AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

Curcumin Reduces the Motility of *Salmonella enterica* Serovar Typhimurium by Binding to the Flagella, Thereby Leading to Flagellar Fragility and Shedding

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ABSTRACT

One of the important virulence properties of the pathogen is its ability to travel to a favorable environment, cross the viscous mucus barrier (intestinal barrier for enteric pathogens), and reach the epithelia to initiate pathogenesis with the help of an appendage, like flagella. Nonetheless, flagella can act as an "Achilles heel," revealing the pathogen's presence to the host through the stimulation of innate and adaptive immune responses. We assessed whether curcumin, a dietary polyphenol, could alter the motility of *Salmonella*, a foodborne pathogen. It reduced the motility of *Salmonella enterica* serovar Typhimurium by shortening the length of the flagellar filament (from ~8 μm to ~5 μm) and decreasing its density (4 or 5 flagella/bacterium instead of 8 or 9 flagella/bacterium). Upon curcumin treatment, the percentage of flagellated bacteria declined from ~84% to 59%. However, no change was detected in the expression of the flagellin gene and protein. A fluorescence binding assay demonstrated binding of curcumin to the flagellar filament. This might make the filament fragile, breaking it into smaller fragments. Computational analysis predicted the binding of curcumin, its analogues, and its degraded products to a flagellin molecule at an interface between domains D1 and D2. Site-directed mutagenesis and a fluorescence binding assay confirmed the binding of curcumin to flagellin at residues ASN120, ASP123, ASN163, SER164, ASN173, and GLN175.

IMPORTANCE

This work, to our knowledge the first report of its kind, examines how curcumin targets flagellar density and affects the pathogenesis of bacteria. We found that curcumin does not affect any of the flagellar synthesis genes. Instead, it binds to the flagellum and makes it fragile. It increases the torsional stress on the flagellar filament that then breaks, leaving fewer flagella around the bacteria. Flagella, which are crucial ligands for Toll-like receptor 5, are some of the most important appendages of *Salmonella*. Curcumin is an important component of turmeric, which is a major spice used in Asian cooking. The loss of flagella can, in turn, change the pathogenesis of bacteria, making them more robust and fit in the host.

The flagellum is one of the important organelles for bacterial motility. It is made up of a hook-basal body, a motor complex, and a filament that is composed of around 20,000 flagellin monomers (1). The formation of the functional flagellar apparatus is highly coordinated and governed by environmental cues (2, 3). Flagella contribute to the virulence of the pathogen by aiding motility, adherence, and invasion of the host cells (4, 5). With the help of flagellar and chemotaxis machinery, the pathogen can cross the mucus barrier and gain access to the epithelial layer (6). *Salmonella* clings onto the epithelial cells with the help of flagella and fimbriae, inducing its uptake into the cells (4, 7–10). The flagellin monomers bind to Toll-like receptor 5 (TLR5) on the epithelial cells (4), initiating signal transduction cascades that induce the expression of the proinflammatory genes via NF-κB and mitogen-activated protein kinases (MAPKs), namely, p38, Jun amino-terminal kinase (JNK), extracellular signal-related kinase 1 (ERK1), and ERK2. Flagellin protein also stimulates antibody production and maturation of dendritic cells (DCs), and it promotes antigen presentation by DCs in a MyD88-dependent manner (4). At the gut mucosal site, signaling through TLR5 is crucial, considering the downregulation of lipopolysaccharide (LPS)-mediated signaling via the TLR4 receptor (11). The proinflammatory responses mediated via TLR5 are essential in recruiting macrophages, dendritic cells, and neutrophils at the infection site. These cells sample the bacteria and disseminate them to systemic organs (4). A similar process occurs during infection with *Salmonella enterica* serovars Typhimurium (in mice) and Typhi (in humans), leading to systemic typhoid fever.

Salmonellosis is endemic in Southeast Asian countries. In these countries, turmeric is widely used as a spice. *Salmonella*, being a

Received 26 January 2016 **Accepted** 11 April 2016

Accepted manuscript posted online 18 April 2016

Citation Marathe SA, Balakrishnan A, Negi VD, Sakorey D, Chandra N, Chakravortty D. 2016. Curcumin reduces the motility of *Salmonella enterica* serovar Typhimurium by binding to the flagella, thereby leading to flagellar fragility and shedding. J Bacteriol 198:1798–1811. doi:10.1128/JB.00092-16.

Editor: A. M. Stock, Rutgers University-Robert Wood Johnson Medical School

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00092-16.

foodborne pathogen, is likely to be exposed to turmeric and hence to its principal component, curcumin. Curcumin is known to modulate the activities of several proteins and the expression of various genes of both the host and the pathogen (12–16). In our study, we have investigated whether curcumin modulates motility, one of the important virulence determinants of *Salmonella*.

MATERIALS AND METHODS

Bacterial strains, chemicals, media, and growth conditions. *S.* Typhimurium strain 12023 with pFPV25.1 and strain 14028 (a kind gift from M. Hensel, Germany) were grown at 37°C in lysogeny broth (LB; HiMedia). The medium was supplemented with 50 μg/ml ampicillin for growing *S.* Typhimurium 12023 with pFPV25.1. *lacZ* transcriptional fusions to *flhD*, *fliL*, and *fliC* or *fljB* in *S.* Typhimurium, a kind gift from Kelly Hughes, University of Utah, were grown in LB containing 50 μg/ml kanamycin. *Escherichia coli* BL21 was grown in LB at 37°C. Solvent (DMSO) for dissolving all the phenolic compounds or curcumin (catalog no. C7727; Sigma) was added to LB wherever mentioned. The phenolic compounds ferulic acid, piperine, and vanillin (Sigma-Aldrich) were added to the medium whenever required.

Mutagenesis of flagellin gene. The DNA sequence that codes for flagellin protein (*fliC* gene) from *S.* Typhimurium 14028 was amplified and cloned into pTrc99A. The cloned *fliC* gene was sequenced to confirm complete identity with the published sequence in the database. The clone was transformed into the *S.* Typhimurium Δ*fliC* mutant to obtain *S.* Typhimurium Δ*fliC*::pTrcfliC. *S.* Typhimurium Δ*fliC*::pTrcfliC was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C, and the whole-cell lysate was probed with anti-FliC antibody (Difco) to confirm the expression of the flagellin protein (see Fig. S1 in the supplemental material). All the mutations were introduced into pTrc99A *fliC* using standard PCR mutagenesis techniques, and mutations were confirmed by sequencing. The mutants generated were the following: (i) pTrc99A *fliC* N120A D123A, (ii) pTrc99A *fliC* N163A S164A, (iii) pTrc99A *fliC* N173A Q175A, and (iv) pTrc99A *fliC* triple mutant (all three mutations were incorporated). For generation of the triple mutant, individual mutations were introduced sequentially.

Swimming and swarming assay. A swimming and swarming assay was performed as described by Kim and Surette (17). Briefly, 2 μl of an overnight grown bacterial culture was spot inoculated at the center of the swim or swarm plates. The plates were dried for 50 min and incubated at 37°C for 6 to 8 h. The plates were imaged, and the diameter of the bacterial growth front was measured using the ImageJ software. The rate of bacterial swimming or swarming was calculated as the growth of a colony per unit of time.

The media used for swimming and swarming assay were (i) swimming medium (LB plus 0.25% agar [bacteriological grade; HiMedia] plus 0.5% glucose), and (ii) swarming medium (LB plus 0.5% agar [bacteriological grade; HiMedia] plus 0.5% glucose).

Curcumin (20 μM), DMSO (volume equivalent to that used for 20 μM curcumin), or the indicated phenolic compounds at different concentrations were added along with the medium (after cooling to 60°C), wherever mentioned.

Immunofluorescence microscopy. The bacteria grown in the presence of curcumin (20 μM) or DMSO were fixed with 2.5% glutaraldehyde and mounted on silane-coated coverslips. The fixed cells were incubated with antiflagellin antibody (1:100 in PBS; Difco) for 1 h. The coverslips were washed twice with PBS, and the cells were incubated with Cy2-conjugated anti-rabbit antibody (1:100 in 2% bovine serum albumin [BSA]; Dianova). The cells were observed under a Carl Zeiss meta 710 microscope, and the images were captured.

Transmission electron microscopy. *S.* Typhimurium cells grown in LB, LB plus 20 μM curcumin, or LB plus DMSO were fixed in 2.5% glutaraldehyde at room temperature for 30 min. One hundred microliters of fixed samples was put onto Parafilm. Formvar-coated nickel grids (3 mm, 200 mesh) were placed on top of the sample drop and left for 1 to 2 min. The grid surface was washed twice with Tris-EDTA (TE) buffer (pH 6.9), blotted dry, and rinsed in distilled water. The bacteria were negatively stained with 2% uranyl acetate solution. The grid surface was washed with TE buffer, air dried, and imaged using a transmission electron microscope (JEM-100 CX II; JEOL).

Enzyme-linked immunosorbent assay for flagellar density on *Salmonella*. The flagellar density of *S.* Typhimurium was determined as described previously (18).

S. Typhimurium grown in the presence or absence of curcumin (20 μM) was pelleted at 10,000 × *g* for 5 min and incubated with rabbit anti-*S.* Typhimurium flagellin antibody (1:100 in 2% BSA; Difco) for 1 h. Bacterial cells were washed with PBS containing 1% BSA by centrifugation at maximum speed, followed by resuspension of the bacterial pellet in PBS. The samples were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2,000; Bangalore Genie) for 1 h. The samples were again washed with PBS, and the peroxidase activity was assessed using 3,3′,5,5′-tetramethylbenzidine as a substrate. The absorbance of the solution was recorded at 450 nm in an enzyme-linked immunosorbent assay (ELISA) microplate reader (ELx 800MS).

Isolation of flagellin and total protein from culture supernatant. (i) Isolation of pure flagellin. Flagellin protein was isolated using a modified protocol from that of Ibrahim et al. (19). Briefly, bacteria were grown in 5 ml of LB at 37°C for 12 h. The bacteria were subcultured at a 1:100 ratio in LB plus 0.5% glucose and incubated at 37°C and 80 rpm for 16 h. The bacteria were harvested by pelleting at 6,000 × *g* for 15 min at 4°C. The pellet obtained from a 1.5-liter culture was resuspended in 5 ml of PBS. The pH of the solution was adjusted to 2 with 1 N HCl. The sample was maintained under shaking conditions for 30 min and then centrifuged at 10,000 × *g* for 15 min. The supernatant thus obtained was spun at 100,000 × *g* for 1 h to pellet any insoluble debris. The pH of the recovered supernatant was adjusted to 7 with 1 N NaOH, and 2.6 M ammonium sulfate was added with vigorous shaking. After overnight incubation at 4°C, the mixture was spun at 14,000 × *g* for 20 min at 4°C. The pellet thus obtained was dissolved in distilled water and the mixture dialyzed against distilled water in the presence of activated charcoal. The water was changed every 1 h for the next 8 h. The solution was stored at −20°C until use. The purity of the sample was assessed by SDS-PAGE, followed by Coomassie brilliant blue staining and mass spectrometry.

In order to obtain purified mutant flagellin protein, *S.* Typhimurium Δ*fliC* was transformed with either pTrc99A *fliC* (wild type [WT]) or the mutant gene. *S.* Typhimurium Δ*fliC*::pTrcfliC was induced with 1 mM IPTG at 30°C. Cells were pelleted 6 h postinduction, and flagellin protein was purified as described above.

(ii) Isolation of total protein from culture supernatant. *S.* Typhimurium was grown in the presence or absence of curcumin for 6 h. The culture was vortexed at 5,000 rpm for 5 min. The cells were pelleted to obtain the culture supernatant. The total protein from the culture supernatant was precipitated using 2.6 M ammonium sulfate. The protein thus obtained was dialyzed using a 10-kDa-cutoff dialysis membrane. The protein solution was heated at 90°C for 10 to 15 min to monomerize the flagellin polymer. The monomerized flagellin was used to treat HEK293 cells.

NF-κB luciferase assay. A total of 5×10^4 HEK293 cells were seeded into a 24-well plate. The medium in each well was replaced with 700 μl of transfection medium containing Opti-MEM (Gibco), polyethylenimine, and 250 ng of each plasmid, pNF-κB-TA-Luc and pCMVβ-gal (both plasmids were a kind gift from Takashi Yokochi, Aichi Medical University, Japan). The final concentration of each plasmid was 50 ng/l0^4 cells. The cells were incubated at 37°C in humidified air (with 5% CO_2) for 6 h. The transfection medium was replaced with complete medium (RPMI with 10% serum), and the cells were further incubated for 36 h. The transfected cells were induced with a concentration of 250 ng/ml of total protein supernatant (heated at 90°C for 10 min) obtained from *S.* Typhimurium grown in the presence of either curcumin (20 μM) or DMSO for 6 h. The luciferase assay was performed 12 h postinduction, in accordance with the

manufacturer's instruction (Promega). A β-galactosidase assay was performed by measuring the hydrolysis of the chromogenic substrate *O*-nitrophenyl-β-D-galactoside to normalize for the transfection efficiency.

Immunoblotting. *S.* Typhimurium culture was grown in the presence of curcumin, phenolic compounds, or DMSO for 6 h. Bacterial cultures were either first vortexed for 3 min and then pelleted or directly pelleted at 10,000 × *g* for 5 min at 4°C. The supernatants from curcumin-treated and untreated samples were processed in one of the following ways and processed for immunoblot analysis: (i) concentrated under a vacuum; (ii) filtered using a 0.2-μm-pore-size filter and concentrated under a vacuum; (iii) acidified to pH 2 and concentrated under a vacuum; or (iv) acidified to pH 2, filtered using a 0.2-μm-pore-size filter, and concentrated under a vacuum.

The whole-bacterium culture was lysed using a lysis solution (0.01 M Tris-HCl, 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol [DTT], 10% [vol/vol] glycerol, 5% [vol/vol] NP-40). Concentrated culture supernatant (400 μg) and the lysed whole-bacterium culture (50 μg) were probed with antiflagellin antibody (1:1,000; Difco). A portion of the gel not used for transfer was stained using the silver staining protocol and used as the loading control.

β-Galactosidase assay. *S.* Typhimurium harboring *lacZ* transcriptional fusions to *flhD*, *fliL*, and *fliC* or *fljB* were used for the assay. The transcriptional activity of each gene was determined by performing a β-galactosidase assay, as described previously (20).

Binding assay. (i) Quenching of tyrosine fluorescence. The change in the intrinsic tyrosine fluorescence was used to estimate the binding affinity of curcumin to flagella. Curcumin (1.5 to 8 μM) or an equivalent volume of DMSO was titrated against a fixed concentration of flagellin protein (4 μM). The solution was excited at 270 nm, and the emission spectrum from 280 to 340 nm was recorded at each curcumin concentration.

(ii) Fluorimetric titration. Various concentrations of curcumin (0.125 to 8 μM) were titrated against a fixed concentration of flagellin (10 μM) in Tris buffer (25 mM [pH 7]) and the curcumin fluorescence estimated at 495 nm by exciting the solution at 425 nm.

(iii) Calculation of inner filter effect. The inner filter effect at a high curcumin concentration was minimized using the following formula (21): $F_{corrected} = F_{observed} \times \text{antilog}\,[(A_{ex} + A_{em})/2]$, where $F_{corrected}$ is the corrected fluorescence intensity, $F_{observed}$ is the observed fluorescence intensity, and A_{ex} and A_{em} are the absorbance of curcumin at excitation and emission wavelengths, respectively.

Agglutination assay. A single isolated bacterial colony (*S.* Typhimurium or *Escherichia coli*) was inoculated in LB and grown at 37°C and 160 rpm for 6 to 7 h. The bacterial cultures were pelleted and resuspended in Tris-HCl buffer (25 mM [pH 7]). These cultures were then incubated in the presence of curcumin (20 μM) or DMSO and antiflagellin antibody (1:200; Difco) for 3 to 5 min. Aliquots of 20 to 50 μl were placed on a slide and observed for agglutination/formation of clumps due to cross-linking of bacteria.

Computational analysis. (i) Preparation of receptor and ligand. The crystal structure of the flagellin monomer, F41 fragment (22), a bacterial flagellar protofilament (Protein Data Bank identifier 1IO1) was obtained at a resolution of 2 Å from RCSB (23). The obtained structure was then prepared for docking by the removal of water molecules and addition of Gasteiger partial charges for adding hydrogens to polar atoms, availed for docking in a .PDBQT format using python script available in the AutoDock Tools package (24, 25). The structure files of curcumin, ferulic acid, vanillin, piperine, and EF-24 were obtained from PubChem in SDF format and translated to the required formats using Open Babel 2.3.0 (26).

(ii) Binding site detection. The possible ligand binding sites were predicted using PocketDepth (27), an in-house geometry-based approach that uses depth-based clustering for the identification of binding sites in proteins. Further support for the predicted sites was obtained by using alternative binding site prediction methods, Q-SiteFinder (28) and SiteHound (29). These two methods are energy-based approaches that use different types of probes to calculate the interaction energy, with SiteHound also including Lennard-Jones and electrostatics energy terms, and LIGSITEcsc (30), which predicts ligand binding sites by using the Connolly surface and degree of conservation of the involved surface residues. The degree of conservation as defined by LIGSITEcsc is the average conservation of all residues within a sphere of a certain radius (8 Å, in this study) of the center of mass of the cluster.

(iii) Sequence analysis. The protein sequences of flagellin from *E. coli* (GenBank accession no. P04949) and *S.* Typhimurium (GenBank accession no. P06179) were retrieved from the UniProtKB (31) database and subjected to a BLASTP search (32) with default BLOSUM62 matrix for pairwise alignment.

(iv) Docking. Autodock4 (AD4) (24) and Vina (33) were employed for docking (22), and the individual parameter files were prepared as required by the program. A 70- by 70- by 70-point grid, centered at the binding site with 0.375-Å and point grid spacing, was used to calculate an atomic affinity map. The conformational space around the binding site was assigned as a cubic grid of 26.6 Å, and the coordinates assigned to the grid center were the same as those used in AD4.

(v) Postdocking analysis. AD4 and Vina generated a docking log file that gives complete information about different binding modes and their corresponding binding free energies of the docked ligand. AD4 gives an additional feature of clustering, which was studied for similarity in binding modes and binding energies. A standalone version of Ligand Protein Contacts (LPC [34]) was used to analyze the interaction profile explaining different kinds of interactions (hydrogen bonds and electrostatic, van der Waals, and hydrophobic interactions) across docked complexes. PyMOL was used for visualization and image construction of the docked complexes (35).

(vi) Multiple-sequence analysis. A database search for sequence homologues of *S.* Typhimurium FliC was carried out in the bacterial genomes of the NR sequence database using the BLASTP server and the BLOSUM62 matrix. An E value of 0.0001 was imposed as the search criterion. BLAST hits having the highest sequence identity, query coverage area, and BLAST score and lowest E value in each species were selected for further analysis. A multiple-sequence alignment was carried out for the hits obtained, using Clustal Omega (36) with default parameters, the results of which were visualized using BoxShade version 3.31, written by K. Hofmann and M. Baron (37).

Statistical analysis. Student's *t* test was used for testing the significance of the differences observed, if any. All analyses were done with the GraphPad Prism (version 5) software.

RESULTS

Curcumin reduced the motility of *Salmonella enterica* serovar Typhimurium. Curcumin (20 μM) decreased the motility of *S.* Typhimurium as visualized under the light microscope. This reduction in motility was confirmed by a swim and swarm assay. There was a significant decrease in bacterial motility, as revealed by the reduction (65 to 80%) in the diameter of the swim and swarm colony of *S.* Typhimurium in the presence of different curcumin concentrations (Fig. 1). Curcumin (20 μM) also reduced the motility of *S.* Typhi strain Ty2 (see Fig. S2A in the supplemental material).

When assessed for cytotoxicity, curcumin (10 to 120 μM) showed no toxic effect on the growth of bacteria (see Fig. S2B in the supplemental material). Thus, curcumin exhibited an apparent effect on the motility of *S.* Typhimurium and not on its growth.

Curcumin decreased flagellar density around *S.* Typhimurium. To address the defect in motility, we investigated curcumin's effect on flagellar machinery. We analyzed the density of flagellar filaments possessed by the bacterium using transmission electron microscopy (TEM) and fluorescence imaging techniques.

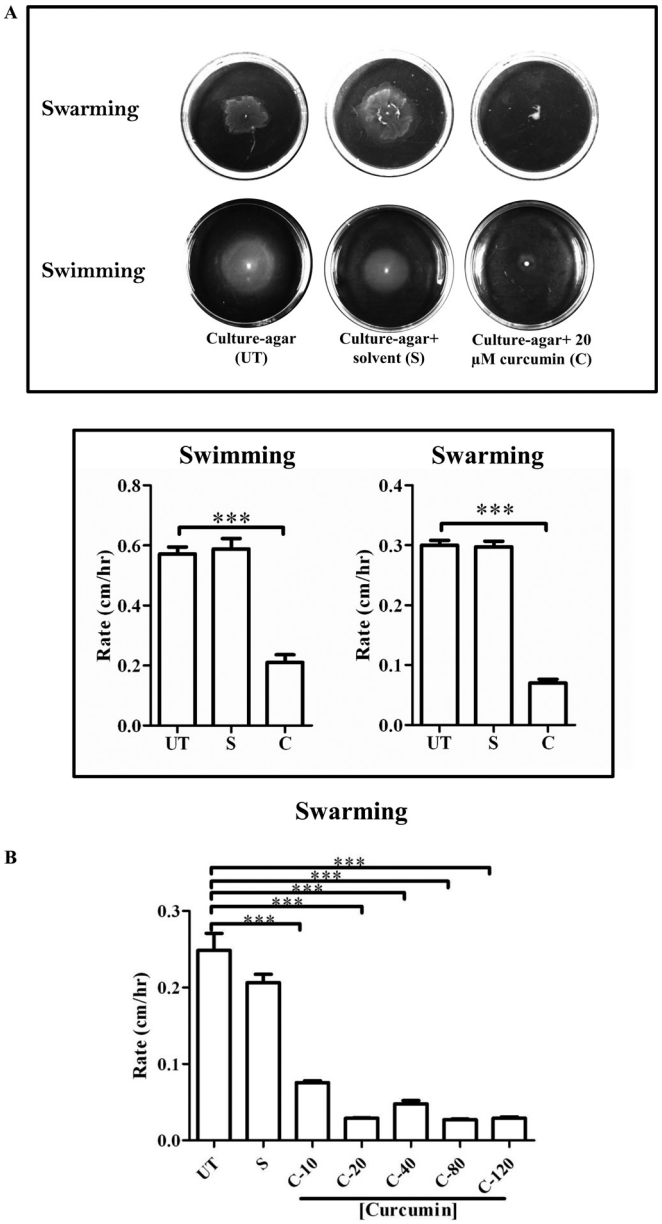

FIG 1 Curcumin reduces swimming and swarming motility of *S.* Typhimurium. (A) *S.* Typhimurium culture plates showing extent of swarming (top) and swimming (bottom) under the indicated conditions after 6 to 7 h of incubation at 37°C. (B) Swarming rate of *S.* Typhimurium under the indicated conditions. The values in the graphs are means and standard errors (SE). UT, swim/swarm agar only; S, swim/swarm agar plus solvent (DMSO); C, swim/swarm agar plus curcumin (numbers refer to curcumin concentrations of 10 to 120 μM). ***, $P < 0.001$.

TEM and fluorescence imaging gave a visual appreciation of the reduction in the filament number around the curcumin-treated (20 μM) bacteria (Fig. 2A). Almost 41% of the curcumin-treated bacteria were aflagellated. The remaining ~59% (flagellated) possessed, on average, five filaments, contrasting with the 8 or 9 filaments possessed by untreated bacteria (Fig. 2A). A modified form of ELISA (see Materials and Methods) that estimates the flagellar density on the bacteria (18) was performed to validate our results. Similar results were obtained (Fig. 2B), confirming the reduction in the number of flagellar filaments upon curcumin treatment.

Curcumin reduced the flagellar density by increasing the shedding of flagellar filament from the bacterial surface. Careful observation of TEM and fluorescence microscopic images revealed detached flagellar filaments in the background of curcumin-treated bacteria (Fig. 2C), indicating that curcumin increased shedding of the flagellar filaments from the bacterial surface. In curcumin-treated samples, the length of the flagellar filaments either attached to or detached from the bacteria (5.37 ± 0.12 μm [mean ± standard error]) was significantly shorter than that for the untreated samples (8.01 ± 0.17 μm) (Fig. 2C). Almost 80% of the filaments in curcumin-treated samples had a length of <6 μm, whereas in curcumin-untreated samples, 80% of them had a length of >6 μm (Fig. 2C), implying that curcumin fragments the flagellar filaments.

Next, we assessed the expression of flagellar genes (temporally divided into three classes, I, II, and III [3]) on curcumin treatment using a reverse transcription-PCR (RT-PCR) and reporter (β-galactosidase) assay. The RT-PCR and reporter assay indicated that there was no effect of curcumin on the expression of the flagellar genes *flhD* (class I gene) and *fliC* and *fljB* (class III genes) (Fig. 3). However, there was an approximately 2-fold increase in the promoter activity of *fliL*, a class II gene (Fig. 3B). *fliL* is the first gene of the operon *fliLMOPQR*, which suggests that the expression of other genes, *viz.*, *fliM*, *fliO*, etc., is also affected by curcumin.

Curcumin made the flagellar filament fragile, thus increasing its shedding from the bacterial surface. Curcumin reduced the number of flagella per bacterium by fragmenting the flagellar filaments without altering the expression of flagellin genes *fliC* and *fljB* (Fig. 2 and 3). To explain these results, we speculated that curcumin might induce some physical changes in the flagella and weaken it, thus leading to its fragmentation. To test this, we applied an external mechanical force in the form of vigorous vortexing and checked for shedding/fragmentation of flagellar filaments. The bacterial cultures grown in the presence and absence of curcumin (20 μM) were vortexed at 5,000 rpm for 5 min to enhance the shedding of flagellar filaments. The amount of flagellin protein in the culture supernatants, bacterial pellet, and whole-cell suspension was estimated by immunoblot analysis. The culture supernatants of curcumin-treated samples had a greater amount of flagellin protein than that of the untreated samples (Fig. 4A). However, the amount of flagellin protein in the whole-cell suspension was unaltered upon curcumin treatment. These results suggest that application of external mechanical force in the presence of curcumin increases the shedding of flagellar filaments (Fig. 4A). Another strategy used was to examine the presence of flagellar fragments in culture supernatant of a curcumin-treated sample. The bacterial cultures were vortexed and the culture supernatants filtered through a 0.2-μm-pore-size filter, with which the monomers or fragments <0.2 μm in length would easily pass through the filter pores. This step would prevent the passage of fragments of >0.2 μm through the filter, unless its orientation is such that it passes through the filter surface lengthwise (filament diameter, ~20 nm, smaller than the filter pore size). Immunoblot analysis revealed that the filtrate obtained from the curcumin-treated samples had a smaller amount of flagellin protein than that of the DMSO-treated samples (Fig. 4B), indicating the presence of flagellar fragments >0.2 μm in length. This also indicates that the

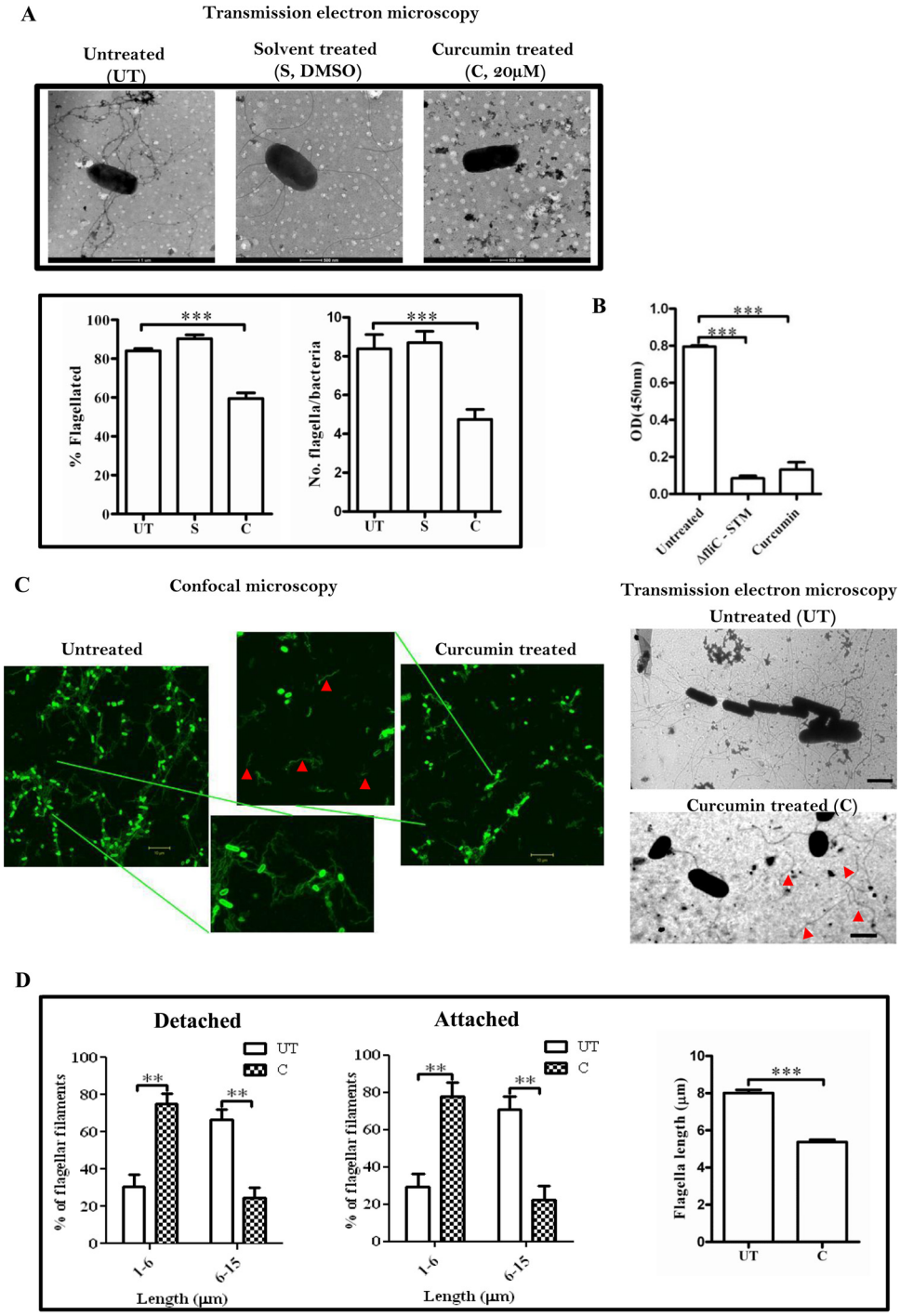

FIG 2 Curcumin reduces the density and length of flagellar filaments around the bacteria. An *S.* Typhimurium overnight culture was subcultured in LB containing curcumin (20 μM) or DMSO at a 1:100 ratio and grown at 37°C and 160 rpm for 6 to 7 h. Bacteria were processed for microscopy and ELISA. (A) TEM images of *S.* Typhimurium showing the presence and absence of flagella under given conditions. (B) ELISA indicating a reduction in flagellar density upon curcumin treatment. The flagellar density of untreated bacteria was considered to be 100%. (C) Confocal microscopy images of *S.* Typhimurium grown under indicated conditions at 37°C for 6 to 7 h. Arrowheads indicate the detached flagellar fragments. (D) Percentages of flagellar filaments with lengths in the ranges of 1 to 6 μM and 6 to 15 μM, calculated using TEM images, under the indicated conditions. The length of flagellar filaments was measured using the ImageJ software. The values in the graphs are means and SE. UT, bacteria grown in LB; S, bacteria grown in LB plus DMSO; C, bacteria grown in LB plus curcumin (20 μM). ***, $P < 0.001$; **, $0.001 \leq P < 0.01$.

flagellin monomers were found in smaller amounts in curcumin-treated samples. This might be due to the spontaneous polymerization of the monomers in the presence of flagellar fragments present in the curcumin-treated cultures (38, 39). The flagellar fragments act as a seed for polymerization, and the monomers become incorporated into a polymer (39). To further confirm the presence of flagellar fragments in curcumin-treated cultures, the culture supernatants were acidified (pH 2) to depolymerize

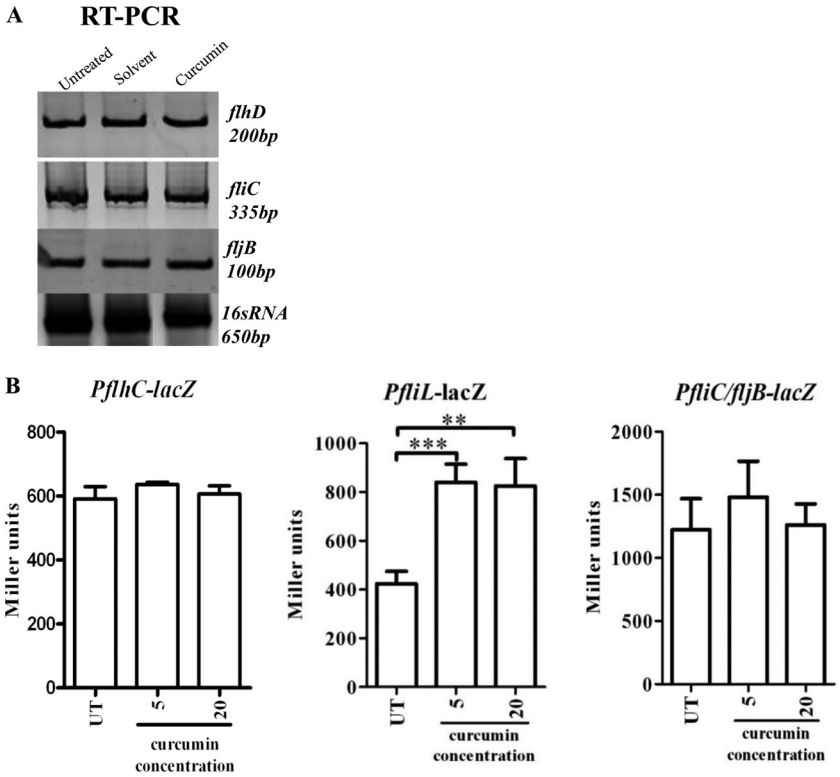

FIG 3 Expression of flagellar genes. An overnight culture of *S.* Typhimurium was subcultured at a 1:100 ratio in LB, LB plus curcumin, or LB plus DMSO. The bacteria were harvested and analyzed for gene expression. (A) Agarose gel electrophoresis of RT-PCR amplicons for the flagellar genes *flhD*, *fliC*, and *fljB* and 16S rRNA under the given conditions. (B) Transcriptional activities of *flhD*, *fliL*, *fliC*, and *fljB* genes under indicated conditions, as analyzed by β-galactosidase assay using respective *lacZ* transcriptional fusions in *S.* Typhimurium. The values in the graphs are means and SE. UT, bacteria grown in LB; S, bacteria grown in LB plus DMSO; C, bacteria grown in LB plus curcumin (20 μM). ***, $P < 0.001$; **, $0.001 \leq P < 0.01$.

the fragments (40) and then passed through the 0.2-μm-pore-size filter. The flagellin monomers thus formed could now pass through the filter. When the filtrates were probed with antiflagellin antibody, the samples of curcumin-treated culture showed higher flagellin content than that in the DMSO-treated control (Fig. 4B).This demonstrates that curcumin-treated bacterial cultures have more flagellar fragments that cannot pass through the filter until they are depolymerized before filtration. Thus, it is clear that curcumin increased the fragmentation and shedding of the flagella from the bacterial surface.

To account for the presence of fragmented flagellar filaments, the culture supernatant from the vortexed sample was spun at 100,000 × *g* for 1 h to pellet the flagellar fragments but not monomers (39) and then probed with antiflagellin antibody. Curcumin-treated samples had a greater amount of flagellin than the DMSO-treated control (Fig. 4C), reaffirming that curcumin treatment leads to the fragmentation of flagellar filaments.

If we assume that curcumin (20 μM) can mechanically modulate the fragmentation of flagellar filaments, transient treatment of bacteria with curcumin might also lead to increased shedding of the flagella. To test this hypothesis, we incubated the bacteria at log phase with curcumin for 10 to 12 min, followed by vigorous vortexing for 5 min, and the amount of flagellar fragments released was analyzed by immunoblotting. The release of fragmented filaments was evident from the greater amount of flagellin protein in the culture supernatant of curcumin-treated samples than that in the DMSO-treated control (Fig. 5A).

Flagellin molecules bind to the TLR5 receptor on the host cell surface, triggering a signal transduction cascade via activation of NF-κB. A functional assay was performed to validate the shedding of flagellar filaments from the bacteria, in which HEK293 cells (transfected with pNF-κB-TA-Luc) were treated with the protein isolated from the culture supernatants of curcumin-treated and untreated cultures. The assay was performed with HEK293 cells, as these cells do not express any TLRs except TLR5 (41, 42). HEK293 cells transfected with pNF-κB-TA-Luc were induced with 250 ng/ml total protein (heated at 90°C for 10 min) from the culture supernatants of curcumin-treated (20 μM) or untreated *S.* Typhimurium cells and assayed for the activation of NF-κB. The NF-κB activity (Fig. 5B) was significantly higher in the cells induced with the protein from the culture supernatant of curcumin-treated *S.* Typhimurium than in the DMSO-treated control. This corroborated our result that curcumin treatment increased the shedding of flagella from the bacteria.

Curcumin-induced expression of *fliL* is dependent on the presence of flagella on the bacteria. It is believed that the FliL protein of *Salmonella* is required for protection of the flagellar rod against the increased torsional stress experienced by the flagella on the swarm agar (43). In our study, we observed increased *fliL* expression (Fig. 3B), along with the increased shedding of flagella in the presence of curcumin (Fig. 2). To test whether the increased expression of *fliL* could be linked to curcumin-induced flagellar fragmentation, we assessed the expression of *fliL* in aflagellated bacteria (*Salmonella* Typhimurium Δ*fliC*) by a reporter (β-galac-

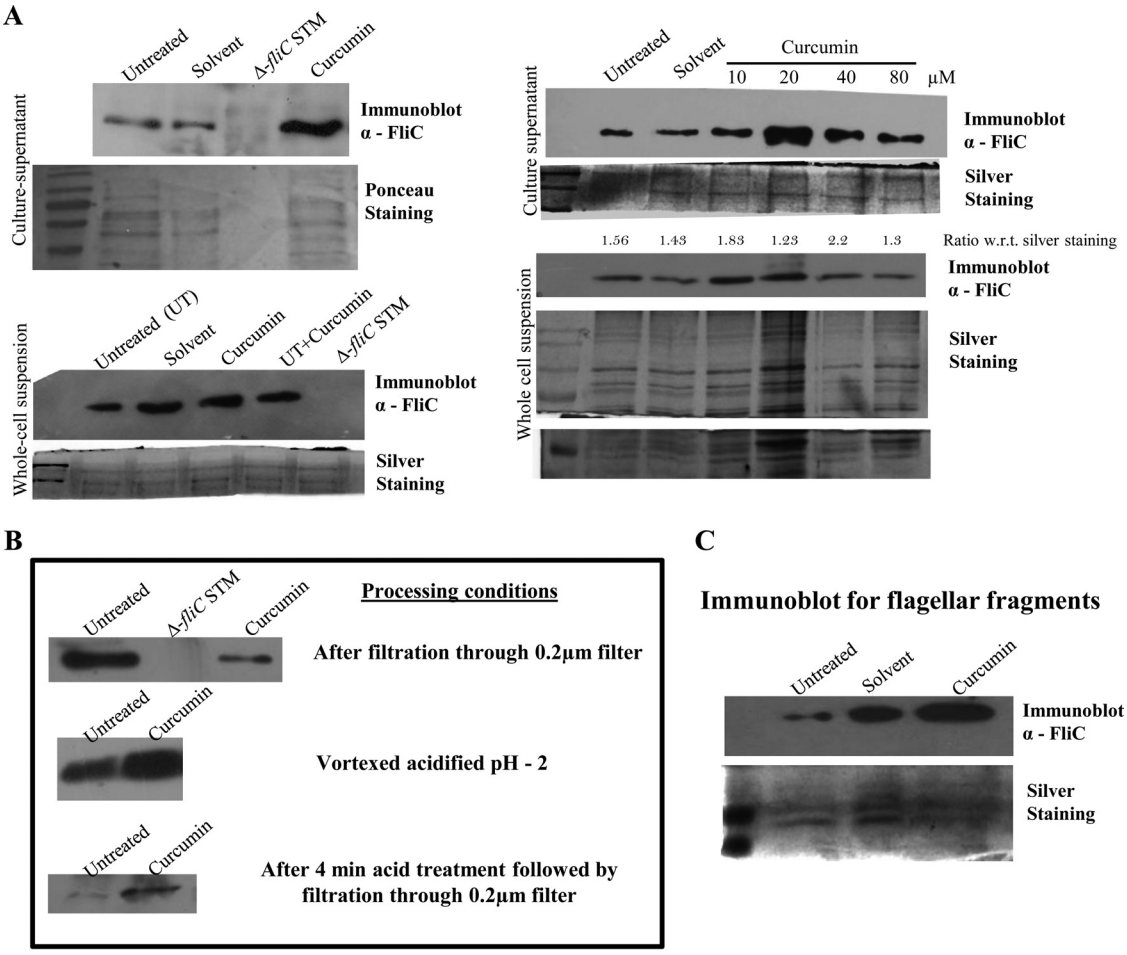

FIG 4 Curcumin increased the shedding of flagellar filaments from the bacteria in the culture supernatant. An *S.* Typhimurium overnight culture was subcultured in LB containing curcumin (20 μm) or DMSO (solvent control) at a 1:100 ratio and grown at 37°C under shaking conditions for 6 h. At 6 h posttreatment, cells were subjected to vortexing at 5,000 rpm for 5 min. (A) Bacteria were pelleted, and flagellin levels in culture supernatant and whole-cell lysate were determined by immunoblotting. Ratio w.r.t. silver staining, ratio of immunoblot intensity with respect to silver staining. (B) Flagellin levels in culture supernatant were assessed after the supernatant was filtered using 0.2-μm-pore-size filters (top), acidified at pH 2 for 4 min (middle), or acidified at pH 2 for 4 min and then filtered using a 0.2-μm-pore-size filter (bottom). (C) Flagellar fragments in culture supernatants were pelleted by ultracentrifugation, and flagellin levels in the pellet were detected by an immunoblot assay against flagellin.

tosidase) assay. We observed no change in the *fliL* promoter activity (β-galactosidase) of the aflagellated strain upon exposure to curcumin (Fig. 6), indicating that curcumin-induced fragmentation could trigger the expression of *fliL*.

Curcumin physically binds to flagella. All the above-mentioned results hint at a physical interaction between curcumin and flagella. To assess the binding of curcumin to flagella, a fluorescence binding assay was performed that measures the change in protein fluorescence on conformational change due to ligand binding. Flagellin protein lacks tryptophan residues in its sequence; hence, the change in the intrinsic fluorescence of tyrosine was monitored at various curcumin concentrations (0.125 to 16 μM). The intrinsic tyrosine fluorescence of flagellin reduced significantly with increasing concentrations of curcumin (Fig. 7A), indicating the binding of curcumin to flagellin molecules. The dissociation constant for specific binding, K_d, which was obtained using a one-site binding hyperbola nonlinear regression analysis (Fig. 7A), was 5.51 ± 0.78 μM (mean ± standard error). We further investigated the binding of curcumin to flagellin by a fluorimetric titration assay, in which the change in the fluorescence of curcumin, if any, in the presence of flagellin protein was monitored. At neutral pH, curcumin shows very weak fluorescence. The addition of flagellin protein to the curcumin (8 μM) solution significantly increased the fluorescence of the solution (Fig. 7B). The dissociation constant obtained using one-site specific hyperbola was 4.84 ± 0.58 μM (mean ± standard error), which is similar to that obtained from the tyrosine fluorescence data. The flagellin protein isolated using the ammonium sulfate precipitation method stays in a polymeric form (19, 44). To investigate whether curcumin interacts with flagellin monomers, a fluorescence binding assay was performed with increasing concentrations of curcumin. The intrinsic fluorescence of flagellin monomers (produced by heat denaturation of flagella at 65°C) was not affected upon the addition of increasing concentrations of curcumin (see Fig. S3 in the supplemental material). These results indicate that curcumin interacts preferentially with flagellar filaments.

Curcumin, a bivalent molecule, does not cross-link flagellar filaments. Curcumin has a bivalent structure. Hence, it could si-

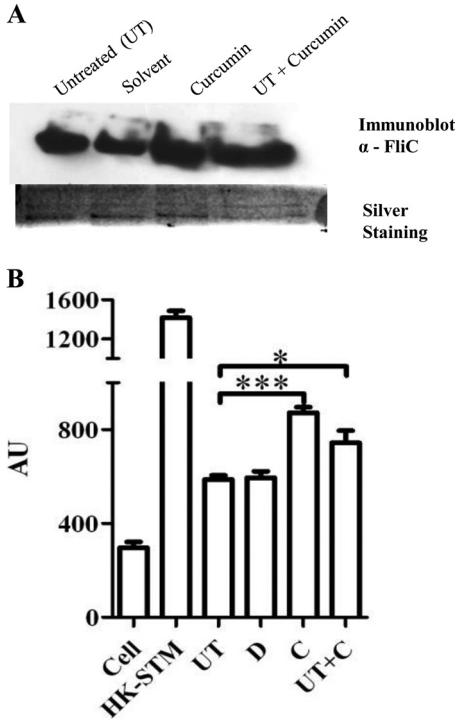

FIG 5 (A) Immunoblot assay for flagellin in culture supernatant after transient exposure of *S.* Typhimurium to curcumin. *S.* Typhimurium log-phase cells were incubated with curcumin for 10 min and then vortexed at 5,000 rpm for 5 min. The cells were pelleted, and the total protein from culture supernatant was isolated. An equal amount of protein in the culture supernatant was processed for immunoblotting against the flagellin protein. (B) NF-κB luciferase activity in HEK293 cells transfected with pNF-κB-TA-Luc after induction with 250 ng/ml protein isolated from *S.* Typhimurium culture supernatant, as explained in the Fig. 4 legend. The values in the graph are means and SE. Cell, uninduced and uninfected HEK293 cells (negative control); HK-STM, HEK293 cells treated with heat-killed *S.* Typhimurium (positive control); UT, D, C, and UT+C, HEK293 cells induced with protein from the culture supernatants of *S.* Typhimurium grown for 6 h in LB, LB plus DMSO, or LB plus curcumin (20 μM) or grown in LB for 6 h and treated with curcumin for 20 min. ***, $P < 0.001$; *, $0.01 \leq P < 0.05$. AU, arbitrary units.

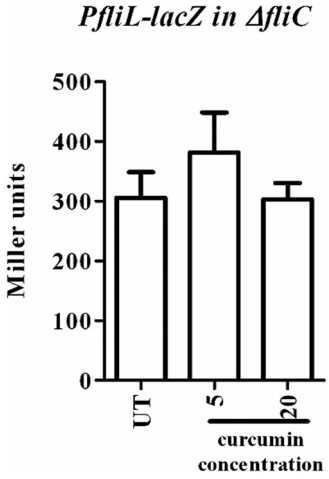

FIG 6 Effect of curcumin on the transcriptional activities of *fliL* in the *S.* Typhimurium Δ*fliC* mutant. Shown is the transcriptional activity of the *fliL* gene, as analyzed by β-galactosidase assay using log-phase cultures of the *S.* Typhimurium Δ*fliC* mutant with the *fliL-lacZ* transcriptional fusion under conditions described in the Fig. 3 legend. The values in the graph are means and SE.

multaneously bind two flagellin monomers. If these monomers happen to be on flagellar filaments of two adjacent bacterial cells, it could cross-link the filaments and cause agglutination (clumping). An agglutination assay revealed that curcumin did not cause any agglutination compared to that observed with antiflagellin antibody (Fig. 8). This indicates that curcumin can either bind directly to residues at the interface of filaments or destabilize filament formation through steric hindrance.

Computational analysis predicts that curcumin might bind to flagellin at the pocket between domains D1 and D2. The binding site of curcumin on flagellin was predicted using computational analysis, as explained in Materials and Methods. The top-ranked pocket predicted by PocketDepth was located at the interface of domains D1 and D2 (see Fig. S4A in the supplemental material). A pocket at the same location was predicted to be the top pocket by all other methods as well, with residues GLN112, THR116, LEU119, ASN120, ASP123, ASN163, SER164, GLN165, LEU169, ASP170, THR171, LEU172, ASN173, GLN175, GLY365, ALA366, THR370, ALA381, LYS384, LEU396, ALA397, GLU398, ALA399, and ALA400 as consensus binding site residues. The grid center of the binding site predicted by PocketDepth, which showed a good match with results from other methods as well, was provided for docking using softwares AD4 and Vina (24, 33).

The possible binding poses of curcumin on the flagellin molecule were determined using AD4 and Vina. The top two conformers of curcumin were selected from AD4 and Vina. The binding energies of the best conformers generated by Vina and AD4 were −7.6 kcal/mol and −9.65 kcal/mol, respectively. Since the searching and scoring processes were implemented differently in the two docking tools using a different scoring function, both have been used for evaluating the binding potential of curcumin to flagellin. With differences in scoring functions, ranking of the poses, and variations in the finer conformations picked, it is useful to explore both. The two programs docked curcumin at similar locations (see Fig. S4A in the supplemental material), mainly in two conformations (a bent conformation and an elongated conformation of curcumin), albeit with slightly different relative ranks. The two poses shared similarity in their binding sites (see Fig. S4B and C in the supplemental material).

Curcumin binds to flagellin molecules at ASN120, ASP123, ASN163, SER164, ASN173, and GLN175 residues. To confirm the binding of curcumin to flagellin at the residues identified using Vina and AD4, the residues predicted to form H bonds with curcumin were mutated, except A397 (see Fig. S4D in the supplemental material), and curcumin-flagellin binding was monitored using the fluorescence binding assay. Mutant proteins were generated with the mutations M1 (N120A and D123A), M2 (N163A and S164A), and M3 (N173A and Q175A) and all of the mutations (M1, M2, and M3) combined together. Mutated protein M3 showed a significant reduction in binding compared to mutant proteins M1 and M2 (Fig. 9). However, when all the residues mentioned above were mutated, there was no change in fluorescence with the addition of increasing concentrations of curcumin (0.125 to 8 μM), indicating abolition of binding of curcumin to a flagellin molecule (Fig. 9). These results suggest that curcumin binds to a flagellin molecule at positions N120, D123, N163, S164, N173, and Q175.

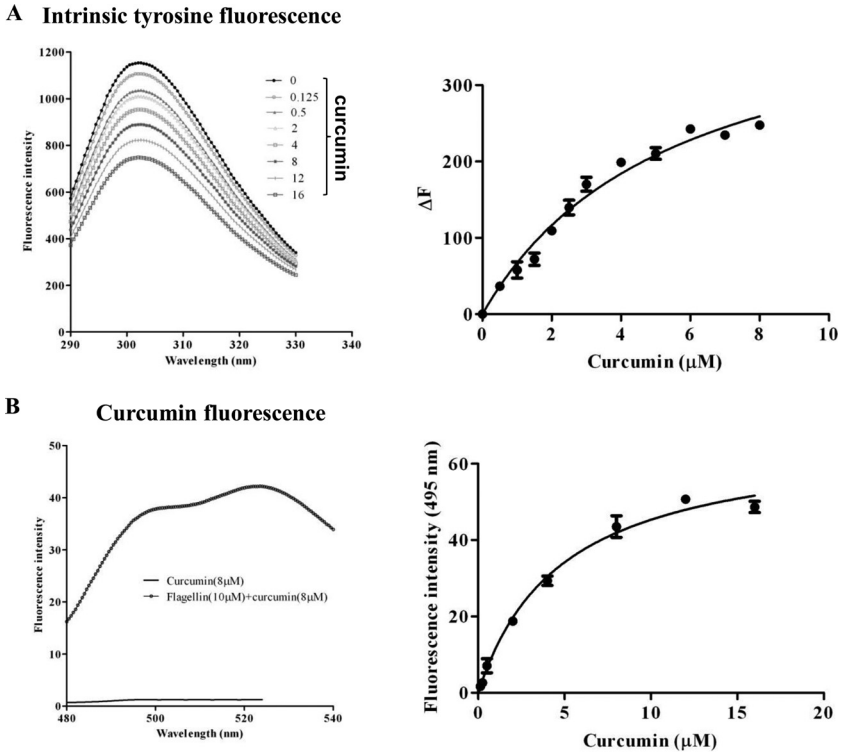

FIG 7 Binding of curcumin to flagella. (A) Emission spectra of flagellin solution in the presence of different concentrations of curcumin (0 to 16 μM) after excitation at 270 nm, showing a reduction in the intrinsic tyrosine fluorescence of flagella with increasing curcumin concentrations. ΔF, change in fluorescence. (B) Fluorescence spectra of curcumin (8 μM) after excitation at 425 nm, showing fluorescence enhancement upon addition of the flagellin protein solution. Data points and error bars represent means and standard deviations.

Degraded products of curcumin and its structural relatives showed effects similar to those of curcumin. To understand whether the effect shown by curcumin is specific to its backbone structure and/or functional groups, we used computational analysis to test the binding of compounds, such as piperine and EF-24 (structural analogue of curcumin), and the degraded products of curcumin, namely, ferulic acid and vanillin, with the flagellin molecule. All the compounds showed an interaction with the flagellin molecule. The binding mode and the interaction profile of all compounds with flagellin are shown in Fig. S5 in the supplemental material.

The effect of these compounds on the swarm motility of *S.* Typhimurium was tested. We found that these compounds inhibited the swarm motility of *S.* Typhimurium (Fig. 10A) without any apparent effect on the growth of the bacteria (see Fig. S6A in the supplemental material). We further evaluated the alteration in the percentage of flagellated bacteria on treatment with these compounds. There was a 10 to 25% reduction in the number of flagellated bacteria on treatment of *S.* Typhimurium with these phenolic compounds (Fig. 10B). Immunoblot analysis of the protein from the culture supernatant and whole-cell lysate suggested that these compounds increased the shedding of flagellar filaments from *S.* Typhimurium and had no effect on the production of flagellin protein (see Fig. S6B in the supplemental material).

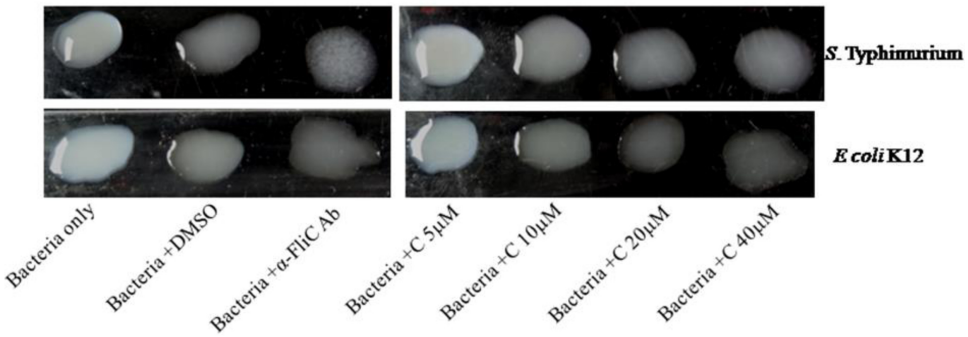

FIG 8 Agglutination of *S.* Typhimurium by curcumin. Log-phase *S.* Typhimurium and *Escherichia coli* cultures were pelleted and resuspended in Tris-HCl buffer (25 mM [pH 7]). The suspensions were incubated with either curcumin (5 to 40 μM) or antiflagellin antibodies (1:200; Difco) for 3 to 5 min and then observed for agglutination. C, curcumin; α-FliC Ab, antiflagellin antibody.

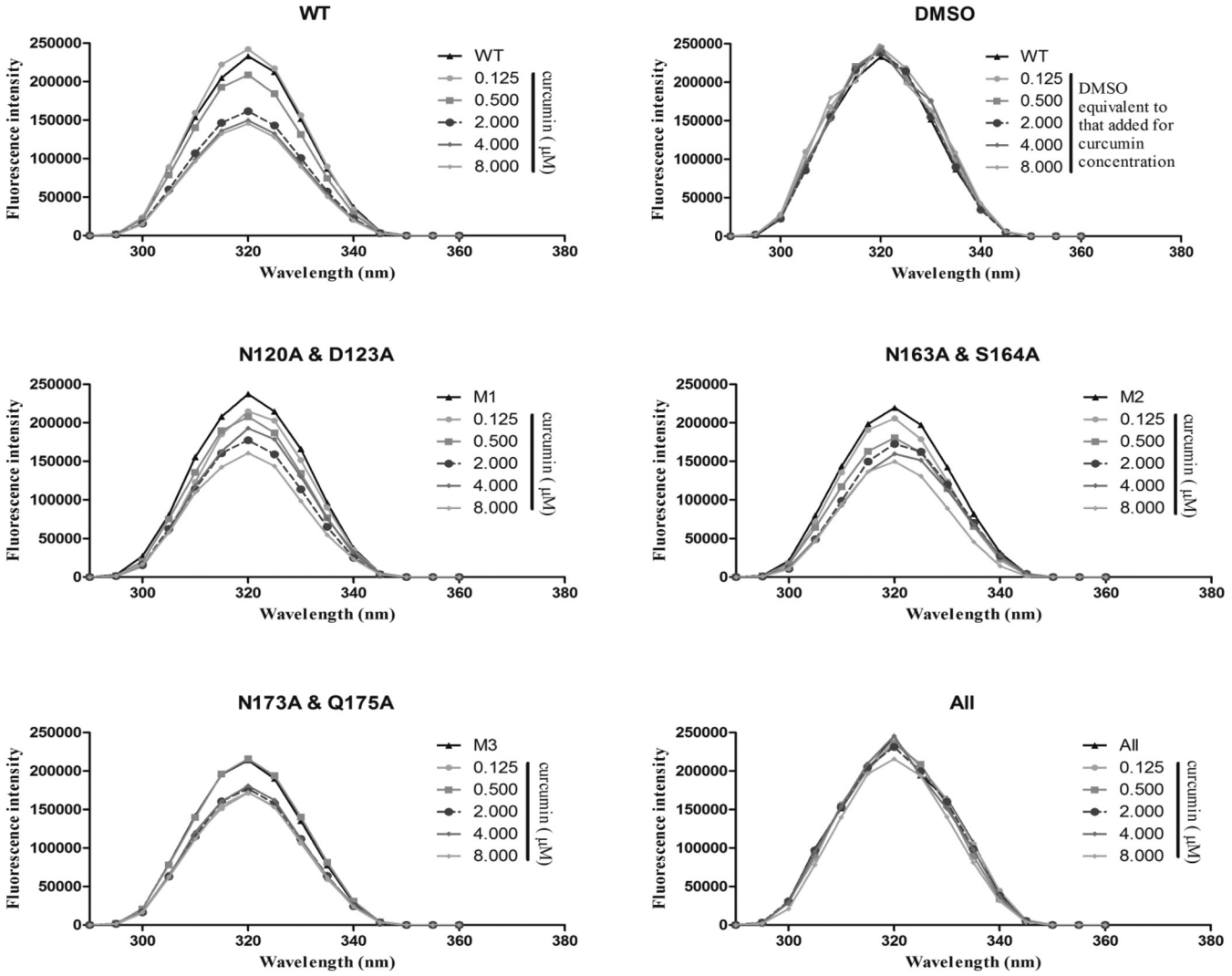

FIG 9 Mutations at selected residues in flagellin reduced its binding to curcumin. Emission spectra of solutions of mutated and wild-type flagellin in the presence of different concentrations of curcumin (0.125 to 8 μM) after excitation at 270 nm. WT, wild-type flagellin; DMSO, DMSO at a volume equal to that of curcumin, in addition to the wild-type flagellin solution; M1, flagellin with mutations at residues ASN120 and ASP123 to ALA; M2, flagellin with mutations at residues ASN163 and SER164 to ALA; M3, flagellin with mutations at residues ASN173 and GLN175 to ALA.

Curcumin reduces the motility of *E. coli*. To determine whether the effect of curcumin is specific to *S.* Typhimurium or is a general phenomenon against flagellated bacteria, we assayed the effect of curcumin on the motility of *E. coli*. Curcumin (20 μM) retarded the spread of the *E. coli* colony by ~50% on swarm agar (see Fig. S7A in the supplemental material). Curcumin did not show any cytotoxic effect on *E. coli* grown in LB (see Fig. S7B in the supplemental material). This indicates that the reduction in the spread of the swarm colony of *E. coli* was due to an effect of curcumin on the motility of *E. coli*.

E. coli and *S.* Typhimurium flagellins showed 55% sequence identity and 67% similarity, as judged by a sequence alignment using the BLOSUM62 substitution matrix. Residues predicted to interact with curcumin, namely, ASP123, ASP170, and THR370, were conserved, whereas others at the putative binding site, including ASN120, ASN163, SER164, ASN173, GLN175, and SER383, had conservative substitutions. Overall, conservation of the binding site between the two species lends further support to the result of the computational analysis.

In order to test whether curcumin could have a generalized effect on the flagella of other gammaproteobacteria, the conservation of the consensus binding site residues across a few species, belonging to both pathogenic and nonpathogenic categories, was assessed using multiple-sequence alignment (see Fig. S8 in the supplemental material). Some of the residues in the expected curcumin-binding site in *S.* Typhimurium FliC are conserved in a flagellin protein of many other bacteria as well, implying that it may bind curcumin in a similar fashion in these species. In particular, GLN112, LEU119, ASP123, SER164, and LEU169 appear to be well conserved.

DISCUSSION

Curcumin affects the physiology of various microorganisms via several mechanisms (12, 15, 16). Our study provides the insight

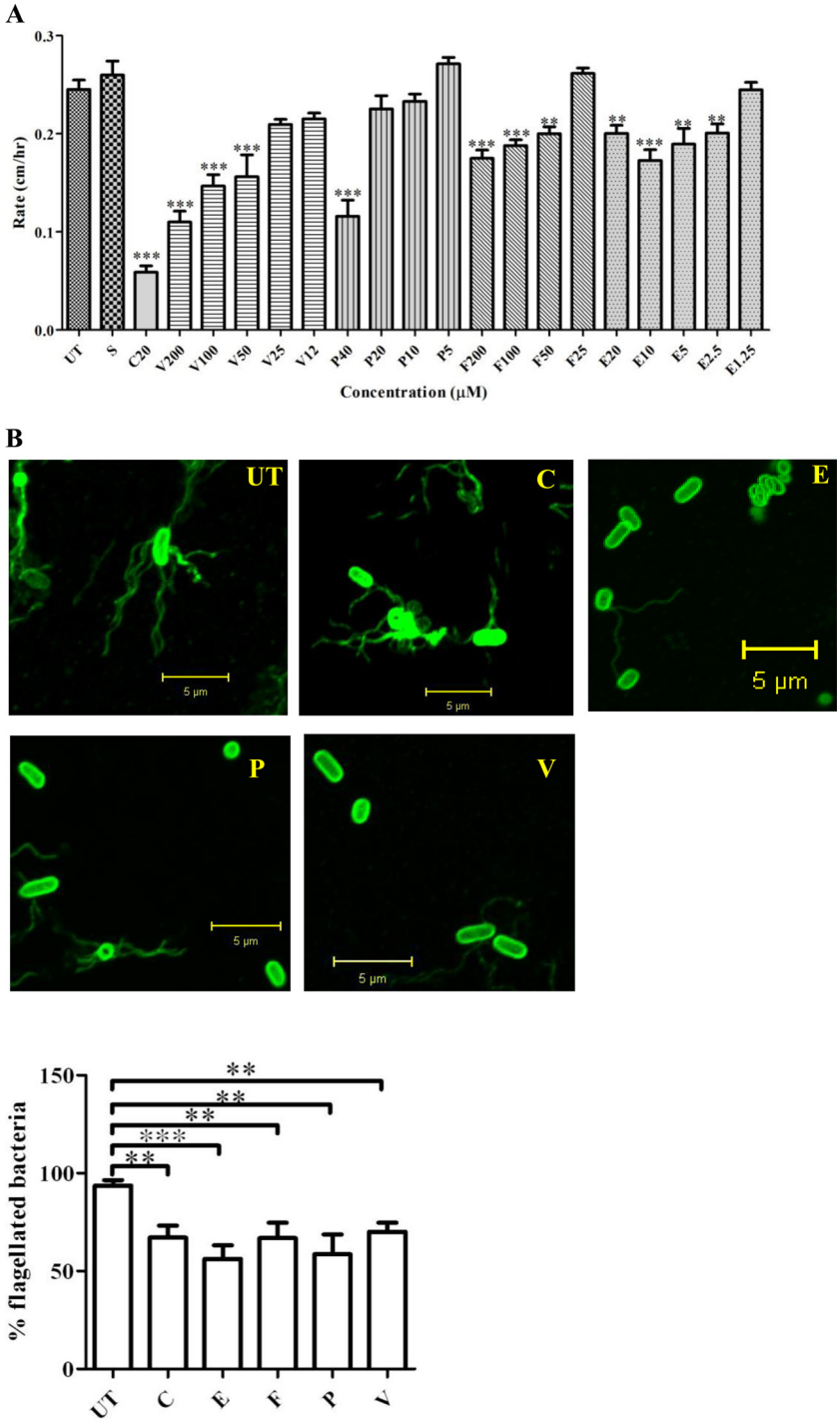

FIG 10 (A) Swarming motility of *S.* Typhimurium in the presence of different phenolic compounds. Shown is the swarming rate of *S.* Typhimurium under indicated conditions after 6 to 7 h of incubation at 37°C postinoculation. UT, swarm agar only; C, E, F, P, and V, swarm agar plus curcumin, EF-14, ferulic acid, piperine, and vanillin, respectively. The values are concentrations. (B) Confocal microscopy images of *S.* Typhimurium grown in the presence of different phenolic compounds. An *S.* Typhimurium overnight culture was subcultured in LB containing different phenolic compounds at a 1:100 ratio and grown at 37°C and 160 rpm for 6 to 7 h. Bacteria were processed for confocal microscopy, and images were acquired. UT, bacteria grown in LB; C, E, F, P, and V, bacteria grown in the presence of curcumin (20 μM), EF-24 (20 μM), ferulic acid (200 μM), piperine (40 μM), and vanillin (200 μM), respectively. The values in the graphs are means and SE. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$.

that curcumin can modulate bacterial motility by altering the physical and/or mechanical properties of the flagellar filament. The flagellar instability and fragmentation induced by curcumin have shed light on the function of this polyphenol at a molecular level. We showed that curcumin negatively modulates the motility of *S.* Typhimurium. The reduction in motility could be ascribed to the reduction in the number and length of the flagellar filaments on bacteria.

In order to form functional flagella, *Salmonella* has 67 genes organized in at least 26 operons that are divided into three classes based on the transcriptional hierarchy (2, 3). Class I consists of an operon coding for master regulatory proteins, class II consists of 9 operons coding for the components of the hook-basal body, and class III consists of 16 operons coding for motor complex, flagellar filament, and chemotaxis proteins (2). Representative genes from each of these classes were analyzed for a change in expression, if any, in the presence of curcumin. We found that curcumin did not alter the expression of class I and III genes tested in our study. However, it increased the expression of *fliL*, a class II gene (Fig. 3). The total amount of flagellin protein produced by the bacteria (Fig. 4) also remained unaffected. Nonetheless, curcumin increased the shedding of the flagellar filaments (Fig. 4). Azithromycin, a macrolide antibiotic, shows results similar to those exhibited by curcumin. However, it inhibits the secretion of flagellin molecules without altering the synthesis of flagellin, thus reducing the swimming and swarming motility of *Salmonella* (45). It also inhibits *Salmonella* invasion of intestinal epithelial cells (45).

During movement in liquid medium, the flagellar filaments form a bundle behind the cell body, acting as a propeller that is driven by the torque generated by the rotary motor at the base (46, 47). The bacteria tumble every few seconds to change their direction (48). To do so, bacteria reverse the motor rotation, generating a torque that apparently collapses the bundle (48). In this process, the filament transforms from a left-handed helix to a right-handed helix (49, 50). The helical form of the filament is determined by supercoiling, which is thought to occur either by switching the conformation or packing interactions of flagellin subunits (51–53). The transformation from a left-handed helix to a right-handed helix occurs by increasing the number of R-type protofilaments in the supercoil. This is achieved by changing the conformation of D1 domains and their packing interactions while keeping the D0 domains rigid (54). Computational modeling predicted the binding of curcumin to a flagellin molecule at a site between domains D1 and D2. Domain D1 is involved in making intersubunit interactions in the filament (22, 55). Hence, we can speculate that binding of curcumin would hinder the intersubunit interactions and the helicity of the flagellar filament. This would further weaken the polymer structure. The propelling and tumbling motion would then increase the pressure on the filament (in the presence of curcumin), leading to its fragmentation. This speculation is supported by the fact that curcumin was observed to bind to the flagellar protein when it is present in the form of a polymeric filament.

Curcumin was observed to bind to flagellin at the interface of two domains, with residues from both D1 and D2 participating in binding. The molecular packing in the crystal structure of the *S.* Typhimurium flagellin fragment resembles a protofilament that was consistent with electron cryomicroscopy and fiber diffraction data. Hence, the conformation of the residues at the binding site in this model can be expected to be similar to that in a filament in solution (55). In the monomeric form, the D0 domain of flagellin, which is absent in the F41 fragment, is present as a random coil (disordered structure). This random coil is converted to an α-helical structure upon polymerization (56, 57). The disordered structure in monomers may prevent the access of curcumin to domains D1and D2, thus preventing their binding. This may explain the inability of curcumin to bind to a monomer in solution.

Curcumin increased the expression of FliL, a protein essential for withstanding the torsional stress experienced by the rod on the swarm agar (43). Differential expression of FliL was abolished in the flagellin-knockout strain, suggesting that the curcumin-induced fragmentation of flagellar filaments might be responsible for the increased expression of FliL in the wild-type strain of *S.* Typhimurium. *fliL* is the first gene of the *fliLMNOPQR* operon that encodes the switch complex, which is important for the generation of torque and reversal of rotor direction (*fliM* and *fliN*) (3, 58). As these genes are part of the same operon, they could be upregulated concurrently upon curcumin treatment to make the flagella rotate more efficiently.

Our speculations are strengthened further by the results obtained from computational and mutational analyses. Docking studies showed that curcumin probably interacts with the interface of domains D1 and D2 of flagellin. The crucial residues showing a favorable H-bond interaction with curcumin were ASN120, ASP123, ASN163, SER164, ASN173, GLN175, and ALA397. When these residues, except ALA397, were mutated, the binding of curcumin to flagellin was lost, validating the computational docking analysis. Of these residues, ASP123 and SER164 seem to be well conserved (~52% of tested species) among gammaproteobacteria (see Fig. S8 in the supplemental material), implying that curcumin may affect the motility of these bacterial species. The phenolic hydroxyl group of curcumin showed major interactions with ASN120, ASP123, ASN163, SER164, GLN175, and ALA397, and the carbonyl carbon interacted with ASN173. A similar interaction of the other phenolic compounds was predicted at the binding site. Residues at the sites (particularly ASP170, THR370, ASN163, GLN175, and ASN173) were predicted to interact with the analogues/by-products of curcumin, including ferulic acid, vanillin, piperine, and EF-24, indicating that they too could bind to the site, throwing light on the features important for recognition of these compounds. The predicted binding site of curcumin and its degraded products on the flagellin molecule is adjacent to the regions important for intersubunit interactions (ND1b) (55, 59). The binding site of curcumin overlaps the TLR5 recognition site on the flagellin molecule (60).

The AutoDock data of curcumin, its analogues, and degraded products suggest a preferable pocket where they can bind (see Fig. S4 and S5 in the supplemental material). EF-24, a structural analogue of curcumin, inhibited motility and induced shedding of flagella, indicating that the backbone structure of curcumin is important for its activity. However, EF-24 does not inhibit motility to the same extent as curcumin. This highlights the importance of the functional groups of curcumin in its activity. The degraded products of curcumin, which possess its functional group, also inhibit motility, albeit at higher concentrations. Thus, it can be concluded that both the backbone structure and the functional groups of curcumin may be important for its activity. Further studies need to be carried out to understand the importance of the backbone structure and functional groups of curcumin in exhibiting the observed phenomenon.

The flagellar system is a type three secretion system (T3SS) and was found to be largely unaltered with respect to gene expression (class I and class III) upon curcumin treatment. However, other T3SSs of *Salmonella*, *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2, are significantly altered upon curcumin treatment (61). Curcumin reduced the expression of SPI-1, a T3SS required for invasion of host cells, but enhanced that of SPI-2, a T3SS required for intramacrophage survival (61). It increased

the pathogenicity of *Salmonella* (61, 62). Thus, although curcumin hinders motility, one of the important virulence determinants of *Salmonella*, it also has a positive effect on *Salmonella* pathogenicity.

ACKNOWLEDGMENTS

We express our thanks to Michael Hensel for the *Salmonella* strains. We thank Hemalatha Balaram, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, for helping us with binding assays. We are very thankful to Kavitha Sarvepalli for critical editing of the manuscript.

This work was supported by a Provision (2A) Tenth Plan (191/MCB) grant from the director of the Indian Institute of Science, Bangalore, India, Department of Biotechnology-IISc partnership program grant 469, and Life Science Research Board grant DRDO-LSRB 229 to D.C. Infrastructure support from ICMR (Center for Advanced Study in Molecular Medicine), DST (FIST), and UGC (special assistance) is acknowledged. S.A.M. and A.B. acknowledge CSIR for a fellowship.

We declare no conflicts of interest.

S.A.M. and D.C. conceived and coordinated the study and wrote the paper. S.A.M. designed, performed (except in the case of Fig. 8), and analyzed the experiments shown in Fig. 1 to 10. A.B. performed the experiments shown in Fig. 5 and designed and performed the experiments shown in Fig. 9. V.D.N. performed the experiments shown in Fig. 1, 4, 5, 8, and 10. D.S. and N.C. designed, performed, and analyzed all the computational data. S.A.M. and V.D.N. provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

FUNDING INFORMATION

This work, including the efforts of Dipshikha Chakravortty, was funded by DBT (DBT469). This work, including the efforts of Dipshikha Chakravortty, was funded by DRDO (LSRB-229).

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