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Exposure of *Salmonella enterica* Serovar Typhimurium to a Protective Monoclonal IgA Triggers Exopolysaccharide Production via a Diguanylate Cyclase-Dependent Pathway

Jayaleka J. Amarasinghe,[a] Rebecca E. D'Hondt,[a] Christopher M. Waters,[c] Nicholas J. Mantis[a,b]

Division of Infectious Diseases, Wadsworth Center, New York State Department of Health, Albany, New York, USA[a]; Biomedical Sciences Program, University at Albany School of Public Health, Albany, New York, USA[b]; Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, USA[c]

Sal4 is a monoclonal polymeric IgA antibody directed against the O antigen (O-Ag) of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), which is sufficient to protect mice against intestinal infections from *S.* Typhimurium. We recently reported that the exposure of *S.* Typhimurium to Sal4 results in the immediate loss of flagellum-based motility, in alterations to the outer membrane (OM) integrity, and in the concomitant appearance of a mucoid phenotype that is reminiscent of cells in the earliest stages of biofilm formation. We demonstrate here that prolonged (>4 h) exposure of *S.* Typhimurium to Sal4 at 37°C (but not at ambient temperature [25°C]) results in measurable exopolysaccharide (EPS) accumulation and biofilm formation on both borosilicate glass surfaces and polystyrene microtiter plates. The polysaccharide produced by *S.* Typhimurium in response to Sal4 contains cellulose, in addition to O-Ag capsule and colanic acid. EPS production was dependent on YeaJ, a proposed inner membrane-localized diguanylate cyclase (DGC) and a known regulator of cellulose biosynthesis. An *S.* Typhimurium Δ*yeaJ* strain was unable to produce cellulose or form a biofilm in response to Sal4. Conversely, the overexpression of *yeaJ* in *S.* Typhimurium enhanced Sal4-induced biofilm formation and resulted in increased intracellular levels of cyclic dimeric guanosine monophosphate (c-di-GMP) compared to that of a wild-type control; this strongly suggests that YeaJ is indeed a functional DGC. Based on these data, we speculate that Sal4, by virtue of its ability to associate with the O-Ag and to induce OM stress, renders *S.* Typhimurium avirulent by triggering a c-di-GMP-dependent signaling pathway via YeaJ that leads to the suppression of bacterial motility while simultaneously stimulating EPS production.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative facultative intracellular bacterium that causes acute gastroenteritis in humans and typhoid-like fever in mice (1). A pivotal step in infection is the invasion of Peyer's patch M cells and villous enterocytes, a highly complex event mediated by the *Salmonella* pathogenicity island 1 (SPI-1) type 3 secretion system (T3SS) (2–8). However, infection of the intestinal epithelium occurs only after a variety of host-associated environmental signals have primed *S.* Typhimurium to express virulence and colonization factors necessary for attachment and invasion (9–13). For example, iron limitation encountered in the intestinal lumen promotes the expression of thin aggregative fimbriae (Tafi), which are involved in epithelial attachment (13), while oxygen limitation activates the expression of the SPI-1 genes involved in invasion (14). *S.* Typhimurium uses a panoply of global regulatory circuits, such as two-component systems, like CpxR-CpxA and PhoP-PhoQ, as a means to adapt to its local environment and to coordinate virulence factor expression levels (15, 16). The transition from a motile and virulent state to a nonmotile and biofilm state, in particular, is regulated by members of the cyclic dimeric guanosine monophosphate (c-di-GMP) regulatory pathways (17–21). Thus, various environmental signals influence the virulence of *S.* Typhimurium and alter the ability of the bacterium to infect a host.

Secretory IgA (SIgA) is the most abundant class of antibodies in intestinal secretions, and it serves as the first line of defense against enteroinvasive pathogens, like *S.* Typhimurium (22). In experimental animal models, IgA antibodies against the O antigen (O-Ag) are highly effective at preventing *Salmonella* infection (23, 24). In fact, O-Ag-specific monoclonal IgA antibodies (MAbs) are more effective than flagellum-specific IgA MAbs at providing protective immunity (25). One of the best examples of this is Sal4, a monoclonal dimeric IgA specific for the *S.* Typhimurium O5 serotype. Using the so-called backpack tumor model, it was shown that, when secreted into the intestinal lumen as SIgA, Sal4 blocks *S.* Typhimurium entry into the intestinal mucosa (23). Invasion of an isogenic O5 mutant was unaffected by Sal4, thereby demonstrating the specificity of the antibody's activity. When examined *in vitro*, Sal4 prevented SPI-1 T3SS-mediated entry into polarized epithelial cell monolayers, but it did not affect bacterial attachment (24, 26).

Although it is now well established that Sal4 prevents *S.* Typhimurium from invading intestinal epithelial cells, the mechanism by which this occurs is unknown. Sal4 does not possess complement-fixing or bactericidal activity (23, 26). Moreover, immune exclusion, a process involving bacterial agglutination, entrapment in mucus, and clearance from the intestinal lumen, cannot fully account for the activity of Sal4 (27). For these reasons, we have focused on the possibility that the association of Sal4 with the

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Address correspondence to Nicholas J. Mantis, nmantis@wadsworth.org.
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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source
Strains		
Salmonella enterica serovar Typhimurium		
ATCC 14028	WT *S.* Typhimurium	ATCC
AMD212	ATCC 14028-Δ*thyA*; WT strain with a deletion in *thyA* gene	34
SJF12	*oafA*126::Tn*10d*-Tc	26
SJF11	*fkpA-lacZ oafA*126::Tn*10d*-Tc; Kan^r, Tc^r	Lab collection
JSG1919	*bscE*::*kan*; kanamycin cassette insertion mutation in *bscE* gene; Kan^r	J. S. Gunn
JSG1546	*wcaA*::*luc*; a mutant of *wcaA* constructed using luciferase-reporter suicide vector pGPL01; Amp^r	59
JSG2897	Δ*yihO*; λ Red deletion of *yihO*	12
STM14_1306	Δ*csgD*; deletion mutant of *csgD*; Kan^r	M. McClelland
JA001	Δ*oafA*; scar-free deletion mutant of *oafA*	34
JA002	Δ*yeaJ*; scar-free mutant of *yeaJ*	This study
JA002+pYeaJ	Δ*yeaJ*(pYeaJ); JA002 strain complemented with pYeaJ plasmid; Amp^r	This study
WT+pYeaJ	WT(pYeaJ); overexpression of YeaJ in WT background with pYeaJ plasmid; Amp^r	This study
WT+pBAD24	WT(pBAD24); WT strain carrying pBAD24 plasmid alone; Amp^r	This study
JSG1919+pYeaJ)	*bscE*::*kan*(pYeaJ); overexpression of YeaJ in JSG1919 background with pYeaJ plasmid; Kan^r Amp^r	This study
Escherichia coli DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* Φ*80* Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
Plasmids		
pBAD24	Arabinose-inducible, pBR322 *ori*; Amp^r	35
pYeaJ	pBAD24 with *yeaJ* insertion at XbaI and HindIII sites	This study
P17	pGEM-T-*thyA*; pGEM-T with *thyA* insertion; Amp^r	34
pWSK29	Amp^r	52
pWYeaJ	pWSK29 with *yeaJ* insertion at SmaI and HindIII sites; Amp^r	This study

[a] Antibiotic resistance phenotypes: Kan^r, kanamycin; Tc^r, tetracycline; Amp^r, ampicillin.

O-Ag may directly affect bacterial virulence. In support of this hypothesis, we demonstrated in a series of recent studies that the association of Sal4 with the O-Ag of *S.* Typhimurium has three immediate effects on bacterial physiology and virulence. First, Sal4 (5 to 15 μg/ml) treatment results in a rapid (<15 min) and dose-dependent arrest of *S.* Typhimurium flagellum-based motility in both liquid and semisolid media (26). Second, the binding of Sal4 to the bacterial outer membrane (OM) causes a significant reduction in SPI-1 T3SS activity, as evidenced by a decrease in SPI-1-dependent pore formation and the diminished delivery of SPI-1 effector proteins into host cells (28). Finally, the exposure of *S.* Typhimurium to Sal4 results in rapid (<15 min) alterations in membrane integrity, including enhanced OM permeability and increased shedding of lipopolysaccharides (LPS), as well as a transient reduction in the proton motive force (PMF) across the inner membrane (28).

In addition to these effects on virulence and physiology, we also observed by cryo-electron microscopy (cryo-EM) that Sal4-treated bacteria were surrounded by an electron-dense material that we have simply referred to as a fuzzy coat (22, 26). The electron-dense material was evident as early as 15 min post-antibody exposure and became more pronounced with time. In fact, within an hour after Sal4 treatment, cells were surrounded by a mucoid capsular material that is remarkably similar in appearance to the exopolysaccharides (EPS) secreted by *S.* Typhimurium during the initial stages of biofilm formation in response to environmental perturbations (22, 28, 29–31).

Based on these data, we postulate that Sal4 treatment generates a form of OM stress and that the early onset of EPS production by *S.* Typhimurium may represent an adaptive response to this sublethal stimulus. In this paper, we sought to characterize in more detail the effects of Sal4 on EPS production by *S.* Typhimurium. We demonstrate here that the prolonged exposure of *S.* Typhimurium to Sal4 results in EPS accumulation and biofilm formation on both glass and plastic surfaces. Moreover, we determined that this response is dependent on a putative diguanylate cyclase (DGC) known as YeaJ. In *Escherichia coli*, YeaJ is involved in regulating the bacterial transition from motility to biofilm formation (32). Based on these data, we propose that the association of Sal4 with the O-Ag induces OM stress, triggering a c-di-GMP signaling pathway in *S.* Typhimurium that effectively promotes the transition of the bacterium from a motile and invasive state to a nonmotile noninvasive biofilm state, thereby rendering the bacterium unable to invade intestinal epithelial cells.

MATERIALS AND METHODS

Bacterial strains, mammalian cell lines, and growth media. The bacterial strains and plasmids used in this study are shown in Table 1. Cultures were grown in Luria-Bertani (LB) or M9 medium with 0.4% glucose at 37°C, or at room temperature (25°C); however, the strains carrying a temperature-sensitive pKD46, a plasmid that encodes λ Red recombinase to enhance homologous recombination, were maintained at 30°C. Cell growth was monitored and measured by the optical density at a wavelength of 600 nm (OD_{600}). Media were supplemented with ampicillin (100 μg/ml) or L-arabinose (0.2%) as needed. HeLa cells were purchased from ATCC (CCL-2) and were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2-95% air atmosphere.

Antibodies. The murine hybridoma secreting Sal4, the monoclonal polymeric IgA antibody, was obtained from Marian Neutra (Children's Hospital, Boston, MA) (23). Hybridomas secreting the IgA antibodies

Sal4 and 23D7 (an isotype control used throughout this study) (33) were maintained as described previously (26). Rabbit polyclonal antiserum against *Salmonella* O antigens (group B factors 1, 4, 5, and 12) was purchased from BD Difco (Franklin Lakes, NJ).

Construction of Δ*yeaJ*, Δ*yeaJ*(pYeaJ), *bscE*::*kan*(pYeaJ), and YeaJ overexpression strains. The *yeaJ* gene of *S.* Typhimurium was deleted using a Flexible Recombineering Using Integration of *thyA* (FRUIT)-based method described previously (34). The primer sequences used to construct the mutant strains used in this study are listed in Table S1 in the supplemental material. Briefly, to construct the Δ*yeaJ* scar-free mutant, the *thyA* cassette was amplified using a primer pair (no. 319 to 320) with a ~40-nucleotide (nt) 5′ sequence that matched the desired site of recombination from the plasmid pGEM-T-*thyA*. PCR products were purified using a MinElute PCR purification kit (Qiagen), electroporated into strain ATCC 14028 containing the pKD46 plasmid but lacking *thyA* (WT14028, Δ*thyA*), and grown on LB agar plates containing ampicillin, thymine (100 μg/ml), and arabinose (0.2%) at 37°C to induce expression of the λ Red genes. After 1 h, recovered cells were plated onto M9 minimal medium (lacking thymine) containing ampicillin at 30°C. Recombinants were verified using colony PCR for *thyA* insertion at the correct genomic location (*yeaJ*::*thyA* intermediate strain). Utilizing splicing by overlap extension (SOEing) PCR, an overlapping PCR product of the desired site of scar-free mutagenesis was constructed and electroporated into the *yeaJ*::*thyA* intermediate strain containing pKD46 and then grown on LB agar containing ampicillin and arabinose (0.2%) to induce expression of the λ Red genes. Recovered cells were plated at 30°C onto M9 minimal medium containing thymine (100 μg/ml), trimethoprim (20 μg/ml), and ampicillin. Recombinants were verified using colony PCR with primers flanking the expected site of mutagenesis and were further confirmed via DNA sequencing; then, *thyA* was reintroduced at its native locus.

The overexpression of *yeaJ* in a wild-type (WT) background (WT+pYeaJ) and *bscE*::*kan* background (*bscE*::*kan*+pYeaJ) and complementation of the Δ*yeaJ* mutant were accomplished as follows. The coding sequence of *yeaJ* from the *S.* Typhimurium ATCC 14028 genome was PCR amplified with *yeaJ*-specific primers containing built-in restriction sites as indicated in Table S1 and cloned into the XbaI and HindIII (New England BioLabs, Ipswich, MA) sites of a pBAD24 vector (35). *E. coli* DH5α competent cells were transformed with the resulting *yeaJ* construct (pYeaJ). Transformants were selected on LB agar plates containing ampicillin, and the sequence of the selected clone was verified by nucleotide sequencing. To construct the WT(pYeaJ) strain, the plasmid (pYeaJ) was isolated using QIAprep spin miniprep columns (Qiagen) and was transformed into *S.* Typhimurium strain ATCC 14028 by electroporation. Transformants were selected on LB agar containing ampicillin and were confirmed using PCR primers (pBAD-F and *yeaJ*-R-XbaI; see Table S1) and nucleic acid sequencing. To induce YeaJ expression, the selected transformants were grown in LB broth containing ampicillin and L-arabinose (0.2%), and the culture was shaken at 37°C overnight. Complementation of Δ*yeaJ* and overexpression of YeaJ in the *bscE*::*kan* background were achieved by transforming pYeaJ plasmid into the Δ*yeaJ* and *bscE*::*kan* mutant strains, respectively. Transformants were selected on LB agar containing ampicillin and were confirmed by PCR using primers (pBAD-F and *yeaJ*-R-XbaI; see Table S1) and by nucleic acid sequencing. To induce the expression of pYeaJ, the selected transformants were grown in LB broth containing ampicillin and L-arabinose (0.2%) with shaking at 37°C overnight.

Crystal violet-based biofilm assays. Sal4-induced biofilms were measured using a previously described crystal violet (CV) staining method (36). Overnight cultures of each test strain were initially standardized in fresh LB broth containing either Sal4 (15 μg/ml), 23D7 (15 μg/ml), an IgA isotype control of irrelevant specificity, or chemically defined medium (CDM) with no antibody, to an OD_{600} of 0.05 (approximately 3×10^7 initial CFU/ml). Biofilm assays were performed either in borosilicate glass tubes or on 96-well polystyrene microtiter plates (Nunc, Denmark). Cultures in borosilicate glass tubes (1.3-ml final volume) were incubated upright on a rotary shaker set at 150 rpm at 37°C. After 24 h, pellicle formation was assessed at the air-liquid interface by visual observation and was digitally photographed. To assess biofilm formation, culture supernatants were decanted and the tubes were washed with phosphate-buffered saline (PBS) (pH 7.4) to remove unbound bacteria; then they were air-dried for 15 min. The remaining cells and cell-associated materials were fixed with 2-volume equivalents of methanol (Macron Chemicals, PA) for 15 min, air-dried for 30 min, and then stained with 0.1% CV (Sigma-Aldrich Co.) for 5 min. After this, the unbound dye was decanted and the tubes were washed with distilled water and digitally photographed.

For polystyrene microtiter plate-based assays, standardized cultures (200 μl per well) were incubated (after being covered and sealed with Parafilm) for 1 to 7 days at 25°C or 37°C. The media were not replaced over the course of these experiments. The OD_{595}s of the bacterial cultures were monitored spectrophotometrically using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). After optical density measurements were taken, culture supernatants were decanted and unbound bacteria were removed by washing with PBS (pH 7.4). The remaining cells and cell-associated materials were fixed (250 μl methanol per well), stained with CV, and solubilized with ethanol; absorbance was quantitated at 570 nm as indicated above.

Calcofluor and Congo red binding assays. Cellulose production and the rdar (red, dry, and rough) phenotype were assessed essentially as described previously (37). The Congo red (CR) binding assay is often used to detect rdar colony morphology that produces cellulose and curli fimbriae (also known as Tafi) in *Salmonella* (38–41), while the calcofluor (CF) binding assay is used to detect the cellulose production of the colonies. Briefly, 5 μl of overnight cultures from WT and *yeaJ* mutant strains was spotted on agar plates containing CF (fluorescence brightener 28; 200 μg/ml) (Sigma) or CR (40 μg/ml) (Sigma) and Coomassie brilliant blue (20 μg/ml) (Sigma), and then was overlaid with 5 μl Sal4 (47 μg/ml) or CDM. Plates were incubated for 1 to 7 days at 25°C or 37°C. The development of the colony morphology and the dye binding activity were analyzed over time.

Isolation of EPS. Isolation of EPS was performed as described previously (42, 43), but with minor modifications. Briefly, bacterial cells were grown in LB broth at 37°C overnight and subcultured into 5-ml fresh LB broth (OD_{600}, 0.05) with Sal4 (15 μg/ml) or 23D7 (15 μg/ml), or simply an equal volume of CDM; then they were grown at 37°C for 4 h without shaking before the removal of 4 ml of bacterial supernatants. The cells in the remaining 1 ml were then collected by centrifugation, washed twice with PBS, and resuspended in sterile PBS to a final OD_{600} of 0.5. One milliliter of each culture was collected by centrifugation, resuspended in 0.5 ml of 50 mM sodium acetate buffer (pH 5.8)-100 mM NaCl, and then transferred to a 1.5-ml microcentrifuge tube. An equal volume containing 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.5% SDS was added, and the tube was vortexed briefly and incubated at 100°C for 5 min. After centrifugation, the resulting pellet containing the crude exopolysaccharide was suspended in 0.5 ml of a solution containing 25 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 0.5% SDS, and 0.5 ml of 2× SDS-PAGE sample buffer; it was incubated at 100°C for 10 min. EPS samples (20 μl each) were separated by 12% SDS-PAGE with an extra-long (10-mm) stacking gel and a 35-mm-long resolving gel. Silver staining and Western blotting were used to determine relative EPS expression levels.

Western blotting. Samples separated by SDS-PAGE were transferred to a pure nitrocellulose membrane (0.45 μM) (Bio-Rad). The membrane was blocked overnight with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBST), washed three times with TBST, and incubated with rabbit anti-*Salmonella* O-antigen antiserum (group B factors 1, 4, 5, and 12) (BD Difco, Franklin Lakes, NJ) diluted (1:500) in blocking buffer. After washing with TBST, the membrane was incubated with secondary antibody (goat anti-rabbit horseradish peroxidase [HRP]-conjugated IgG) (SouthernBiotech) diluted 1:1,000 in blocking buffer. Antibody bound to the target antigen was detected by en-

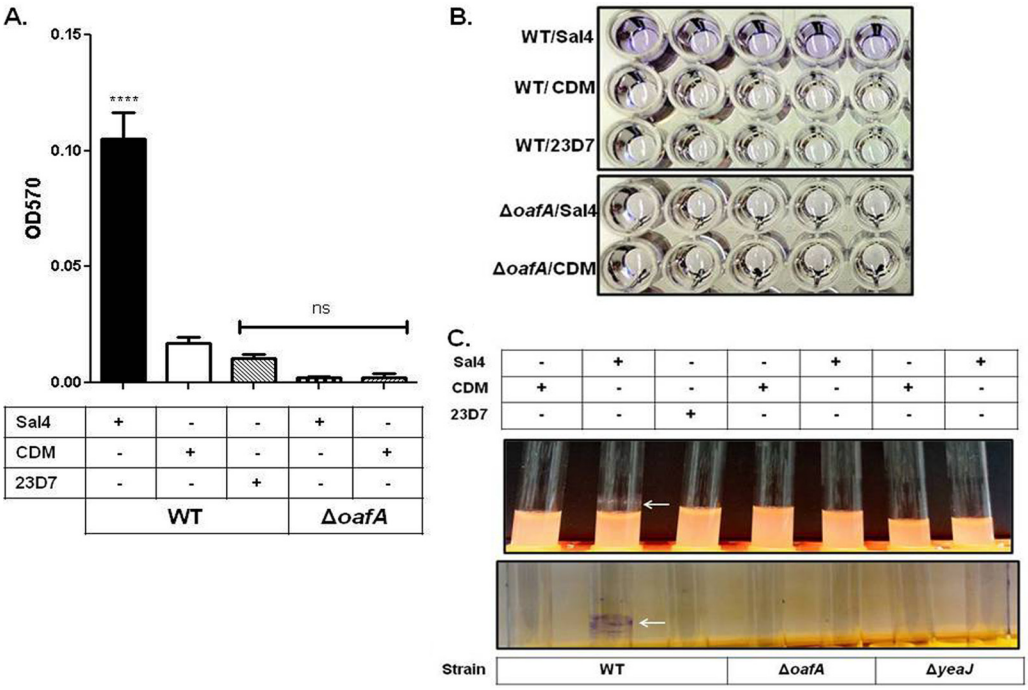

FIG 1 *S.* Typhimurium biofilm formation in response to Sal4. WT and JA001 (Δ*oafA*) strains of *S.* Typhimurium in LB broth were incubated on microtiter plates or in borosilicate glass culture tubes at 37°C for 24 h in the presence of Sal4 (15 μg/ml), 23D7 (15 μg/ml), or chemically defined medium (CDM). The cultures were then assayed for EPS production using CV, as described in Materials and Methods. (A) Quantification of biofilm mass, as measured by absorbance (optical density) at 570 nm (OD_{570}). Statistical significance was determined by a one-way ANOVA, followed by Bonferroni's correction, compared to the WT strain treated with CDM. ****, $P < 0.0001$; ns, nonsignificant. Each bar represents the mean ± the standard error of the mean (SEM). (B) Representative images of CV-stained microtiter plates highlighting the effect of Sal4 on WT *S.* Typhimurium (top panel, top row). In contrast, there was no EPS production by strain JA001 (Δ*oafA*) in response to Sal4 (bottom panel), demonstrating the specificity of the antibody response. (C) Representative images demonstrating pellicle formation (top panel) and EPS production (bottom panel) on borosilicate culture tubes in response to Sal4 (arrows). The data in each panel represent the averages of three independent assays. In each assay, experimental samples were performed in five-replicate (quintuplicate) wells or culture tubes.

hanced chemiluminescent (ECL) Western blotting substrate (Pierce Chemicals, Rockford, IL).

c-di-GMP quantification. To determine the levels of c-di-GMP produced by YeaJ, the amount of c-di-GMP produced by specific strains of *S.* Typhimurium was quantified by using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) as described previously (44, 45). Briefly, standardized cultures of WT, Δ*yeaJ*, WT(pYeaJ), and WT(pBAD24) strains were grown at 37°C with shaking at 150 rpm for 3 h in M9 minimal medium containing 0.4% glucose until the cultures reached a density of ~2.8×10^8 CFU/ml (equivalent to an OD_{600} of 0.4). The final CFU/ml for each strain was determined by plating serial dilutions of the cultures onto nonselective LB medium. Bacteria were collected by centrifugation and nucleotides were extracted by resuspending the pellet in extraction buffer (MeOH to acetonitrile to distilled water [dH_2O] [40:40:20] plus 0.1 N formic acid) as described previously (45). Ten microliters of each sample was analyzed by UPLC-MS-MS on a Quattro Premier XE mass spectrometer (Waters Corporation) coupled with an Acquity UPLC system (Waters Corporation). c-di-GMP was detected with electrospray ionization using multiple reaction monitoring in negative ion mode at *m*/*z* 689.16 to 344.31. Chemically synthesized c-di-GMP (Axxora) was used to generate a standard curve for calculating the c-di-GMP concentration in each extract.

HeLa cell invasion assay. HeLa cell invasion and gentamicin protection assays were done as described previously (24, 26, 28). *S.* Typhimurium ATCC 14028, SJF11 (Δ*oafA*), Δ*yeaJ*, and WT(pYeaJ) strains were grown to mid-log phase in LB with aeration (~2.8×10^8 CFU/ml), diluted 1:10, and then mixed at equal proportions. The exact ratio of the two strains was determined by replica plating. The bacteria were then incubated with Sal4 (5 μg/ml) for 45 min before being applied to HeLa cells in 12-well microtiter plates. The microtiter plates were subjected to low-speed centrifugation (10 min at 1,000 × *g*) at 4°C to promote bacterium-epithelial cell contact and then were incubated at 37°C for 1 h, followed by gentamicin treatment and cell lysis, as described previously (24). The competitive index (CI) was calculated as CI = ([% strain A recovered/% strain B recovered]/[% strain A inoculated/% strain B inoculated]) (46). Statistical significances were determined by one-way analysis of variance (ANOVA).

RESULTS

Sal4 induces biofilm formation in *S.* Typhimurium. To determine whether Sal4 treatment induces EPS production and biofilm formation, we performed quantitative biofilm assays using uncoated polystyrene microtiter plates (Fig. 1A and B) or borosilicate glass tubes (Fig. 1C). After incubation at 37°C for 24 h, there was a significant increase in CV staining on both the polystyrene microtiter plates and glass tubes in the presence of Sal4 (15 μg/ml) compared to that on 23D7, an IgA isotype control MAb (15 μg/ml), or CDM alone (Fig. 1). As an additional control, we used an *S.* Typhimurium *oafA* deletion (Δ*oafA*) mutant (strain JA001), which lacks the Sal4 epitope due to the loss of *O*-acetyltransferase activity (47). The Δ*oafA* mutant did not exhibit biofilm formation on polystyrene microtiter plates or glass tubes in response to Sal4 (Fig. 1), demonstrating that the effects of Sal4 are specific and related to its ability to associate with the bacterial O-Ag.

A modified SDS-PAGE and silver-staining protocol (see Materials and Methods) confirmed that there were elevated amounts of

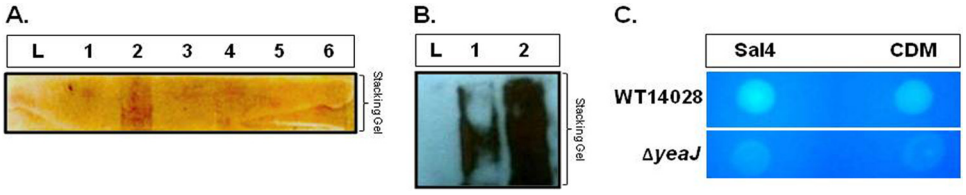

FIG 2 Expression of an EPS containing O-Ag capsule-like material by *S.* Typhimurium in response to Sal4. (A and B) EPS preparations from WT strain 14028 or strain SJF12 (Δ*oafA*) treated with Sal4 (10 μg/ml), 23D7 (10 μg/ml), or CDM for 4 h at 37°C were separated by SDS-PAGE on 12% gels with an extra-long (35-mm) stacking gel. (A) Silver stain. Lane L, molecular weight ladder; lane 1, WT treated with CDM; lane 2, WT treated with Sal4; lane 3, WT treated with 23D7; lane 4, heat-killed WT treated with Sal4; lane 5, SJF12 treated with CDM; and lane 6, SJF12 treated with Sal4. (B) Following SDS-PAGE, EPS preparations were transferred onto nitrocellulose membranes and subjected to Western blotting using *Salmonella*-specific O-Ag polyclonal antisera, as described in Materials and Methods. Lane L, ladder; lane 1, WT treated with CDM; lane 2, WT treated with Sal4. (C) Cellulose production in response to Sal4. WT and Δ*yeaJ* strains were spotted on CF agar plates and then overlaid with 5 μl Sal4 (47 μg/ml) or CDM. Plates were then incubated at 37°C for 24 h and photographed on a UV light box.

EPS associated with Sal4-treated WT cells compared to that with 23D7-treated cells or the CDM-treated control (Fig. 2A). As expected, EPS production in response to the Sal4 antibody was not observed with either heat-killed bacteria or the Δ*oafA* mutant (Fig. 2A). Furthermore, Western blot analysis revealed that the EPS material secreted by *S.* Typhimurium in response to Sal4 was reactive with O-Ag polyclonal antiserum, suggesting that, in part, the EPS may be composed of O-Ag capsule (Fig. 2B) (31). Sal4-treated colonies also bound to CF, a specific and sensitive probe for β-1,4-linked D-glucopyranosyl residues of cellulose (Fig. 2C) (48). Together, these data demonstrate that EPS material is produced in response to the Sal4 antibody.

To examine the complexity of the EPS induced by Sal4, we assessed the Sal4-induced EPS production by strains carrying mutations in genes known to be important in *S.* Typhimurium exopolysaccharide synthesis, including Δ*yihO* (O-Ag capsule), *wcaA*::*luc* (colanic acid), and *bscE*::*kan* (cellulose). The *bcsE*::*kan* mutant was completely defective in Sal4-induced biofilm formation, while the Δ*yihO* and *wcaA*::*luc* strains each demonstrated reduced biofilms compared to that shown by the WT strain (see Fig. S1 in the supplemental material). These data further support the notion that the EPS elicited in response to Sal4 consists primarily of cellulose, and likely also O-Ag capsule and colanic acid.

YeaJ is important in Sal4 biofilm formation at 37°C. In an effort to identify the specific genes and proteins of *S.* Typhimurium involved in biofilm formation in response to Sal4, we screened a library of chromosomal deletion mutants for those that were incapable of forming a biofilm in the presence of Sal4 (J. Amarasinghe, K. Levinson, and N. Mantis, unpublished data). One of the mutants we identified carried a chromosomal deletion in *yeaJ* (STM1283), a gene that encodes a putative inner membrane-localized DGC (49). DGCs synthesize c-di-GMP to regulate cellulose production, biofilm formation, and virulence in *S.* Typhimurium (50). In *E. coli*, *yeaJ* regulates the transition between motility and biofilm formation states (32). In *S.* Typhimurium, *yeaJ* as expressed on a plasmid was capable of complementing the biofilm deficiency of a strain lacking *gcpA*, a known DGC (51). Therefore, we hypothesized that *yeaJ* is involved in Sal4-induced biofilm formation in *S.* Typhimurium.

To determine the role of YeaJ in the induction of biofilm formation by Sal4, we constructed a nonpolar chromosomal *yeaJ* deletion (Δ*yeaJ*) in an otherwise WT background. To investigate the effect of this mutation on Sal4-induced biofilm formation, Δ*yeaJ* was tested using a quantitative microtiter biofilm assay in the presence or absence of Sal4. In this assay, the Δ*yeaJ* strain was unable to form a biofilm in response to Sal4 (Fig. 3A and B), or to produce cellulose in response to Sal4 (Fig. 2C). In an effort to complement this mutation, we cloned the wild-type allele of *yeaJ*, under the control of its own promoter, into a low-copy-number plasmid (pWSK29) (52), and introduced the resulting plasmid (pYeaJ) into the Δ*yeaJ* strain. Although we confirmed by nucleotide sequencing that *yeaJ* was properly cloned into this vector, the resulting low-copy-number plasmid, pWYeaJ, did not restore the Sal4-induced biofilm phenotype or cellulose production. We speculated that the failure of this plasmid to complement the Δ*yeaJ* mutant might be due to the insufficient (or improper) expression of YeaJ possibly because of the absence of *cis* or *trans*-acting elements involved in the regulation of *yeaJ*. We therefore cloned the native *yeaJ* gene into a commonly used medium-copy-number plasmid (pBAD24), in which the expression of *yeaJ* was now under the control of the arabinose-inducible *araC* promoter. The introduction of *yeaJ* as expressed from this pBAD-based plasmid resulted in the full restoration of cellulose production, as indicated by CF (Fig. 4A) and CR binding (Fig. 4A), as well as in the partial restoration of biofilm formation in response to Sal4 (Fig. 3A and B).

If *yeaJ* encodes a bona fide DGC, then we postulated that overexpression of the *yeaJ* gene via an inducible promoter in an otherwise WT background will enhance baseline (as well as Sal4-induced) biofilm formation and EPS production. To test this, we introduced the plasmid carrying the full-length *yeaJ* (pYeaJ) cloned under the control of the arabinose-inducible *araC* promoter into wild-type *S.* Typhimurium. As expected, the ectopic expression of YeaJ resulted in enhanced Sal4-induced biofilm formation at 37°C on borosilicate glass and microtiter plates (Fig. 3A and B). Ectopic expression of YeaJ in the absence of Sal4, however, did not significantly induce biofilm formation. This result suggests that the DGC activity of YeaJ is dependent on Sal4-mediated changes to *S.* Typhimurium, arguing that Sal4 and YeaJ are components of a common signaling pathway. Based on these data, we concluded that YeaJ is involved in Sal4-induced biofilm formation at 37°C, likely functioning as a DGC enzyme to synthesize c-di-GMP in the presence of Sal4.

YeaJ is also necessary for biofilm formation at 25°C. Our above-mentioned data indicate that the activity of YeaJ is Sal4-dependent at 37°C. However, we noticed that Sal4 does not induce biofilm formation at 25°C (data not shown), a temperature that has been shown to induce robust biofilm formation by *S.* Typhimurium due to the optimal production of cellulose and fimbriae under this condition (53, 54). Therefore, we wondered whether

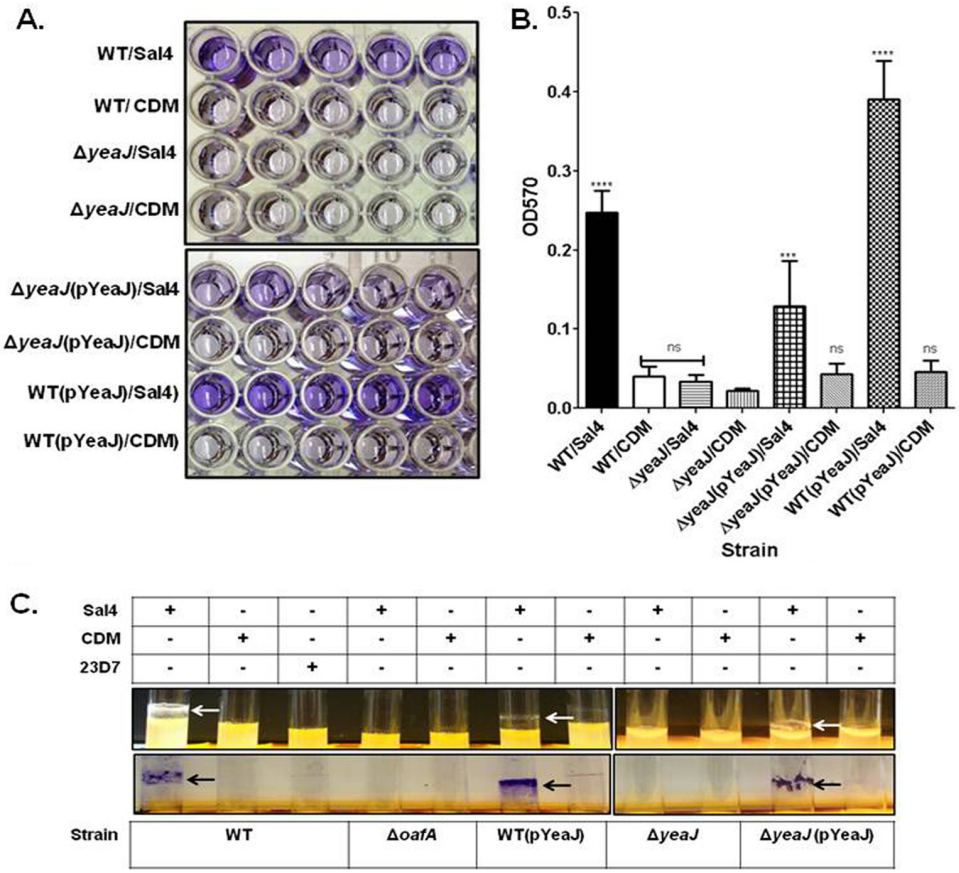

FIG 3 *S.* Typhimurium biofilm formation in response to Sal4 is dependent on YeaJ. WT, JA002 (Δ*yeaJ*), JA002 complemented with pYeaJ, and WT (pYeaJ) strains of *S.* Typhimurium in LB broth were incubated on microtiter plates (A and B) or in borosilicate glass (C) culture tubes at 37°C for 24 h in the presence of Sal4 (15 μg/ml), 23D7 (15 μg/ml), or CDM. The cultures were then assayed for biofilm formation using CV, as described in Materials and Methods. (A) Representative images of CV-stained microtiter plate demonstrating that Sal4-induced biofilm formation is dependent on YeaJ. (B) Quantification of biofilm mass, as measured by absorbance (optical density) at 570 nm (OD_{570}). Statistical significance was determined by a one-way ANOVA, followed by Bonferroni's correction, compared to the WT strain treated with CDM. ****, $P < 0.0001$; ***, $P < 0.001$; ns, nonsignificant. Each bar represents the mean ± the standard error of the mean (SEM). (C) Representative images demonstrating that pellicle formation (top panel) and biofilm formation (bottom panel) on borosilicate culture tubes in response to Sal4 (arrows) are dependent on YeaJ. The data in each panel represent the average of three independent assays. In each assay, experimental samples were performed in quintuplicate wells and duplicate culture tubes.

the activity of YeaJ can transpire at 25°C in the absence of Sal4. We first tested whether the disruption of *yeaJ* impacts cellulose production at room temperature, as assessed using the CF binding assay. In this assay, the WT strain produced bright colonies (by 3 days), an indicator of cellulose production (Fig. 4A, top). In contrast, the Δ*yeaJ* mutant colonies appeared dull under a UV light source, even when they had been incubated at room temperature for as long as 3 days (Fig. 4A, top). Cellulose production was restored to WT levels when the Δ*yeaJ* strain was transformed with a plasmid carrying a functional copy of *yeaJ* (Fig. 4A, top). Furthermore, the overexpression of YeaJ in an otherwise WT background accelerated the appearance of CF-positive colonies (Fig. 4B, top) and biofilm formation (Fig. 4D and F).

In *S.* Typhimurium, the rdar (red, dry, and rough) colony morphotype is linked to biofilm formation at low ambient temperatures (≤28°C). To examine the effect of the *yeaJ* mutation on the rdar morphotype, the *yeaJ* mutant and the WT control were plated on CR agar and incubated at room temperature for 7 days. The WT strain displayed CR-positive colonies within 48 h, and it assumed the rdar morphotype within 3 days (Fig. 4A, bottom). In contrast, the Δ*yeaJ* mutant was defective in CR binding and in the formation of rdar colonies, as evidenced by smooth moist colonies, not rough and dry colonies, at day 3 (Fig. 4A, bottom). Complementation of the Δ*yeaJ* mutant partially restored the rdar phenotype in that the colonies remained mildly rough and not dry at day 3 (Fig. 4A, bottom) or even after prolonged incubation (>14 days) (data not shown). On the other hand, the overexpression of YeaJ in a WT background (WT+pYeaJ) accelerated and accentuated the rdar morphotype, as CR-bound colonies appeared within 24 h (Fig. 4B, bottom) and colony roughness was notably pronounced at 7 days compared to that of the WT control at the same time point (Fig. 4C, top). The WT strain with pBAD24 vector alone exhibited similar levels of CR and CF binding as the WT strain, indicating no evidence of a deleterious effect or the metabolic burden of pBAD24 plasmid (see Fig. S4 in the supplemental material). From these data, we conclude that YeaJ is required for the production of cellulose, and possibly fimbriae, in *S.* Typhimurium at low ambient temperatures.

Next, we evaluated the capacity of the Δ*yeaJ* mutant to produce a biofilm at room temperature over a 1-week period. The WT *S.*

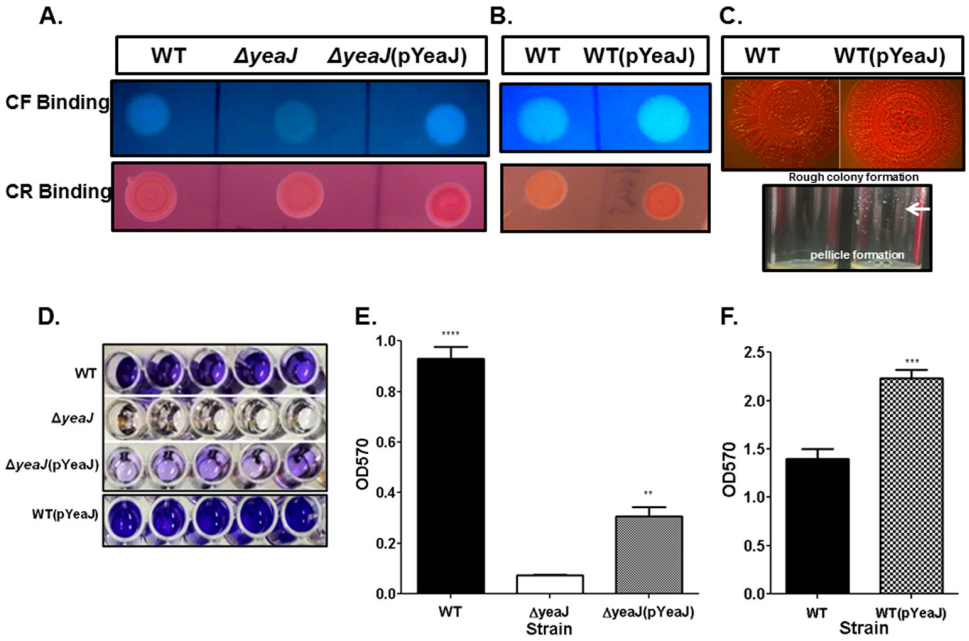

FIG 4 Cellulose production and biofilm formation at 25°C by *S.* Typhimurium are dependent on YeaJ. (A) WT, JA002 (Δ*yeaJ*), and JA002+pYeaJ [Δ*yeaJ*(pYeaJ)] strains were spotted on CF and CR agar plates and incubated at 25°C for 72 h to assess cellulose production and rough colony formation. Under these conditions, Δ*yeaJ* was deficient in cellulose production and failed to form rough colonies compared to the WT strain. See the text for details. (B) Phenotype of WT and WT(pYeaJ) on CR (top panel) and CF (bottom panel) agar plates following incubation at 25°C for 24 h. (C) WT and WT(pYeaJ) rough colony formation on CR agar after 7 days at 25°C (top panel) and pellicle formation (arrow) after 3 h of incubation in LB at 37°C (bottom panel). (D to F) WT, JA002 (Δ*yeaJ*), JA002 + pYeaJ [Δ*yeaJ*(pYeaJ)], and WT(pYeaJ) strains incubated in microtiter plates for 7 days at 25°C. The cultures were then assayed for biofilm formation using CV, as described in Materials and Methods. (D) Representative images of CV-stained microtiter plates highlighting the role of YeaJ in biofilm formation at 25°C; (E and F) quantification of biofilm mass, as measured by absorbance (optical density) at 570 nm (OD570). The data in each panel represent the average of three independent assays. In each assay, experimental samples were performed in quintuplicate wells and duplicate culture tubes. In panel E, statistical significance was determined by a one-way ANOVA, followed by Bonferroni's correction, compared to the WT strain treated with CDM. ****, $P < 0.0001$; **, $P < 0.01$. In panel F, significance was determined by paired Student's *t* test, compared to the control (WT). ***, $P = 0.0003$. In panels E and F, each bar represents the mean with the SEM.

Typhimurium strain 14028 formed a robust biofilm at 25°C within 7 days, as evidenced by CV staining in untreated polystyrene 96-well plates (Fig. 4D). In contrast, at the same time point, wells inoculated with the Δ*yeaJ* mutant were CV-negative (Fig. 4D), suggesting that the mutant is defective in biofilm production under these conditions. The failure of the Δ*yeaJ* mutant to produce a biofilm was not due to a growth defect, because it achieved the same optical density as the WT strain (data not shown). Complementation of the Δ*yeaJ* strain resulted in partial restoration of the biofilm phenotype (Fig. 4D), while overexpression of YeaJ in a WT background resulted in pronounced pellicle formation at the air-liquid interface within 3 h of growth in LB broth and in significantly greater biofilm formation (Fig. 4C, D, and F). These data demonstrate that YeaJ, a putative DGC, is critical in *S.* Typhimurium biofilm formation and is possibly a major regulator of cellulose production at 25°C and 37°C in response to Sal4.

YeaJ is an active DGC in *S.* Typhimurium. To determine whether YeaJ is indeed an active DGC responsible for c-di-GMP production at 37°C in *S.* Typhimurium, bacterial cell lysates from the WT, Δ*yeaJ*, WT(pYeaJ), or WT(pBAD24) strains grown at 37°C were subjected to UPLC-MS-MS. c-di-GMP levels in lysates from WT(pYeaJ) cells were significantly higher (8.3-fold) than WT levels (Fig. 5; $P < 0.001$). In contrast, c-di-GMP levels in the Δ*yeaJ* strain of *S.* Typhimurium were reduced ~0.6-fold compared to the WT levels, although this difference was not statistically significant (Fig. 5). Finally, c-di-GMP levels were identical between the wild-type strains with and without the pBAD24 vector, confirming that the plasmid itself did not influence c-di-GMP production (see Fig. S3 in the supplemental material). These data demonstrate that YeaJ contributes to the total c-di-GMP pool at 37°C in *S.* Typhimurium, likely by functioning as an active DGC.

A role for YeaJ in *S.* Typhimurium invasion of epithelial cells *in vitro*. A number of DGCs, including STM1283, have been implicated in the regulation of the capacity of *S.* Typhimurium to invade intestinal epithelial cells (19, 21). In order to assess the specific role of YeaJ in this process, as well as to evaluate the impact of Sal4, we performed competitive uptake assays in HeLa cells. We expected that if YeaJ does indeed contribute to the overall pool of c-di-GMP in the cell, then a Δ*yeaJ* mutant would be expected to be more invasive than the WT *S.* Typhimurium, while a strain overexpressing YeaJ would be expected to be less invasive. To test this, WT strain 14028, the Δ*yeaJ* mutant, or the WT strain overexpressing YeaJ (WT+pYeaJ) was mixed one-to-one with SJF11 (Δ*oafA*) and incubated with or without Sal4 (5 μg/ml) for 45 min before being applied to HeLa cell cultures. As predicted, the Δ*yeaJ* mutant was slightly more invasive than the wild-type strain, whereas the YeaJ-overexpressing strain was slightly less invasive (Fig. 6). In the presence of Sal4, the invasion of all three strains was reduced by >80%, demonstrating that neither deletion nor overexpression of YeaJ bypasses the effects of Sal4 on bacterial invasion.

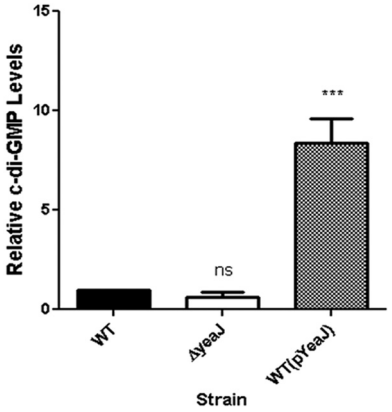

FIG 5 YeaJ possesses DGC activity shown by c-di-GMP levels in *S.* Typhimurium. WT, JA002 (Δ*yeaJ*), and WT+pYeaJ strains of *S.* Typhimurium were grown on M9 medium supplemented with 0.4% glucose to mid-log phase and were collected by centrifugation. Nucleotides were extracted from the cell pellets as described in Materials and Methods and then were subjected to UPLC-MS-MS. Levels of c-di-GMP were normalized relative to the WT strain (set to a value of 1). Statistical significance was determined by a one-way ANOVA, followed by Bonferroni's correction, compared to the control (WT): ***, $P < 0.001$; ns, not significant. Each bar represents the mean with the SEM.

DISCUSSION

Sal4 was described more than 20 years ago as an IgA MAb against the O5 epitope of LPS of *S.* Typhimurium that was protective against intestinal *Salmonella* infection, although the exact mechanism for this protection remains unclear (23, 24). Sal4 is neither bactericidal nor bacteriostatic (23, 55, 56). Sal4 does not activate complement nor does it promote Fc (fragment, crystallizable)-mediated clearance of opsonized bacteria (23, 57). Furthermore, although Sal4 is effective at promoting bacterial agglutination and entrapment in mucus, a phenomenon known as immune exclusion, these properties alone cannot fully account for the protective capacity of Sal4 (22, 26). We recently demonstrated that Sal4 treatment leads to an immediate inhibition in motility and decreased T3S activity in *S.* Typhimurium (28). Sal4 also triggers changes in OM integrity, accompanied by the appearance of a mucoid capsular material (28). In this study, we have demonstrated that prolonged exposure (>4 h) of *S.* Typhimurium to Sal4 results in detectable levels of EPS production that ultimately result in biofilm formation, at least *in vitro*. Sal4-induced EPS production and biofilm formation were found to be dependent on YeaJ, a member of the c-di-GMP network in *S.* Typhimurium. YeaJ has been demonstrated to regulate the bacterial transition from a motile and invasive state to a biofilm and noninvasive state in *E. coli* (32). Based on these data, we speculate that Sal4, by virtue of its ability to associate with the O-Ag and to induce OM stress, renders *S.* Typhimurium avirulent by triggering the c-di-GMP-dependent signaling pathway via YeaJ that leads to the suppression of bacterial motility and T3S, while simultaneously stimulating EPS production and biofilm formation.

In *S.* Typhimurium, EPS production is proposed to facilitate the ability of the bacterium to colonize inert and host-associated surfaces and to respond to various environmental stress conditions (58–61). For example, *S.* Typhimurium produces biofilms on abiotic and biotic surfaces, including glass, plastic (62), gall-

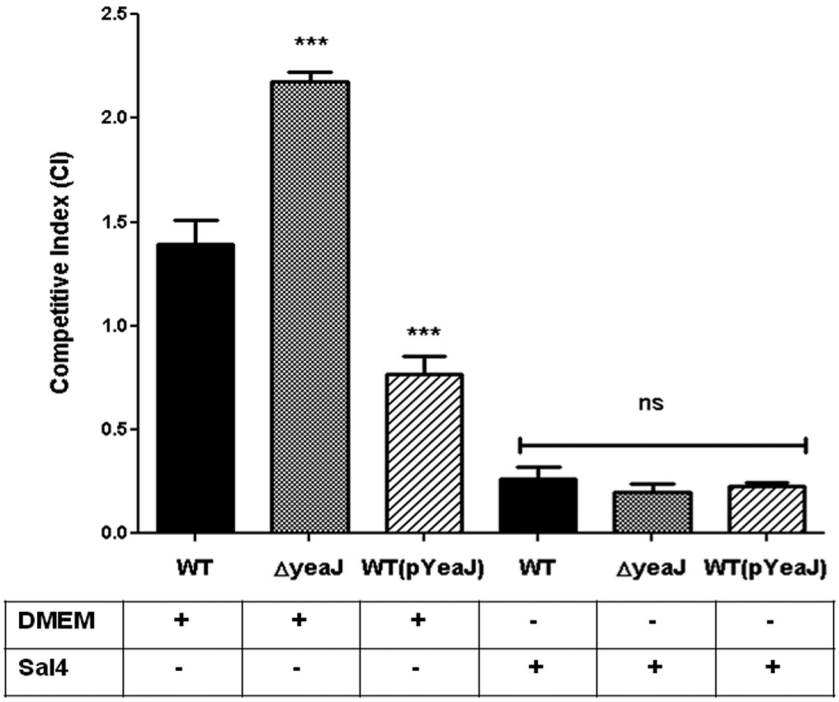

FIG 6 YeaJ plays a role in invasion of epithelial cells. The WT, Δ*yeaJ*, and WT+pYeaJ strains of serovar Typhimurium were mixed with the *oafA* mutant (SJF11) at a 1:1 ratio, incubated without antibody (DMEM alone) or with Sal4 (5 μg/ml) for 45 min, applied to HeLa cells seeded in 24-well plates, and subjected to centrifugation (10 min at 4°C) to promote bacterium-epithelial cell adherence, followed by incubation for 1 h at 37°C. The number of invasive bacteria was determined via a gentamicin protection assay (24). The competitive index (CI) reflects the invasion of WT, Δ*yeaJ*, or WT+pYeaJ strain cells to HeLa cells relative to the invasion of *oafA* mutant cells (data not shown). Statistical significance was determined by a one-way ANOVA, followed by Bonferroni's correction, compared to the control (WT/Δ*oafA*+DMEM): ***, $P < 0.001$. Each bar represents the mean with the SEM.

stones (12, 59), epithelial cells, and chicken intestinal tissues (63). In addition, *S.* Typhimurium has been shown to form biofilms as a defense against phagocytosis by host immune cells (64). Gunn and colleagues have demonstrated that *S.* Typhimurium is capable of producing biofilms *in vitro* in response to prolonged exposure to bile (e.g., 14 days), revealing that host factors can stimulate *S.* Typhimurium to initiate biofilm formation (30). In *S.* Typhimurium, EPS is primarily composed of three major polysaccharides: cellulose, O-Ag capsule, and colanic acid (29, 31, 40, 41, 65). In addition, fimbriae and the surface protein BapA are known to be critical components of the extracellular matrix (13, 65) (for review, see 20). The actual composition of the EPS produced by *S.* Typhimurium, however, varies based on environmental conditions and the nature of the colonized surfaces. For example, O-Ag capsule, but not cellulose or colanic acid, is a vital part of the biofilm formed on gallstones in response to bile (12). On the other hand, cellulose and colanic acid (as well as fimbriae), but not O-Ag capsule, are critical factors in biofilm formation on epithelial surfaces (12, 66).

EPS production in response to Sal4 is unique in at least two aspects. First, the onset of EPS production is virtually instantaneous and appears to persist for hours. The appearance of Sal4-triggered EPS production was initially observed by cryo-EM as being like a fuzzy coat within 15 min of antibody exposure (28). Scanning and transmission electron microscopy revealed that by 1 h, Sal4-treated cells were surrounded by a mucoid layer and formed a meshwork that is reminiscent of the earliest stages of biofilm formation (28). We speculate that the appearance of CV-positive staining at the air-liquid interface of *S.* Typhimurium grown for 24 h in the presence of Sal4 on borosilicate glass tubes and polystyrene microtiter plates represents a continuation of EPS production. Furthermore, our data suggest that Sal4-induced EPS consists of cellulose, O-Ag capsule, and colanic acid, because mutations in any one of these polysaccharide biosynthesis pathways resulted in reduced EPS production based on Western blot analysis of the EPS preparations and indicator agar plates. However, we cannot exclude the possibility that Sal4 triggers the production of an additional unidentified EPS. Therefore, studies are under way to determine the structure and exact composition of the EPS secreted by *S.* Typhimurium in response to Sal4.

Second, EPS production in response to Sal4 occurs at 37°C, not at ambient (25°C) temperature. This is unique because, in general, *S.* Typhimurium biofilm production is largely suppressed at elevated temperatures (i.e., 37°C), due to the temperature-dependent regulation of biofilm determinant genes by CsgD (53, 54). The fact that Sal4-induced biofilm production requires a functional CsgD (see Fig. S2 in the supplemental material) is consistent with the previous findings that show that CsgD is essential for the c-di-GMP-mediated induction of biofilm formation (67). This finding also suggests that the antibody stimulates a signaling pathway(s) in *S.* Typhimurium that overrides the temperature-dependent regulation by CsgD. This would not be unprecedented, as the temperature-dependent regulation by CsgD can be circumvented (at least genetically) at 37°C in order to express curli fimbriae and cellulose (13, 68–70). In addition, Römling and colleagues have demonstrated that upon iron starvation, the temperature-independent expression of Tafi occurred at 37°C (13). Thus, Sal4 is a unique environmental signal that induces biogenesis of EPS in *S.* Typhimurium at host temperatures. To our knowledge, these data represent the first demonstration of a specific host-derived molecule that stimulates a c-di-GMP network in *S.* Typhimurium.

It has been demonstrated that SIgA can promote the formation of biofilms by commensal bacteria, like *E. coli*, on intestinal epithelial surfaces (22, 71). Bacteria grown *in vitro* in the presence of high SIgA concentrations (e.g., 0.5 mg/ml) were capable of colonizing epithelial cell surfaces and of forming a microbial mat, whereas cells grown in the absence of SIgA were not (71). This phenomenon was determined to be the result of SIgA serving as a scaffold for bacterial colonization, not as a response of *E. coli* to SIgA per se. Specifically, *E. coli* bound to oligosaccharide side chains on SIgA by virtue of its mannose-specific type-1 pilli. Thus, the interaction between *E. coli* and IgA is bacterially mediated and is independent of the variable (Fv) regions of IgA. This is in contrast to the interaction between *S.* Typhimurium and Sal4, which occurs solely through the association of Sal4 with O5-Ag, as a strain of *S.* Typhimurium that lacks the O5-Ag does not produce EPS or form a biofilm in response to Sal4.

Sal4 promotes the transition of *S.* Typhimurium from a virulent to an avirulent modality, in which the loss of motility and T3S is accompanied by increased EPS production. This inversely proportional regulation of motility and EPS production is reminiscent of that of the c-di-GMP regulatory pathways in enteric bacteria (32, 50, 72–75). Thus, it is intriguing that we found that EPS production and biofilm formation in response to Sal4 are dependent on YeaJ, a suspected member of the DGC family. The *S.* Typhimurium YeaJ was annotated as a DGC based on its sequence similarity with known DGCs (49). Garcia and colleagues provided the first functional evidence to support YeaJ as a possible DGC, in that they demonstrated that YeaJ expressed on a plasmid complemented the biofilm deficiency of a Δ*gcpA* mutant (*gcpA* is a known member of the c-di-GMP network) (51). Furthermore, recent genetic studies have indicated YeaJ as being involved in rdar colony formation, flagellum-based motility, and cellulose production in *S.* Typhimurium (21, 76).

We put forth several lines of evidence that demonstrate that the YeaJ protein does indeed possess DGC activity that ultimately contributes to c-di-GMP levels in *S.* Typhimurium. We found that the ectopic overexpression of YeaJ in *S.* Typhimurium at room temperature results in pronounced pellicle formation at the air-liquid interface and in significantly greater biofilm formation at 7 days. Overexpression of YeaJ in the WT background also enhanced EPS production and biofilm formation at 37°C in response to Sal4. Another line of evidence for the significant capacity of YeaJ to govern the Sal4-induced biofilm formation at 37°C is its ability to complement the biofilm defect of a *bscE*::*kan* mutant, a cellulose mutant. We observed that when a functional copy of *yeaJ* was introduced in *trans* to the *bscE*::*kan* mutant, the Sal4-induced biofilm defect of this mutant was fully restored (see Fig. S1 in the supplemental material). Finally, we found that the overexpression of YeaJ resulted in an 8-fold increase in baseline c-di-GMP levels in *S.* Typhimurium compared to that in a WT control (Fig. 5). It should be noted that we quantitated c-di-GMP levels using UPLC-MS-MS, a technique that is considerably more sensitive than standard high-performance liquid chromatography (HPLC), which likely explains why others have not noted YeaJ-induced changes in c-di-GMP levels (21).

Interestingly, although the overexpression of YeaJ in *S.* Typhimurium resulted in an increase in the intracellular pool of c-di-GMP at 37°C, this itself was not sufficient to promote biofilm formation at this elevated temperature. We think that this finding is significant because it may imply that EPS production and bio-

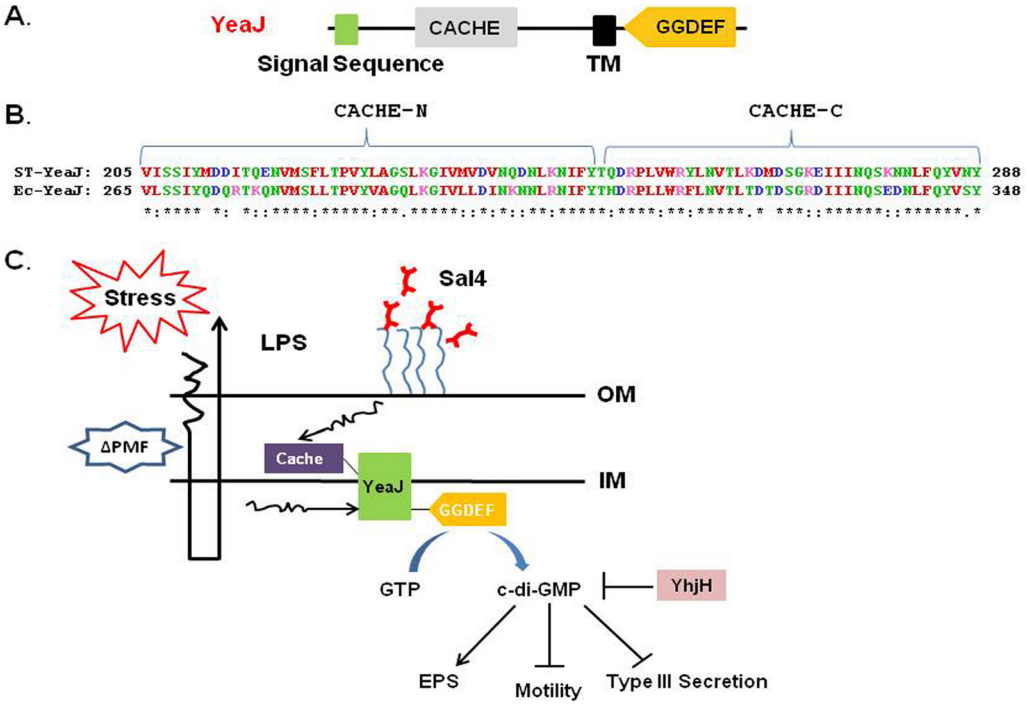

FIG 7 Model for Sal4-induced EPS production and biofilm formation. (A) Schematic linear depiction of the structure of YeaJ, highlighting the putative N-terminal signal sequence, periplasmic Cache domain, a transmembrane (TM) domain, and the GGDEF domain; (B) ClustalW-sequence alignment of the Cache domains of YeaJ from *S.* Typhimurium (ST) and *E. coli* (Ec). (C) Proposed mechanism for YeaJ-mediated c-di-GMP signal transduction in *S.* Typhimurium in response to Sal4. Sal4 binding to the O-Ag results in OM stress, including a transient reduction in the PMF. We propose that the Cache domain of YeaJ senses Sal4-induced changes in membrane integrity and, in turn, activates the GGDEF domain of YeaJ to produce c-di-GMP. Elevated levels of c-di-GMP stimulate EPS production and reduce both bacterial motility and T3S. We speculate that the cognate phosphodiesterase (PDE) YhjH degrades the c-di-GMP produced by YeaJ.

film formation by *S.* Typhimurium in response to Sal4 require two signals. In addition to elevated intracellular c-di-GMP levels, we propose that a second signal related to Sal4-induced changes to the OM may be required. We recently reported that the association of Sal4 with the O-Ag of *S.* Typhimurium results in the shedding of LPS, a transient reduction in the PMF, and a corresponding decrease in intracellular ATP levels (28). Any one of these physiologic changes in the OM can serve as the secondary cue to initiate EPS production and biofilm formation by *S.* Typhimurium.

Indeed, it is interesting to speculate that YeaJ may sense Sal4 binding to the bacterial surface, possibly as a consequence of antibody-mediated OM stress (28). In addition to its conserved GGDEF domain that is involved in diguanylate cyclase activity, YeaJ has a putative extracellular sensory Cache (Ca^{2+} channels, chemotaxis receptors) domain that is virtually identical to that of YeaJ in *E. coli* (Fig. 7A and B). In general, Cache domains sense stimuli present in the periplasm and transmit signals to an output domain, such as GGDEF, resulting in a specific adaptive response (77–80). It has been proposed that Cache domains sense changes in the periplasm or inner membrane. Current studies are under way to understand the role of the Cache domain of YeaJ in sensing Sal4 antibody and in inducing EPS and biofilm formation.

In conclusion, we propose that the binding of Sal4 to the O-Ag of *S.* Typhimurium activates YeaJ, which in turn stimulates intracellular levels of c-di-GMP, thereby leading to a loss in motility, a reduction in T3S activity, and an increase in EPS production (Fig. 7C). Moreover, we speculate that Sal4-induced EPS production represents an adaptive mechanism employed by *S.* Typhimurium to shed antibody and/or to increase resistance to secondary insults, such as antimicrobial peptides and other factors present in intestinal secretions. This study provides further insight into how SIgA-mediated immunity may occur in the gut, and it underscores how antibodies may exert their effects differently depending on the environmental conditions (27). In systemic compartments, for example, the opsonization of *Salmonella* by surface antigen-specific IgG leads to complement-mediated killing and Fc-dependent phagocytosis (81). In the gut, we propose that the opsonization of *S.* Typhimurium by Sal4 (or another IgA antibody of similar specificity) induces a form of outer membrane stress that is sufficient to incapacitate both SPI-1 T3S and flagellum-based motility (28). Moreover, we would now argue that prolonged exposure to Sal4 promotes the transition of *S.* Typhimurium from a motile and invasive state to a nonmotile and noninvasive state. Once *S.* Typhimurium has been coated with SIgA and effectively immobilized in the intestinal lumen, it is likely cleared through peristalsis. Additionally, there is evidence that SIgA-coated microbes are actively sampled by intestinal M cells and are transported to underlying gut-associated lymphoid tissues (22, 82). Taken together, our study advances our fundamental understanding of how SIgA may exert its protective effects at mucosal surfaces, without the assistance of Fc-mediated effector functions.

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We declare no conflicts of interest.

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