, and these are often severe and may be fatal, especially in AIDS patients and other immunocompromised persons (10–12). The bacterial factors responsible for this invasive phenotype of *S.* Dublin in humans are unknown.

The difference in clinical outcome between typhoidal salmonellae and NTS is thought to be due partly to the way both groups of serovars interact with the gut epithelium, with the NTS causing an acute local inflammatory reaction with a strong neutrophil influx that prevents systemic spread of the bacteria (2, 13, 14).

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TABLE 1 *S.* Dublin field isolates used in this study

Isolate	Yr of isolation	Source[b]	Patient information[b]
Human isolates[a]			
SDu1	1995	Blood	ND
SDu2	2004	Blood	ND
SDu3	2006	Blood	Female, 41 years old, asthmatic, diabetic
SDu4	2008	Blood	Young man, drug addict
SDu5	2000	Feces	ND
SDu6	2005	Feces	Male, 40 years old
SDu7	2008	Blood	Male, 1 year old
SDu8	2011	Blood	Male, 76 years old
SDu9	2011	Blood	Female, 50 years old
SDu10	2011	Urine	Male
Bovine isolates			
75/95	1995	ND	
56/96	1996	Calf lymph	
57/96	1996	Calf lung	
69/96	1996	Calf	
72/96	1996	Calf	
73/96	1996	Bile	
74/96	1996	Liver	
75/96	1996	Calf	
76/96	1996	Calf	
79/98	1998	ND	
210/00	2000	Calf	
73/04	2004	Calf	
74/04	2004	Calf	
75/04	2004	Calf	
99/04	2004	Fetus	

[a] All human isolates were of ST10 according to MLST.
[b] ND, no data are available.

TABLE 2 Strain constructs used in this study

Strain	Description
SDu5 *fliC*::*kan*	SDu5 derivative containing *fliC*::*kan* (Kan^r)
SDu3 Str^r	SDu3 derivative containing *aadA* (Str^r)
SDu5 Str^r	SDu5 derivative containing *aadA* (Str^r)
SDu5 Str^r *fliC*::*kan*	SDu5 derivative containing *aadA* and *fliC*::*kan* (Kan^r Str^r)

For *Salmonella enterica* serovar Typhimurium, several bacterial factors have been described as important for triggering the inflammatory response at the gut in calves and streptomycin-pretreated mice, including SPI-1 (15–17) and SPI-2 (18–20) effectors and flagella (21, 22).

The flagellin protein (FliC) is the main structural component of the flagellar filament and is also the agonist of the pattern recognition receptors Toll-like receptor 5 (TLR5) and Naip5/NLRC4 (23–27). In model human intestinal epithelia, it has been demonstrated that *S.* Typhimurium FliC binding to TLR5 triggers a proinflammatory response characterized by upregulation of expression of interleukin 8 (IL-8), CCL20 (macrophage inflammatory protein 3α [MIP-3α]), and several other proinflammatory chemokines (25, 28) that recruit neutrophils and dendritic cells into the subepithelial compartment (29, 30). *In vivo*, it has been reported that in streptomycin-pretreated mice, *S.* Typhimurium flagellin knockout mutants trigger diminished intestinal inflammation compared to that with wild-type bacteria at early time points postinfection (p.i.) (21, 31). Similar findings were obtained with calf ligated ileal loops (22, 32) and chickens (33). Interestingly, a mechanism has been described for the invasive serovar *S.* Typhi through which *fliC* expression is repressed when the bacterium encounters conditions of tissue osmolarity (but not the higher osmolarity present in the intestinal lumen), thus enabling it to overcome the mucosal barrier at the intestine through innate immunity evasion (34).

The aim of this work was to elucidate bacterial factors responsible for the unusual invasiveness exhibited by *S.* Dublin in humans, focusing on the role of flagella. Thus, we characterized the motility, flagellar expression, and proinflammatory properties of a set of *S.* Dublin clinical isolates obtained at different times and from invasive or localized infections and evaluated the correlation between flagellar expression and the source of the isolate (blood versus nonblood). Our results revealed the existence of a large proportion of aflagellate strains among the clinical isolates of this serovar, a phenotype that correlated with impaired proinflammatory properties *in vitro* and *in vivo*. Note that all aflagellate strains were isolated from invasive cases of salmonellosis.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Uruguayan clinical isolates of *S.* Dublin were obtained from the NSC and the Bacteriology Unit of the Ministry of Public Health (MPH) collections (Table 1). These were all the human isolates of this serovar available in both collections and included seven isolated from blood and three from other sources (feces or urine). "Invasive" cases were defined as those in which *Salmonella* had been isolated from the bloodstream. An isolate obtained from urine was not considered invasive because we lacked the clinical data to distinguish between invasive infection and colonization or contamination of urine. Where available, data about the patients from whom the isolates were obtained are depicted in Table 1.

S. Dublin cattle isolates were obtained from the NSC and are shown in Table 1. All isolates were confirmed biochemically and serologically at the NSC and the Bacteriology Unit of the MPH. In those cases where no agglutination with anti-H serum was obtained, the sequence of the *fliC* hypervariable region was determined using primers Gfor and Grev (see Table 3) and compared with those available at the GenBank database to confirm the Dublin serotype. In addition, multilocus sequence typing (MLST) was performed as described at http://mlst.ucc.ie/mlst/dbs/Senterica/documents/primersEnterica_html (35).

The hypervariable *fliC* sequences of the aflagellate *S.* Dublin isolates described in this study were identical to those of the two sequenced *S.* Dublin strains available in the GenBank database (strains CT_02021853 and SD3246) and differed in three single nucleotide polymorphisms (SNPs) from the same region of *S.* Enteritidis strain P125109, whose complete genome is also available in GenBank. Moreover, MLST analysis revealed that all 10 *S.* Dublin human isolates used in this study belong to ST10 (Table 1), which is the predominant ST reported for *S.* Dublin (35).

Luria-Bertani (LB) broth and LB agar (Sigma) were used for routine cultures of *S.* Dublin at 37°C in an orbital shaking incubator (200 rpm). Isolates were stored in replicates at −80°C in LB broth containing 25% glycerol.

Bacterial constructs are depicted in Table 2. An SDu5 derivative *fliC* knockout mutant was constructed by P22 transduction of a *fliC*::*kan* cassette from *S.* Enteritidis strain PT4 P125109 *fliC*::*kan* (36). The resulting strain, SDu5 *fliC*::*kan*, was resistant to kanamycin and lacked motility, as verified by plating on soft agar and microscopic visualization.

For animal studies, field isolates were made streptomycin resistant by P22 phage transduction of the *aadA* gene from the streptomycin-resistant *S.* Typhimurium strain SL1344 (http://www.sanger.ac.uk/Projects/Microbes/). The resulting strains were able to grow in 500 μg/ml

TABLE 3 Primers used in this study

Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Reference
Gfor	GTGATCTGAAATCCAGCTTCAAG	509	58
Grev	AAGTTTCGCACTCTCGTTTTTGG		
icdA F	TGGTATCGGTGTTGATGTCACTC	140	Modified from reference 59
icdA R	CATCCTGGCCGTAAACCTGTGTG		
fliA F	GCATCGAACTGGTGACTGAAGAAC	149	59
fliA R	GAGCTCTTCCTGGTAATACAGCGT		
fliC F	AGATCACCTTAGCTGGCAAAACC	164	This work
fliC R	CCCCAGAGAAGAACGAACTGC		
mB-actin_F	GCTTCTTTGCAGCTCCTTCGT	68	60
mB-actin_R	CGTCATCCATGGCGAACTG		
mCXCL1_F	CTTGGTTCAGAAAATTGTCCAAAA	84	60
mCXCL1_R	ACGGTGCCATCAGAGCAGTCT		
mIL-17A_F	CTCCAGAAGGCCCTCAGACTAC	69	60
mIL-17A_R	GGGTCTTCATTGCGGTGG		
mIFNg_F	TCAGCAACAGCAAGGCGAAA	143	61
mIFNg_R	CCGCTTCCTGAGGCTGGAT		
mCCL20_F	TTTTGGGATGGAATTGGACAC	69	60
mCCL20_R	TGCAGGTGAAGCCTTCAACC		
mTNFalfa_F	CATCTTCTCAAAATTCGAGTGACAA	63	60
mTNFalfa_R	CCTCCACTTGGTGGTTTGCT		
mLcn2_F	CCATCTATGAGCTACAAGAGAACAAT	89	62
mLcn2_R	TCTGATCCAGTAGCGACAGC		
mS100.A9_F	CACCCTGAGCAAGAAGGAAT	95	62
mS100.A9_R	TGTCATTTATGAGGGCTTCATTT		

of streptomycin and were verified by anti-O-antigen serum agglutination and motility testing. In addition, growth curves were performed to verify that genetic manipulation did not affect the growth properties of the original isolates. An SDu5 *fliC*::*kan* derivative of the previously constructed streptomycin-resistant SDu5 strain was obtained by P22 transduction. The resulting strain was resistant to kanamycin and lacked motility; the rest of the analyzed parameters (growth rate and O-antigen agglutination) were unchanged compared to those of the parental strain.

For mouse infection experiments, bacteria were grown overnight (o/n) at 200 rpm and 37°C in LB broth containing 50 μg/ml of streptomycin; the o/n cultures were diluted 1:20 in the same medium plus 0.3 M NaCl and subcultured for 4 h with mild aeration (100 rpm).

Motility tests. Motility tests were performed as described by Yim et al. (37). Briefly, 2 μl of overnight culture grown in LB broth was spotted onto the surface of an LB plate containing 0.3% agar (semisolid agar) and incubated for 6 h at 37°C. Those isolates showing no halo of growth (indistinguishable from a *fliC* knockout strain) were considered nonmotile. Values are expressed as diameters of growth (in mm) obtained after 6 h of incubation at 37°C. The assays were repeated three times, and the results were confirmed by phase-contrast microscopic visualization of mid-log-phase bacterial cultures grown in LB broth.

Protein analysis. For preparation of total protein extracts, log-phase bacterial cultures (optical density at 600 nm [OD_{600}] = 0.4 to 0.6) were centrifuged, resuspended in phosphate-buffered saline (PBS) containing 0.15 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM EDTA, sonicated, and centrifuged again to remove unbroken cells. The supernatants (cleared lysates) were quantified by the Bradford assay (Sigma).

For Western blot analysis, 25 μg of total protein extract was loaded onto a 12% SDS-PAGE gel and analyzed by Western blotting using mouse anti-flagellar antigen Hg antibody (Bio-Stat, United Kingdom) and ECL Prime Western blotting detection reagent (GE Healthcare). As an internal control, an anti-DnaK monoclonal antibody was used (AbCam, United Kingdom).

Flagellar staining. For detection of flagella in live cells, we performed a previously described method using Alexa Fluor 594-carboxylic acid succinimidyl ester (Molecular Probes), an amino-specific fluorescent dye (37). Briefly, overnight cultures of bacteria grown in LB broth at 37°C and 200 rpm were diluted 1/100 in fresh medium and grown under the same conditions to mid-log phase (OD_{600} = 0.4 to 0.6). The protocol was then followed exactly as described previously (37). The samples were visualized on an Olympus FV300 confocal laser scanning microscope with Fluoview software 4.3, a 100× oil immersion objective (numerical aperture [NA] = 1.35), and 543/610-nm excitation/emission wavelengths. A minimum of 10 fields were recorded for each isolate, using an image size of 1,024 by 1,024 pixels in the *x-y* plane, with a pixel size of 70 nm. Images were deconvolved with Huygens Professional software.

Cell lines, media, and growth conditions. The human colon carcinoma Caco-2 cell line was obtained from the American Type Culture Collection (ATCC). The cells were maintained in minimal essential medium with Earle's salts (high glucose; 4.5 g/liter) (PAA Laboratories) supplemented with 2 mM L-glutamine and 20% fetal calf serum (FCS) at 37°C in 5% CO_2, at up to 80% confluence.

The Caco-Rumbo reporter cell line consists of the Caco-2 cell line stably transfected with a plasmid harboring the luciferase gene under the control of the human *CCL20* promoter (38) and was kindly provided by J. C. Sirard (Université de Lille Nord, France). It was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories) supplemented with 10% FCS, 1 mM sodium pyruvate, 1× nonessential amino acids, and 0.7 mg/ml G418 (Invitrogen) at 37°C in 5% CO_2.

Analysis of the nucleotide sequence of *fliC* and its promoter region. To analyze the nucleotide sequence of *fliC* and its promoter region, we purified genomic DNA from overnight bacterial cultures grown in LB by using a DNeasy Blood and Tissue kit (Qiagen). One hundred nanograms of this DNA was used as the template to PCR amplify a 1,713-bp region comprising the entire *fliC* coding sequence, as well as 152 bp upstream from the start codon and 42 bp downstream from the stop codon, using primers fliC1 and fliC4 (Table 3), and the resulting amplicon was sequenced using primers fliC1, fliC2, fliC3, and fliC4.

Quantitative real-time PCR (qRT-PCR). For bacterial mRNA quantifications, strains were grown to mid-log phase and total RNA was extracted using an RNeasy minikit (Qiagen), with a pretreatment with RNAprotect bacterial reagent (Qiagen). One microgram of this RNA was

treated with DNase (Invitrogen) and reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) and random primers in a 20-μl reaction mixture, and 2 μl of a 1/32 dilution of this reaction mixture was used for real-time PCR using Sybr green (QuantiTect; Qiagen) in a Corbett thermocycler. Primer sequences used are presented in Table 3. The cycling program was as follows: 15 min at 95°C and 45 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 72°C. For the analysis, we used the comparative threshold cycle (C_T) method for relative mRNA quantitation (39). Briefly, the C_T obtained for the *fliC* or *fliA* gene was normalized to the C_T obtained for *icdA* in each sample, giving the ΔC_T ($\Delta C_T = C_T$ for *fliC* or *fliA* − C_T for *icdA*). The corresponding $2^{-\Delta CT}$ value was calculated for each strain, and the $2^{-\Delta CT}$ values were compared to the $2^{-\Delta CT}$ value obtained for an arbitrary selected motile reference strain (SDu5) to obtain the fold variation in mRNA abundance of the sample relative to the reference (fold variation = $2^{-\Delta CT}$ for sample/$2^{-\Delta CT}$ for reference). Each isolate was assayed in triplicate. Non-reverse-transcribed controls rendered no detectable C_T values or were amplified at least 10 cycles later than the corresponding reverse-transcribed samples.

For Caco-2 mRNA quantifications, cells were infected with log-phase bacteria at a multiplicity of infection (MOI) of ~30:1, the plates were centrifuged for 5 min at 200 × *g*, and invasion was allowed to proceed for 1 h at 37°C in 5% CO_2. After three PBS washes, gentamicin (100 μg/ml)-containing medium was added for 1.5 h. The antibiotic concentration was then changed to 10 μg/ml, and the cells were incubated for an additional 1.5 h. At 3 h postinfection, the cells were gently washed 3 times with prewarmed PBS and resuspended in TRIzol (Invitrogen) for extraction of total RNA. After reverse transcription with random hexamers and real-time PCR using specific primers, C_T values were normalized to the values for the 18S rRNA gene and referred to values for uninfected cells. Total RNA extraction, reverse transcription, and qRT-PCR were carried out as previously reported (36).

For mRNA quantification in the ceca of infected mice, fractions of the middle cecum were immediately removed after sacrifice, embedded in TRIzol (Invitrogen), and stored at −80°C. For total RNA extraction, the samples were homogenized using an Ultraturrax instrument, and then the protocol indicated by the manufacturer was followed. One microgram of the resulting RNA was treated with DNase and reverse transcribed using MMLV reverse transcriptase as described above. Two microliters of a 1/5 dilution of this reaction mixture was used for real-time PCR using Sybr green (QuantiTect; Qiagen) in an ABI 7400 thermocycler (Applied Biosystems). Primer sequences used are shown in Table 3; the final concentration of primers in the reaction mixture was 0.9 μM. The cycling program was as follows: 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. We applied the C_T method for relative mRNA quantitation using the β-actin housekeeping gene, and the mRNA levels for each group of infected mice were referred to the levels obtained for the uninfected (streptomycin-pretreated) group. The experiment was done two times, using five animals per group each time.

Luciferase assays. The Caco-Rumbo cell line was stimulated with log-phase bacteria at an MOI of ~100 to 200:1, and the infection was allowed to proceed similarly to that described for Caco-2 mRNA measurements. At 6 h p.i., the luciferase activity in cell extracts was measured using the One Glo luciferase assay (Promega) and a Luminoskan Ascent microplate luminometer (Thermo Scientific). Purified FliC (1.0 μg/ml) from *S.* Typhimurium, generously provided by J. C. Sirard, was used as a positive control. The luminescence obtained for the stimulated cells was normalized to the luminescence obtained for the unstimulated cells, with the latter value set to 1. Each isolate was assayed in quadruplicate.

Animal experiments. Animal experiments were performed as described by Barthel et al. (40). Briefly, groups of five 6- to 8-week-old female C57BL/6 mice (provided by the National Division of Veterinary Laboratories, Uruguay) were pretreated with 25 mg of streptomycin 24 h prior to infection with ~5×10^7 CFU of the indicated bacterial strain. At 24 h p.i., mice were sacrificed, and fractions of the distal cecum were embedded in OCT compound (Sakura), immediately frozen in liquid nitrogen, and stored at −80°C. Cecal pathology was evaluated in hematoxylin and eosin (H&E)-stained cryosections (5 to 8 μm thick), in a blinded manner, by following exactly the histopathological score scheme described by Stecher et al. (21). The parameters evaluated were destruction of the epithelial cell layer, submucosal edema, reduction in number of goblet cells, and infiltration of polymorphonuclear (PMN) cells. In addition, fractions of the middle cecum were immediately removed after sacrifice, embedded in TRIzol (Invitrogen), and stored at −80°C for subsequent total RNA extraction and qRT-PCR analysis as described above. Bacterial loads in ceca and spleens were analyzed by homogenizing the organ contents in PBS containing 0.5% Tergitol and plating appropriate dilutions on MacConkey agar plates containing 50 μg/ml streptomycin.

Experiments with animals were performed according to national guidelines for animal experimentation that meet the international guiding principles for biomedical research involving animals, and all protocols were approved by the university ethics committee.

Statistical analysis. For analysis of differences in motility, transcriptional responses to infection of Caco-2 cells, and *fliC* mRNA levels, one-way analysis of variance (ANOVA) with Dunnet's multiple-comparison posttest was used (GraphPad Prism 4.0 software), with P values of <0.05 considered to be statistically significant. For analysis of differences in transcriptional responses to infection of mice, we used the Mann-Whitney U test (GraphPad Prism 4.0 software), with P values (two-tailed) of <0.05 considered to be statistically significant.

RESULTS

Motility characterization of *S.* Dublin clinical isolates and determination of flagellar expression. Ten *S.* Dublin isolates recovered from human sources in Uruguay from 1995 to 2011 were evaluated (Table 1). These were all *S.* Dublin human isolates available in the two national *Salmonella* collections.

Four of the 10 analyzed isolates were nonmotile (Fig. 1A), and all of these were isolated from blood. In order to evaluate flagellation, we performed fluorescence microscopic analysis of two selected isolates (SDu3 and SDu5, which are nonmotile and motile, respectively) stained with an amino-specific fluorescent dye. The peritrichous flagellar filaments of isolate SDu5 were readily visualized, while no filaments at all were observed in SDu3 (Fig. 1B). Furthermore, Western blotting revealed undetectable levels of flagellin in total bacterial extracts of the nonmotile isolates (Fig. 1C), indicating that the absence of flagella was due to reduced flagellin synthesis or increased degradation, not to defects in export of subunits or in filament assembly. To gain further information about this phenotype, we quantified *fliC* mRNA levels by qRT-PCR analysis of a selected set of isolates (SDu1 to SDu7). The results indicated that the levels of *fliC* mRNA were significantly reduced in the nonmotile isolates compared to the motile ones (about 50- to 100-fold reductions) (Fig. 1D), which is consistent with the hypothesis of reduced flagellin synthesis. Interestingly, the complete nucleotide sequences of *fliC* and its promoter region in isolates SDu1 to SDu7 were identical (data not shown). We also quantified the mRNA levels for *fliA*, coding for the sigma factor responsible for transcription of flagellar class III genes (which include *fliC* [41]), in isolates SDu1 to SDu7 (Fig. 1D). There were no significant differences among isolates, suggesting that the repression occurred at the last level of the flagellar regulatory hierarchy.

To investigate the reversibility of the nonmotile phenotype, we performed successive daily passages of the SDu3 isolate in semisolid agar, but this strain did not recover motility, at least after 12 passages, suggesting an irreversible mechanism.

In summary, all these results indicate that the absence of motility observed in *S.* Dublin blood isolates is due to a significant

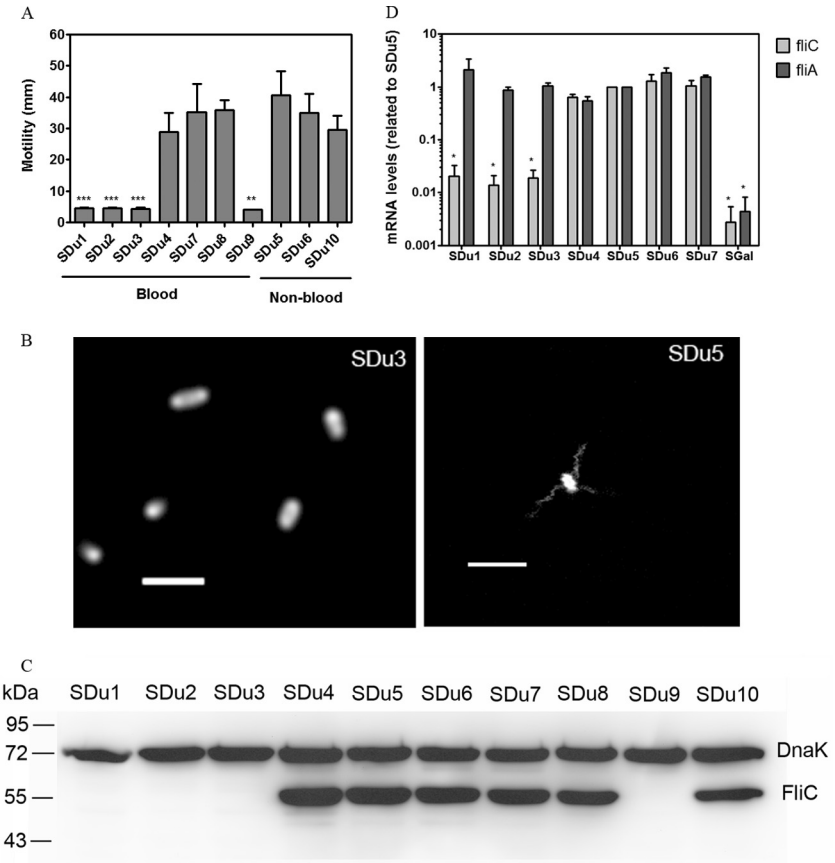

FIG 1 (A) Motility measurement of *S.* Dublin isolates. The diameter of the halo of growth (in mm) after 6 h at 37°C is plotted for each isolate. Results (means and standard deviations [SD]) from three independent experiments are shown. Asterisks indicate significant differences relative to SDu5. **, $P < 0.01$; ***, $P < 0.001$. (B) Fluorescence labeling of flagellar filaments in live cells of isolates SDu3 and SDu5. Bars, 2 μm. (C) Western blot analysis of total protein extracts of *S.* Dublin isolates grown in LB to mid-log phase, using anti-flagellin antibody. Detection of DnaK was used to verify equal loading of samples. Sizes of molecular mass markers are indicated in kDa. (D) *fliC* and *fliA* mRNA level quantification in *S.* Dublin isolates grown in LB to mid-log phase. Results (means and standard errors [SE]) from three independent experiments are shown. *, significant difference relative to SDu5 ($P < 0.05$). SGal is a strain of the aflagellate serovar *S.* Gallinarum.

reduction in *fliC* expression which results in impaired synthesis of flagellin and, consequently, a lack of flagellar filaments at the bacterial surface.

***In vitro* evaluation of the proinflammatory properties of *S.* Dublin clinical isolates.** Since *Salmonella* flagellin is regarded as the major proinflammatory determinant in epithelial cells (28, 42), and because all four aflagellate strains were isolated from invasive infections, we hypothesized that the lack of flagella would impair the proinflammatory properties of *S.* Dublin on the gut epithelium, therefore promoting its systemic dissemination. First, we sought to determine if the different *S.* Dublin isolates would trigger different proinflammatory responses in model human epithelia. Thus, we quantified the mRNA levels for *IL-8* and *CCL20* in cultured intestinal epithelial Caco-2 cells infected with four selected *S.* Dublin isolates (two aflagellate and two flagellated isolates) and compared them to the levels in uninfected cells. While the two flagellated isolates elicited transcriptional induction of *IL-8* and *CCL20* in Caco-2 cells, the two aflagellate isolates failed to do so (Fig. 2A). We further analyzed the induction of *CCL20* expression in Caco-2 cells in response to infection by all *S.* Dublin isolates by using a CCL20-luciferase reporter cell line. As expected, a *fliC* knockout mutant triggered no luciferase activity (Fig. 2B). A perfect correlation between the presence of flagellin in *S.* Dublin isolates and upregulation of expression from the *CCL20* promoter was found (compare Fig. 2B with Fig. 1C), suggesting that repression of *fliC* expression in aflagellate *S.* Dublin isolates is maintained during interaction with epithelial cells. Quantification of *fliC* mRNA levels in *S.* Dublin isolates incubated in the presence of Caco-2 cells by qRT-PCR confirmed this suggestion (data not shown).

Evaluation of the proinflammatory properties of *S.* Dublin clinical isolates *in vivo*. Results of *in vitro* studies showed a correlation between FliC production and induction of the intestinal epithelial proinflammatory gene program and indicated that the lack of *fliC* expression observed when *S.* Dublin isolates were grown in LB broth remained in the presence of epithelial cells. To evaluate if these phenotypes were reproduced *in vivo*, we analyzed proinflammatory gene expression and histological pathology in the ceca of streptomycin-pretreated mice 24 h after oral infection with two selected *S.* Dublin isolates (one nonflagellated and one flagellated isolate) and compared the results to those for mock-infected animals. We decided to analyze the ceca of streptomycin-pretreated mice because severe pathological inflammatory changes are restricted to this organ (40). We selected the time point of 24 h

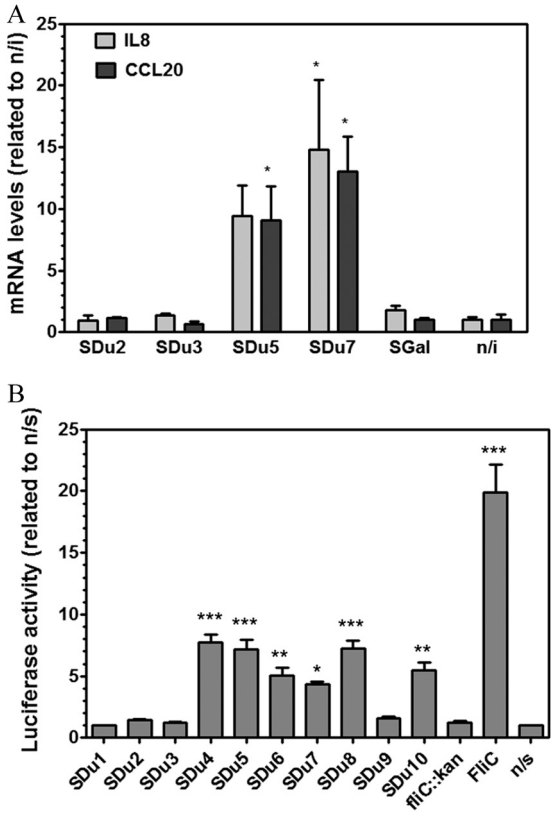

FIG 2 (A) Analysis of the Caco-2 transcriptional response to infection by *S.* Dublin flagellated or aflagellate isolates. Caco-2 cells were infected with isolate SDu2, SDu3, SDu5, or SDu7, and at 3 h p.i., the levels of mRNA transcripts for CCL20 and IL-8 were measured by qRT-PCR. SGal is a strain of the aflagellate serovar S. Gallinarum. n/i, noninfected cells. *, statistical difference relative to noninfected cells ($P < 0.05$). Means and SE for two independent experiments are shown. (B) Analysis of Caco-2 transcriptional response (*CCL20* expression) to infection by all *S.* Dublin clinical isolates. Purified *S.* Typhimurium flagellin (FliC) and an SDu5 derivative *fliC* knockout mutant (*fliC*::*kan*) were used as positive and negative controls, respectively; induction was performed for 6 h. Luciferase activity was determined and normalized to the activity obtained for the nonstimulated control (n/s), with this value considered to be 1. Means and SE for one representative experiment performed in quadruplicate are shown. Asterisks indicate significant differences relative to the nonstimulated control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

postinfection because it has been shown that the differences in pathology caused by a nonflagellated mutant of *S.* Typhimurium compared to the wild type are more pronounced at early times p.i. (21). Both *S.* Dublin isolates efficiently colonized the cecum within 24 h p.i. (for SDu3, $1.97 \times 10^9 \pm 5.14 \times 10^8$ CFU/g of cecum content; and for SDu5, $1.32 \times 10^9 \pm 3.24 \times 10^8$ CFU/g of cecum content), and no statistically significant differences in bacterial numbers were observed between the isolates. Mice infected with the flagellated isolate SDu5 showed a dramatic and statistically significant increase in cecal mRNA levels for IL-17 (70-fold), keratinocyte-derived chemokine (encoded by *Kc*, the murine homologue of human *IL-8*) (56-fold), tumor necrosis factor alpha (TNF-α) (27-fold), and gamma interferon (IFN-γ) (470-fold) compared to mock-infected animals (Fig. 3A). Infection with the aflagellate isolate SDu3 also elicited significant induction of expression of IL-17 (2-fold), Kc (4-fold), TNF-α (3.6-fold), and IFN-γ (15-fold) compared to the case in mock-infected mice, indicating that despite the absence of flagella and motility, the SDu3 isolate was still able to elicit an innate immune response. However, the increases in the cecal mRNA levels for all four cytokines were significantly lower than those elicited by infection with SDu5. SDu3 bacteria recovered from the cecum contents of infected mice were still devoid of motility (data not shown), supporting the hypothesis that the aflagellate phenotype is maintained during interaction with host tissues. In contrast to the observation in cell culture, Mip-3a (*Ccl20*) expression was not significantly induced in the ceca of streptomycin-pretreated mice infected with either *S.* Dublin isolate (Fig. 3A). Although this may be considered surprising, because it is postulated that induction of CCL20 production may contribute to orchestrating host responses during *Salmonella* infection through dendritic cell recruitment to the follicle-associated epithelium, similar results were indeed previously reported by Winter et al. (32).

Other genes involved in defense responses, such as those encoding antimicrobial peptides and proteins, have also been reported to be induced in the murine intestine upon *S.* Typhimurium infection (43). Thus, we also quantified the mRNA levels for two antimicrobial proteins involved in iron and zinc deprivation, i.e., lipocalin-2 (encoded by *Lcn2*) and calprotectin (one of whose subunits is encoded by *S100.a9*), respectively. Transcript levels for lipocalin-2 were significantly upregulated in the ceca of mice infected with the flagellated isolate (29-fold) compared to those of uninfected mice, whereas those animals infected with the aflagellate isolate did not show significant upregulation of this antimicrobial protein (Fig. 3B). Concerning S100.A9, its expression was significantly induced in mice infected with both isolates relative to that in mock-infected animals, but the induction was significantly lower with SDu3 (22-fold) than with SDu5 (1,441-fold).

Cecal pathology in *S.* Dublin-infected animals was also analyzed by scoring the signs of inflammation in H&E-stained cryosections. Despite the similar colonization levels, mice infected with SDu5 developed a severe colitis at 24 h p.i., with high levels of PMN cell recruitment, epithelial damage, and submucosal edema, whereas the ceca of SDu3-infected mice showed milder signs of inflammation (Fig. 4A and B). Taken together, these results suggest that lack of flagella results in *S.* Dublin isolates still being able to trigger intestinal proinflammatory and antimicrobial responses *in vivo*, but this ability is severely impaired compared to the responses induced by flagellated isolates.

In order to verify if flagellation was the underlying cause of the observed differences in mucosal innate immune responses elicited by isolates SDu5 and SDu3, we constructed an isogenic mutant of SDu5 carrying *fliC* inactivated by a kanamycin cassette and tested it in the mouse model of colitis. Since *S.* Dublin has monophasic flagella, inactivation of the *fliC* gene renders it a completely aflagellate strain. Similar to the previously tested isolates, the mutant strain efficiently colonized the ceca of infected mice within 24 h p.i. ($1.09 \times 10^9 \pm 2.86 \times 10^8$ CFU/g of cecum content). As shown in Fig. 3A and B, the aflagellate SDu5 mutant induced significantly less cecal proinflammatory and antimicrobial gene expression than the parental strain. Moreover, there were no significant differences between cecal mRNA levels for Kc, IFN-γ, Lcn2, and S100.A9 in mice infected with SDu5 *fliC*::*kan* and those infected with SDu3, indicating that the aflagellate phenotype results in impaired proinflammatory properties regardless of the genetic background. In addition, the ceca of mice infected with the SDu5 *fliC*::*kan* mutant revealed no significant pathological

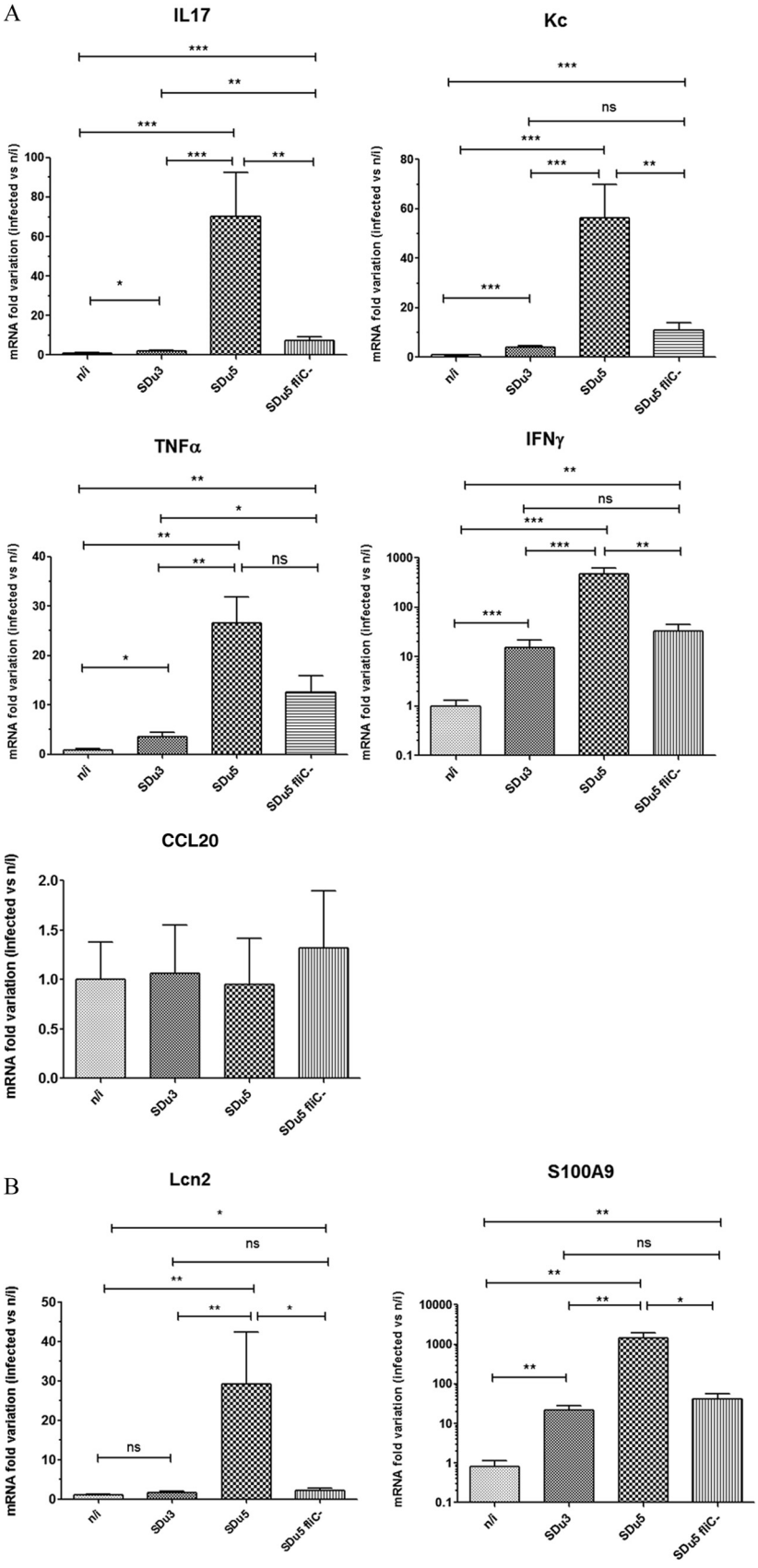

FIG 3 mRNA variation at 24 h p.i. in the cecal mucosa of mice infected with *S.* Dublin, determined by qRT-PCR. Values are expressed as fold changes of mRNA levels for the indicated genes in infected mice relative to their levels in mock-infected animals. Note that the *y* axes of the IFN-γ and S100.A9 graphs show $\log_{10}$ values. Means and SE for two independent experiments, with five animals per group in each experiment, are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant. (A) mRNA quantification of genes coding for proinflammatory cytokines and chemokines. (B) mRNA quantification of genes coding for antimicrobial proteins.

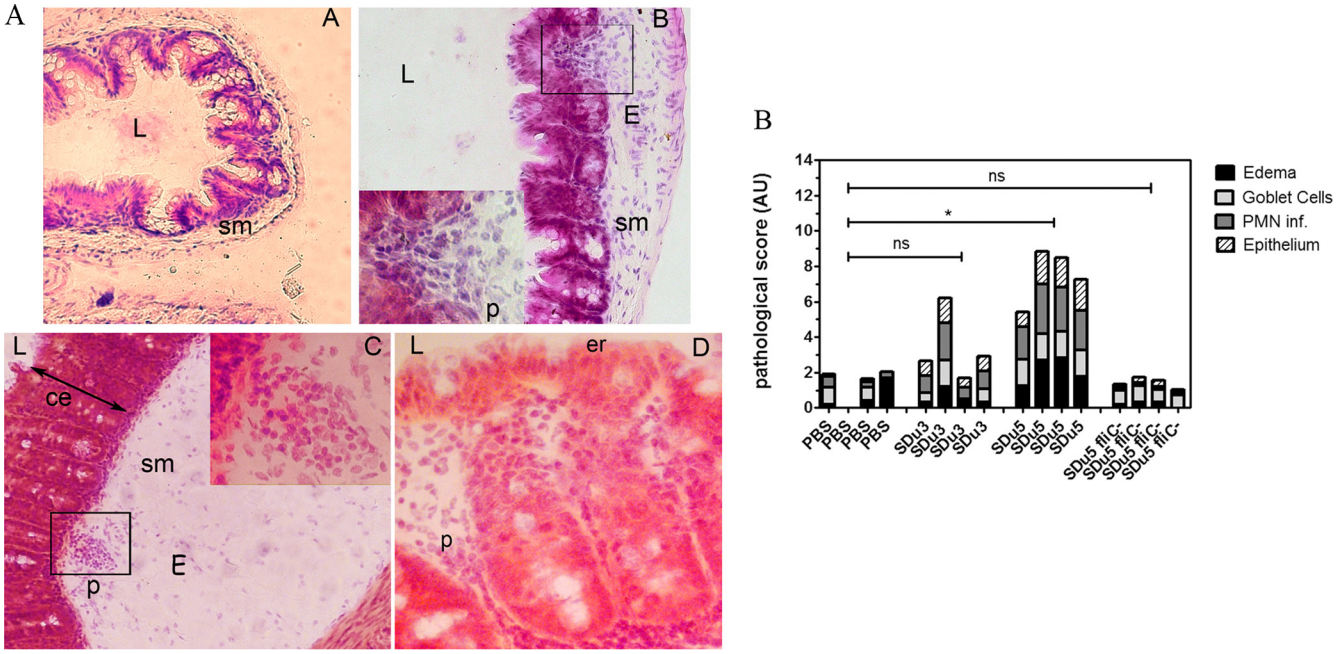

FIG 4 Cecal pathology in H&E-stained tissue cryosections of streptomycin-pretreated *S.* Dublin-infected mice at 24 h p.i. (A) Representative histology of the cecum of a mock-infected mouse (A), a mouse infected with SDu3 (B), and a mouse infected with SDu5 (C and D). L, intestinal lumen; sm, submucosa; E, edema; p, PMN cell; ce, crypt elongation; er, erosion of the epithelial layer. Note the larger extent of edema in the submucosa, as well as PMN cell infiltration and epithelial erosion, in the cecum of the SDu5-infected mouse than in that of the mock- or SDu3-infected mouse. Magnification, ×200 (A to C) or ×400 (D and insets of parts B and C). (B) Histological scores for changes in the cecum. Tissue cryosections were scored for edema in the submucosa (black bars), reduction in the number of goblet cells (light gray bars), PMN cell infiltration (dark gray bars), and desquamation and erosion of the epithelial monolayer (dashed bars). Values are expressed as stacked vertical bars; each bar corresponds to one animal. PBS, mock-infected mice. The results of statistical analysis of the total score value (the sum of the separate scores) for each isolate- infected group relative to that of the mock-infected group are shown (Mann-Whitney test). *, $P < 0.05$; ns, not statistically significant.

changes compared to SDu3- and mock-infected mice (Fig. 4B). These results demonstrate that flagellation is indeed the major property underlying the differences in the ability of *S.* Dublin isolates to induce mucosal immune responses. However, the SDu5 *fliC*::*kan* mutant and SDu3 elicited significantly different levels of expression of IL-17 and TNF-α (Fig. 3A), suggesting that other factors could also have minor contributions to *S.* Dublin's proinflammatory properties.

Concerning the ability to spread to systemic sites, while slightly larger numbers of SDu5 than SDu3 were recovered from spleens of streptomycin-pretreated mice at day 3 p.i., the differences were not statistically significant (for SDu5, $2.00 \times 10^5 \pm 6.94 \times 10^4$ CFU/organ; and for SDu3, $7.93 \times 10^4 \pm 3.06 \times 10^4$ CFU/organ).

Motility properties of *S.* Dublin isolates from cattle. Since *S.* Dublin is a cattle-adapted serovar, we decided to analyze the presence/absence of flagella in a collection of *S.* Dublin isolates obtained from cattle in Uruguay in order to evaluate whether the aflagellate phenotype is a specific trait of human isolates or is widely distributed in nature. Ten of 15 analyzed isolates, obtained from 1995 through 2004, showed a total absence of motility (Fig. 5A). Western blotting using an anti-FliC antibody indicated that 7 of these 10 nonmotile isolates did not produce flagellin, similar to the case observed in human isolates (Fig. 5B). However, the remaining 3 nonmotile cattle isolates did produce flagellin yet were unable to move.

DISCUSSION

Flagella have classically been regarded as important virulence factors, mainly because they allow bacteria to move toward favorable environments and to colonize host tissues (44). More recently, evidence has indicated that flagella also participate actively in other processes, such as adhesion, biofilm formation, translocation of virulence factors, and modulation of both the innate and adaptive immune systems (44).

The role of flagella in the pathogenesis of *Salmonella* has been investigated extensively, and the results vary depending on the experimental model and serovar involved. It has been reported that *S.* Typhimurium mutants harboring paralyzed flagella, in contrast to aflagellate mutants, are slightly attenuated in the mouse typhoid model upon oral infection but not when the mice are challenged via the intraperitoneal route (45). In another study, an *S.* Typhimurium *flhD* mutant (the master flagellar regulator gene), but not a *fliC fljB* mutant (which lacks flagella but not the flagellar secretion apparatus), was significantly more virulent than the parental strain in the mouse model of typhoid fever (22). Moreover, an *S.* Typhimurium mutant deficient in the anti-sigma factor *flgM* gene, which overproduces flagellin, was attenuated *in vivo* in mice (46). More recently, Lai et al. reported that the attenuation of *flgM*-deficient *Salmonella in vivo* is due to recognition of flagellin by the innate immune system, predominantly by caspase-1-dependent pathways (47).

Using the model of murine colitis, it has been demonstrated

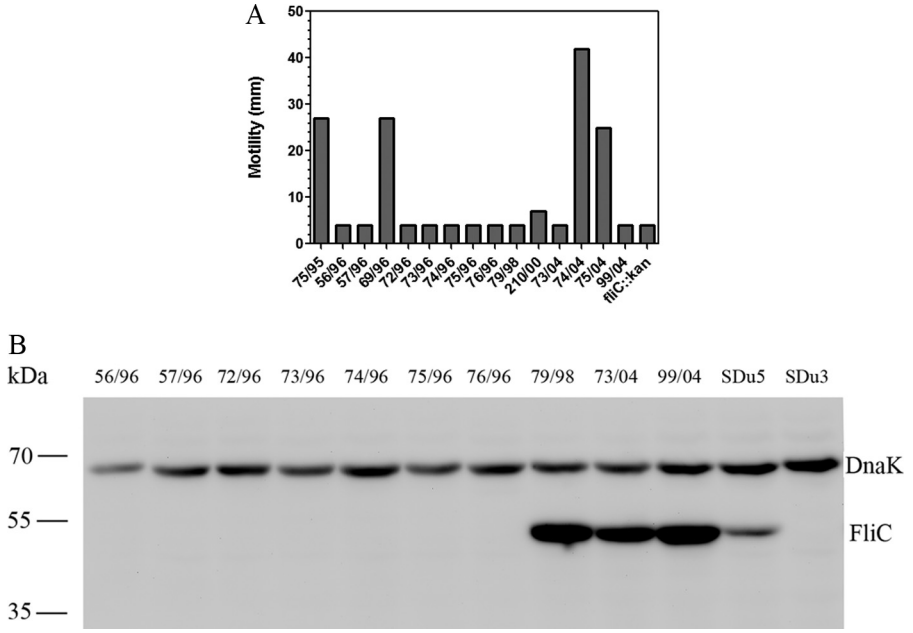

FIG 5 (A) Motility analysis of 15 isolates of *S.* Dublin obtained from cattle. An SDu5 derivative *fliC* knockout mutant (*fliC*::*kan*) was included as a negative control. (B) Western blotting of total protein extracts from the 10 nonmotile *S.* Dublin isolates obtained from cattle, using a specific anti-FliC antibody. Detection of DnaK was used to verify equal loading of samples. Sizes of molecular mass markers are indicated in kDa.

that *S.* Typhimurium flagella contribute to early cecal inflammation, predominantly by providing motility, and promote enhanced bacterial growth by allowing *S.* Typhimurium to benefit from the nutrients released in the inflamed intestine (21, 48). However, the systemic infection which occurred parallel to the enterocolitis did not depend significantly on flagellum function (21). More recently, Winter and colleagues reported that *S.* Typhimurium flagellin pattern recognition contributes to initiating inflammatory responses in the bovine ileal mucosa but not the mouse mucosa (32).

Most studies have been conducted using artificially constructed mutants of *S.* Typhimurium and animal models, mainly murine models, and little is known about the role of flagella in naturally occurring isolates of less prevalent *Salmonella* serovars. In this work, we report that 4 of 10 human isolates and 7 of 15 cattle isolates of *Salmonella enterica* serovar Dublin lack flagella, are therefore nonmotile, and have the antigenic formula 9,12:−:−. These isolates had a *fliC* hypervariable region sequence identical to that of *S.* Dublin strains available in the GenBank database and belonged to the same ST as most *S.* Dublin isolates worldwide (ST10), and they were therefore confirmed as belonging to this serovar. The lack of flagella in human isolates was due to an impaired *fliC* expression that resulted in undetectable levels of FliC protein in total bacterial extracts. However, comparative analysis of the nucleotide sequence of *fliC* and its promoter region between flagellated and nonflagellated isolates revealed no differences, nor did the mRNA levels coding for FliA, the alternate sigma factor responsible for *fliC* transcription. These results suggest that the repression of flagellar gene expression occurs at the last level of the regulatory cascade and may be associated with impairment of the synthesis/stability of FliA or its interaction with FlgM, the corresponding anti-sigma factor (41).

It has been reported that the TviA protein of *S.* Typhi, encoded in SPI-7, is able to repress *fliC* expression under tissue osmolarity conditions (34). We previously analyzed the SDu1 to SDu3 and SDu5 isolates by comparative genomic hybridization using a *Salmonella* pan-array, but no signal for *tviA* was detected in the genomes of these isolates (49). This indicates that a different mechanism mediates *fliC* repression in *S.* Dublin.

In a previous work, we found that among a collection of 266 *S.* Enteritidis field isolates, 27% of the strains were nonmotile (37). This percentage included strains that had structurally normal but paralyzed flagella, meaning that the percentage of aflagellate isolates was even lower. The results presented in this work showing 11 aflagellate strains among 25 field isolates (considering human and cattle isolates together) of *S.* Dublin indicate an unusually high frequency of naturally occurring aflagellate isolates in this *Salmonella* serovar.

Lack of flagella does not seem to be a specific trait of *S.* Dublin field isolates from Uruguay. Selander et al. reported that all but 1 of 114 nonmotile isolates of serotype 1,9,12:−:− recovered from cattle and swine in the United States in 1988 and 1989 were Du1 (the major type of electrophoretic type assigned for *S.* Dublin according to multilocus enzyme electrophoresis [MLEE] analysis) and had been identified provisionally as *S.* Dublin at the National Veterinary Services Laboratories (50). Similarly, Franklin et al. reported that five 1,9,12:−:− *Salmonella* isolates recovered from cattle in Sweden were identified as *S.* Dublin based on restriction plasmid profiles (51). Taken together, these results support the hypothesis of a nonessential role for flagella in the infectious cycle of *S.* Dublin. In line with this, a previous study using a *fliC* transposon mutant showed that flagella play a minor role in extra-animal survival of *S.* Dublin, a feature considered important for zoonotic pathogens (52). More recently, the same group investigated the role of flagella during *S.* Dublin infection in the murine model, using the *fliC* knockout mutant to-

gether with the wild-type parental strain in coinfection assays (53). They found that 4 to 5 days after oral infection, the *fliC* mutant was recovered from the spleens of infected animals in significantly smaller numbers than those of the wild type, but this difference was modest, and no effect was observed after intraperitoneal challenge. The authors concluded that these small differences were sufficient enough to provide the selective force for stable maintenance of the flagellar apparatus in this serovar. To our knowledge, no studies have addressed the role of *S.* Dublin flagella during infection in cattle, the natural host, but results of the present study indicate that 7 of 15 isolates recovered from cattle were indeed nonflagellated. Based on the results of others and the present study, it is tempting to speculate that the flagellar apparatus may be suffering a process of loss in this serovar, maybe because its function is not essential for the infectious cycle or its absence is advantageous under specific circumstances. Notably, the aflagellate phenotype we observed in *S.* Dublin clinical isolates was irreversible in semisolid agar, an environmental condition where the selective pressure is in favor of a motile phenotype. Moreover, it remained during bacterial interaction with cultured epithelial cells and host tissues, suggesting that *fliC* expression is permanently repressed in these isolates and is not due to a regulatory mechanism specific for a particular environmental condition.

Host-restricted and host-adapted serovars seem to have lost gene functions that were previously active in their broad-host-range ancestors, and they are generally more prone to cause invasive disease than ubiquitous serovars (9, 54, 55). Indeed, we have shown by comparative genomics that *S.* Dublin has a larger number of pseudogenes than its close relative but ubiquitous serovar *S.* Enteritidis (49). Moreover, *Salmonella enterica* serovar Gallinarum, a serovar closely related to *S.* Dublin and *S.* Enteritidis that is avian restricted and causes an invasive typhoid-like disease in chickens, is nonflagellated due to pseudogene accumulation in flagellar genes (54). It has been postulated that the lack of flagella may promote *S.* Gallinarum's ability to disseminate systemically by avoiding the flagellin-TLR5-induced proinflammatory responses in the gut (56). Consistent with this hypothesis, Iqbal et al. reported that an aflagellate *S.* Typhimurium *fliM* mutant established early enhanced systemic infection in chickens, as well as inducing less IL-1β mRNA and PMN cell infiltration at the gut during the early stages of infection than the wild type (33). Conversely, a flagellated mutant of *S.* Gallinarum colonized efficiently and caused pathological changes in the ceca of infected birds, as well as producing less mortality than the wild-type strain (57). Similarly, for *S.* Typhi, a molecular mechanism mediated by TviA has been described that represses *fliC* expression when the bacterium encounters human tissue osmolarity, i.e., during interaction with the epithelium, where it could be sensed by pattern recognition receptors (34). In the same study, the authors reported that an *S.* Typhimurium mutant expressing *tviA* (which is normally absent in this serovar) induced lower levels of CXCL-1 chemokine expression than the wild type in human model epithelia and showed more invasiveness in an avian model of infection (34). In the present work, we showed that *S.* Dublin isolates lacking flagella were not able to induce the expression of the CCL20 and IL-8 chemokines in cultured human intestinal epithelial cells, confirming the results obtained by Eaves-Pyles et al. (42) (i.e., similar to what has been described for *S.* Typhimurium, FliC is also the main determinant in *S.* Dublin for induction of these chemokines in intestinal epithelial cells). In addition, a naturally aflagellate isolate as well as a flagellin knockout mutant caused milder pathological changes and significantly less proinflammatory cytokine and antimicrobial gene expression in the ceca of infected mice than a flagellated isolate at early phases of infection. These data indicate, as reported for *S.* Typhimurium (21), that *S.* Dublin flagella are necessary for efficient induction of early cecal inflammation in streptomycin-pretreated mice.

In spite of the remarkably different mucosal responses elicited by SDu3 and SDu5, no significant differences were observed in their ability to colonize the spleens of infected animals. This result is consistent with those obtained by Stecher et al. (21), who reported that a nonflagellated mutant of *S.* Typhimurium caused significantly reduced levels of cecal inflammation but showed no differences in mesenteric lymph node (MLN), liver, and spleen colonization compared to the wild-type strain in the mouse model of colitis. These results strengthen the notion that the streptomycin-pretreated model is not suitable for studying the correlation between intestinal inflammatory responses and translocation of *Salmonella* to internal organs, since NTS rapidly disseminate to the liver and spleen of mice, establishing a systemic infection that resembles typhoid, regardless of the intestinal inflammatory response generated (34).

Interestingly, all aflagellate clinical isolates included in this study were recovered from invasive cases of salmonellosis (i.e., were isolated from blood), whereas all nonblood isolates harbored flagella. Thus, it is tempting to speculate that in *S.* Dublin, the lack of flagella promotes systemic dissemination in the human host through dampening of the gut immune response. Confirmation of such a hypothesis would require analysis of a larger collection of human isolates obtained at a worldwide level. Note that isolates SDu4, SDu7, and SDu8 were isolated from blood yet harbored flagella and were motile. However, these strains were isolated from a drug-addicted individual, a toddler, and an elderly person, respectively, suggesting that in these cases the immune status of the host may have influenced the disease outcome.

In summary, the data presented here indicate that the aflagellate phenotype is frequent among field isolates of *S.* Dublin and renders strains significantly less proinflammatory than flagellated ones. The fact that all aflagellate isolates recovered from humans were isolated from invasive infections suggests that this phenotype may render strains more prone to systemic dissemination in humans. Our data also indicate that the aflagellate phenotype is a consequence of abrogated *fliC* but not *fliA* transcription and is irreversible in the *S.* Dublin blood isolates studied here. The molecular mechanism responsible for such a phenotype is currently being investigated.

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