Bistable Expression of CsgD in *Salmonella enterica* Serovar Typhimurium Connects Virulence to Persistence

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Pathogenic bacteria often need to survive in the host and the environment, and it is not well understood how cells transition between these equally challenging situations. For the human and animal pathogen *Salmonella enterica* serovar Typhimurium, biofilm formation is correlated with persistence outside a host, but the connection to virulence is unknown. In this study, we analyzed multicellular-aggregate and planktonic-cell subpopulations that coexist when *S. Typhimurium* is grown under biofilm-inducing conditions. These cell types arise due to bistable expression of CsgD, the central biofilm regulator. Despite being exposed to the same stresses, the two cell subpopulations had 1,856 genes that were differentially expressed, as determined by transcriptome sequencing (RNA-seq). Aggregated cells displayed the characteristic gene expression of biofilms, whereas planktonic cells had enhanced expression of numerous virulence genes. Increased type three secretion synthesis in planktonic cells correlated with enhanced invasion of a human intestinal cell line and significantly increased virulence in mice compared to the aggregates. However, when the same groups of cells were exposed to desiccation, the aggregates survived better, and the competitive advantage of planktonic cells was lost. We hypothesize that CsgD-based differentiation is a form of bet hedging, with single cells primed for host cell invasion and aggregated cells adapted for persistence in the environment. This allows *S. Typhimurium* to spread the risks of transmission and ensures a smooth transition between the host and the environment.

Salmonella persistence, or the ability of cells to survive under stressful conditions, has traditionally been studied separately from virulence. The continued success of these pathogens, however, indicates that both traits must be inherently connected. Most of the >2,000 serovars of *Salmonella* collectively account for approximately 94 million annual cases of human gastroenteritis (1), a disease characterized by intestinal inflammation and diarrhea (2). The gastroenteritis-causing serovars are referred to as nontyphoidal *Salmonella* (NTS), and *Salmonella enterica* serovar Typhimurium is one of the serovars most commonly isolated in the world. Most NTS isolates are considered to be host generalists, with a cyclical lifestyle involving exposure to the environment between host colonization events (3). This is in contrast to typhoidal *Salmonella* serovars (i.e., *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi A), which are human restricted (4, 5). Despite their differences, both NTS and typhoidal *Salmonella* strains rely on type three secretion systems (T3SS) to stimulate invasion, uptake, and survival within human epithelial cells and macrophages (6). While our understanding of pathogenesis is improving, less is known about the strategies that pathogenic *Salmonella* strains employ to survive outside the host (7, 8). It is generally assumed that people become infected with *Salmonella* through ingestion of contaminated food and water, but there are also numerous examples of sporadic human cases where the causative source is unknown (9).

One strategy for bacterial survival is through the formation of biofilms, where cells aggregate and become embedded in a self-produced extracellular matrix, usually in contact with a surface. This has been described in *Salmonella* as the rdar (red, dry, and rough) morphotype (10, 11). The extracellular matrix surrounding rdar-positive cells is comprised of protein polymers (curli fim-

briae) and extracellular polysaccharides (cellulose and an O-anti-gen capsule) that physically link cells together (10–13). The genes coding for these polymers are conserved throughout *Salmonella* (14, 15) and *Escherichia coli* (16, 17). Comparisons between rdar-positive and rdar-negative *S. Typhimurium* and *E. coli* strains have shown that embedded cells have enhanced persistence upon exposure to conditions of starvation, desiccation, and treatment with disinfectants (15, 18–21), as well as an increased ability to adhere to both biotic and abiotic surfaces (22, 23). Rdar-positive cells can also cause infections after long periods (24) but do not appear to be specifically adapted for virulence (25). Rdar-negative *Salmonella* and *E. coli* isolates tend to cause more invasive forms of disease (26, 27).

Most NTS isolates are rdar positive, and most typhoidal *Salmonella* isolates are rdar negative (21, 25, 26). One possible explanation for this correlation is that the rdar morphotype is an adap-

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tation necessary for life outside the host. However, there have been recent links between the rdar morphotype, immune stimulation, and invasion (28, 29), making it difficult to fully understand this physiology. Although they are informative, a potential problem with strain-to-strain comparisons is that they could miss fluctuations that exist within individual populations of cells. It was recently demonstrated that when *S. Typhimurium* is grown under biofilm-inducing conditions in liquid medium, the population of cells in the fluid phase differentiates into large, multicellular aggregates and single (planktonic) cells (25, 30). This bifurcation is caused by the bistable synthesis of CsgD, the central transcriptional regulator of the rdar morphotype (31). Aggregated cells are in a CsgD-on state, whereas the nonaggregative planktonic cells are primarily in a CsgD-off state. The multicellular aggregates can be thought of as noncanonical biofilms, as the cells strongly express extracellular matrix components (25, 30) but are not in direct contact with a surface. While it is assumed that the aggregates retain the resistance properties of classic adherent biofilms, the planktonic cells that result from CsgD bistability have never been analyzed in detail, and it is unclear what role they may have in *S. Typhimurium* physiology.

In this study, we isolated and examined these two groups of cells and demonstrate for the first time that they could represent a critical link between the virulence and persistence of *Salmonella*. Bistability and the formation of specialized cell types are predicted to contribute to *S. Typhimurium* transmission success, with single cells adapted for host cell invasion and multicellular aggregates adapted for persistence in the environment.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *S. enterica* serovar Typhimurium strain ATCC 14028 was the wild-type strain used in this study. In general, strains were inoculated from frozen stocks onto LB agar and grown for 20 h at 37°C. One isolated colony was used to inoculate 5 ml LB broth (containing 1% NaCl), and the culture was incubated for 12 h at 37°C with agitation at 200 rpm. To analyze growth under biofilm-inducing conditions, approximately 4×10^9 CFU was inoculated into 400 ml of 1% tryptone, pH 7.4, and the culture was distributed in 100-ml volumes into four 250-ml flasks that were incubated at 28°C for 8 h, 13 h, 18 h, or 32 h with agitation at 200 rpm. The *csgD* promoter (*P_{csgD}*) *luxCDABE* fusion plasmid used for bioluminescence assays has been previously described (21). To measure luminescence, 200- μ l aliquots of culture were removed at hourly intervals in triplicate using a wide-bore pipette, transferred into individual wells of a 96-well clear-bottom black plate (product 9520; Corning Life Sciences), and assayed on a Victor X³ multilabel plate reader (Perkin-Elmer Life Sciences).

Generation of *S. Typhimurium* 14028 Δ csgD and Δ SPI-1 mutant strains. Δ csgD and Δ SPI-1 strains were generated using the lambda red recombinase knockout procedure (32). Primers containing a 50-nucleotide sequence on either side of the entire *Salmonella* pathogenicity island 1 (SPI-1) region (SPI-1_F and SPI-1_R) (33) or *csgD* (*csgD*ko sense [GCAGC TGTCAGATGTGCGATTAAAAAGTGGAGTTTCATCATGTTTAA TGTGTAGGCTGTAGCTGCTTC] and *csgD*ko antisense [CTCTGCTG CTACAATCCAGGTCAGATAGCGTTTCATGGCCTTACCGCCTGCC TCCTTAGTTCCTATTCGG]) were used to amplify the *cat* gene from pKD3 using Phusion High-Fidelity DNA polymerase (New England Biolabs). The PCR products were purified (AxyPrep Mag PCR cleanup kit; Axygen) and electroporated into *S. Typhimurium* 14028 cells containing pKD46. Mutant strains were first selected by growth at 37°C on LB agar supplemented with 7 μ g ml⁻¹ chloramphenicol (Cm) before restreaking onto LB agar containing 30 μ g ml⁻¹ Cm. The mutations were moved into clean wild-type backgrounds by transduction with P22 phage (34). The *cat* gene was resolved from the chromosome using pCP20 (32). DNA

sequencing of PCR products amplified from the chromosome of the Δ csgD or Δ SPI-1 strain confirmed the loss of the majority of *csgD* or SPI-1, respectively.

Generation of Kn^r or Cm^r *S. Typhimurium* strains. sig70_16 is a reporter plasmid that contains a synthetic σ^{70} -dependent promoter driving *luxCDABE* expression (35). A chloramphenicol-resistant (Cm^r) version of the sig70_16 plasmid was generated, and the sig70_16 kanamycin-resistant (Kn^r) or Cm^r fragments were used for Tn7 integration (36) into the *S. Typhimurium* ATCC 14028 chromosome, using a modified protocol (D. Shivak, K. MacKenzie, B. Jones, and A. White, submitted for publication). To confirm that the fragments had been properly inserted into the *att* Tn7 site downstream of the *glmS* gene of *S. Typhimurium*, the chromosomal region was PCR amplified and the resulting products were sequenced. To ensure the absence of secondary mutations, the Kn^r and Cm^r sig70_16 fragments were moved into a clean wild-type or Δ csgD background by P22 transduction.

Isolation of multicellular-aggregate and planktonic-cell subpopulations. *S. Typhimurium* biofilm cultures were transferred into 15-ml conical tubes and subjected to low-speed centrifugation (210 $\times$ g; 2 min; 4°C). The supernatant fractions were transferred into new 15-ml tubes, and the cell aggregates were transferred into 1.5-ml tubes using a sterile loop. After brief centrifugation (10,000 $\times$ g; 30 s), the culture medium was removed and the wet weight of the aggregates was determined. The aggregates were resuspended in 1 ml of phosphate-buffered saline (PBS) (for direct counting) or 1 ml cell-free 1% tryptone culture supernatant (for subsequent applications) and homogenized for approximately 30 s (25 Dounce homogenizations) with a glass tissue grinder (product 7727-2; Corning Life Sciences). For CFU determination, serial dilutions of planktonic cells or homogenized aggregates were plated in 4- μ l drops onto LB agar prior to incubation overnight at 37°C.

Visualizing the homogenization of multicellular aggregates. Multicellular aggregates were harvested from an *S. Typhimurium* 14028 reporter strain containing a *csgBAC* promoter-green fluorescent protein (GFP) fusion (25) and grown for 13 h (time point 2 [TP2]). Ten milligrams of aggregates was resuspended in 1 ml of cell-free culture supernatant and homogenized in a tissue grinder, and two successive 1-in-10 dilutions were used to prepare a solution of 1.2×10^7 CFU/ml. Ten 200- μ l aliquots were individually concentrated onto the surfaces of 3-in. by 1-in. by 1.0-mm glass slides (Fisher Scientific; 12-550-343) using a Cytospin-4 cytocentrifuge (Thermo Shandon, Asheville, NC). Samples were centrifuged at 500 rpm for 5 min with low acceleration. Five microliters of Mowiol mounting medium (Sigma-Aldrich; 81381) was added to the cells on each slide, and each mixture was topped with a 12-mm-diameter micro-cover glass (72230-01; Electron Microscopy Sciences). The slides were air dried overnight prior to visualization on a Leica TCS SP5 confocal microscope, using a 63 $\times$ oil immersion objective with the laser operating at 488 nm.

SDS-PAGE and Western blotting. Planktonic cells were sedimented (11,000 $\times$ g; 10 min; 4°C) to concentrate the samples before suspending them in 1 ml of SDS-PAGE sample buffer (without 2-mercaptoethanol and bromophenol blue). Samples (100 mg) of multicellular aggregates were resuspended in 1 ml of water and homogenized with a glass tissue grinder. The cell material was sedimented (10,000 $\times$ g; 1 min) before resuspending it in 1 ml of SDS-PAGE sample buffer and boiling it for 5 min. Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories), samples were normalized to 30 μ g of total protein per lane, and bromophenol blue (0.0002% final concentration) and 2-mercaptoethanol (0.2% final concentration) were added. SDS-PAGE was performed with a 5% stacking gel and a 12% resolving gel or with 10%, 12%, or 4 to 15% precast Mini-Protean TGX gels (Bio-Rad Laboratories). For immunoblots, proteins were transferred to nitrocellulose for 40 min at 25 V using a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories) in buffer recommended by the manufacturer. For detection of curli fimbriae, cell samples were boiled for 5 min in SDS-PAGE sample buffer with 2-mercaptoethanol, and the cell debris was sedi-

mented ($14,000 \times g$; 2 min) and washed twice in 500 μ l of distilled water before suspension in 250 μ l of 90% formic acid, freezing at -80°C , and lyophilization (10). The dried samples were resuspended in SDS-PAGE sample buffer and loaded directly, without boiling, into each gel lane prior to electrophoresis.

CsgD was detected using the monoclonal antibody described below. CsgA, the major subunit of curli fimbriae, was detected using rabbit polyclonal immune serum raised to whole purified curli (10). GroEL was used as a protein-loading control and was detected with rabbit polyclonal immune serum used at a 1-in-10,000 dilution (Sigma-Aldrich; G6532). SPI-1 T3SS proteins (InvG, SipD, SopE2, and SopB) were detected with rabbit polyclonal immune sera generated against each of the individual proteins (33). The secondary antibodies were goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase or human-adsorbed goat-anti-mouse IgG-alkaline phosphatase conjugates (KPL). Proteins were detected by dye precipitation using the substrate 5-bromo-4-chloro-3-indolylphosphate with 4-nitroblue tetrazolium chloride (Sigma-Aldrich) as an enhancer.

Generation of CsgD-specific monoclonal antibodies. *csgD* was PCR amplified from *S. Typhimurium* 14028 genomic DNA using Phusion High-Fidelity DNA polymerase (New England BioLabs) and primers *csgD_expFOR* (GGCTCATATGTTTAATGAAGTCCATAG) and *csgD_expREV2* (ATTCTCTGAGCCGCTGAGATTATCGTTTG). The PCR product was digested with NdeI and XhoI and ligated into NdeI/XhoI-digested pET-21a (Novagen), using T4 DNA ligase (NEB), to generate a vector encoding CsgD with a 6 $\times$ His tag at the C-terminal end. *E. coli* BL21(DE3) plus pET21a-*csgDHis* was grown at 37°C with agitation at 200 rpm in LB broth supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin until an optical density at 600 nm (OD_{600}) of 0.7 was reached; expression of CsgD-His was induced by adding IPTG (isopropyl- β -D-thiogalactopyranoside) to a final concentration of 1 mM and growing the cells for 2 h. Cells were harvested and lysed, and CsgD-His was purified using a Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen), following procedures outlined by the manufacturer (Qiagen; QIAexpressionist, protocol 9). Approximately 5 mg of purified CsgD-His was used for Rapid Prime custom monoclonal antibody development (ImmunoPrecise Antibodies Ltd., Victoria, BC, Canada). Concentrated tissue culture supernatants containing a CsgD-specific monoclonal antibody were used for Western blotting experiments.

Measurement of c-di-GMP levels inside cells. Planktonic cells and multicellular aggregates were harvested from 100-ml *S. Typhimurium* biofilm cultures grown for 8 h (TP1) or 13 h (TP2). Planktonic-cell samples were prepared by centrifugation ($10,000 \times g$; 10 min), and multicellular-aggregate samples were homogenized as described above in cell-free culture supernatant. Cyclic-di-GMP (c-di-GMP) extractions were performed with 100 μ l of 40% methanol-40% acetone in 0.1 N formic acid, used for every 48 mg (wet weight) of cells (37). Cell samples were transferred into screw-cap tubes containing 0.2 g of 0.1-mm zirconia beads, and homogenization was performed in a high-speed mixer mill (Retsch; MM400) for 2 min at 30 Hz. After centrifugation ($16,000 \times g$; 2 min), the supernatant was neutralized by the addition of 4 μ l of 15% ammonium bicarbonate per 100 μ l of sample. Aliquots (50 μ l) of each sample were dried down in a speed vacuum concentrator, and the residues were dissolved in 300 μ l of 30% methanol, followed by solid-phase extraction with Agilent OPT 30-mg/ml reversed-phase cartridges (Agilent; 5982-3013) to remove lipids and other lipophilic compounds. The flowthrough fractions were collected under a 3- to 5-lb/in² vacuum, and the cartridges were washed with 400 μ l of 30% methanol. The flowthrough fractions were collected and pooled for each sample and dried down in a speed vacuum concentrator. The residues were resuspended in 50 μ l of 80% acetonitrile, and 20- μ l aliquots were injected into a Dionex UPLC 3000 system coupled to an AB Sciex 4000 QTrap mass spectrometer, which was operated in the electrospray ionization and negative-ion multiple-reaction-monitoring (MRM) mode. The MRM transition for quantitation was 689.2/344.2. Ultra-performance liquid chromatography (UPLC) separation was carried

out on a 10-cm-long UPLC column, which was run in a hydrophilic interaction (HILIC) mode. The mobile phase was ammonium acetate-acetonitrile for binary gradient elution. The column temperature was 30°C , and the flow rate was 0.40 ml/min. A standard curve for cyclic-di-GMP (Axxora; BLG-C057) was prepared within a concentration range of 2 to 400 fmol/ml. The concentration of cyclic-di-GMP in each sample was calculated with external calibration. Since multicellular aggregates consist of both cells and extracellular matrix, a conversion factor was applied to all c-di-GMP concentrations. One milligram (wet weight) of planktonic cells was equivalent to 6.94×10^8 CFU, whereas 1 mg (wet weight) of multicellular aggregates was equivalent to 1.73×10^8 CFU. This conversion was thought to represent a more accurate cell-to-cell comparison between samples.

RNA purification procedure. Total RNA was isolated using a modified procedure based on the SV Total RNA Isolation kit (Promega). At TP1, cells were sedimented by centrifugation ($10,000 \times g$; 2 min; 4°C) and resuspended in 1 ml of RNeasy Lysis Buffer (Life Technologies). At later time points, cell subpopulations were first separated by low-speed centrifugation, as described above. Planktonic-cell fractions were sedimented as described above and resuspended in 1 ml of RNeasy Lysis Buffer (Life Technologies). Multicellular-aggregate samples were resuspended in 1 ml of RNeasy Lysis Buffer and homogenized for approximately 30 s using a tissue grinder. Cells in 1 ml of RNeasy Lysis Buffer were sedimented by centrifugation ($13,200 \times g$; 1 min) and resuspended in 700 μ l of RNA lysis buffer A (RLA). This cell suspension was transferred to a 1.5-ml screw-cap tube containing 0.2 g of 0.1-mm zirconia beads and homogenized in a high-speed mixer mill (Retsch; MM400) for 5 min at 30 Hz. Approximately 300 to 400 μ l RLA solution was recovered from the beads, and 600 to 800 μ l of RNA dilution buffer was added, followed by 0.4 volume of 95% ethanol. Samples were centrifuged ($13,200 \times g$; 10 min) to remove extracellular debris that precipitated upon addition of ethanol prior to loading onto the RNA purification column. This step was necessary to avoid plugging the column, especially with the multicellular-aggregate samples. The column-washing steps and on-column DNase I treatment were performed according to the manufacturer's instructions. Bound RNA was eluted from each column twice with 50 μ l of RNase-free water for a final volume of 100 μ l. After elution, samples were DNase treated a second time in solution using a Turbo DNA-free kit (Life Technologies; AM1907). The MicroExpress bacterial mRNA enrichment kit (Life Technologies; AM1905) was used for mRNA enrichment. To assess the quantity, purity, and integrity of RNA, samples were analyzed using a NanoDrop ND-1000 spectrophotometer (Fisher Scientific) and an Agilent 2100 Bioanalyzer with a Prokaryote Total RNA Nano chip.

Directional transcriptome sequencing (RNA-seq). For sequencing on the Illumina HiSeq 2000, first-strand cDNA was synthesized from RNA using random-hexamer priming and reverse transcriptase. Second-strand cDNA synthesis was performed with DNA polymerase I in the presence of dATP, dGTP, dCTP, and dUTP. Double-stranded cDNA was fragmented by sonication, and 70- to 200-bp fragments were purified with AMPure magnetic beads (Beckman-Coulter). After the standard addition of 18 μ l of AMPure beads to every 10 μ l of sample, 9.3 μ l isopropanol was added. This step allows recovery of fragments below 100 bp, as described in the AxyPrep Mag PCR Cleanup kit protocol (Axygen Biosciences Inc.). cDNA fragments were ligated to Illumina adapters and primers containing bar-coded sequences; fragment sizes in the final library ranged from approximately 196 to 326 bp. The second cDNA strand containing UTP was selectively removed by uridine digestion (37°C ; 15 min) in 5 μ l of 1 $\times$ Tris-EDTA (TE) buffer, pH 7.5, with 1 unit of uracil-N-glycosylase (UNG) (Applied Biosystems) prior to library amplification and sequencing (38). In total, 21 libraries were sequenced (75-bp paired-end tag [PET] sequencing), prepared from three biological replicates of planktonic cells (samples at TP1, TP2, TP3, and TP4) and multicellular aggregates (samples at TP2, TP3, and TP4).

For sequencing on the Illumina MiSeq, cDNA libraries prepared from *S. Typhimurium* 14028 Δ *csgD* at TP2 were constructed using the

NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England BioLabs) according to the manufacturer's protocol. This differs from the above-mentioned protocol in that fragmentation occurs through autocatalysis of RNA before first-strand cDNA synthesis. The final library fragment sizes were 200 to 300 bp. The approach also incorporates dUTP into the second cDNA strand synthesis, followed by digestion of the second strand before PCR amplification. The 75-bp PET sequencing was performed on three libraries using the MiSeq reagent kit version 3 (Illumina).

Mapping and comparison of sequencing reads. Sequencing reads were mapped onto the *S. Typhimurium* ATCC 14028 chromosome (NC_016856) and plasmid (NC_016855) sequence using Geneious v5.6.5 (Biomatters). The mapping parameters were set as follows: (i) gaps not allowed, (ii) words repeated more than twice ignored, and (iii) maximum mismatches per read set as 4%. The other parameters conformed to the default settings of Geneious. The coordinates and strand of each putative protein-encoding gene were according to genome annotations (39), and in-house perl scripts were used for counting the mapping reads to each protein-encoding gene and its antisense strand. The small-RNA (sRNA) coverage was determined in a similar way. The full set of known *Salmonella* sRNAs (40) were retrieved, and the orthologous counterparts in *S. Typhimurium* 14028 were found by direct sequence alignment.

Before comparing the gene expression levels between samples, the counts of mapped reads were normalized with edgeR, which, instead of using RPKM notation, assumes that the total RNA amount could vary among different samples and that expression of more genes should be stable among libraries (41). A negative binomial distribution was fitted for the normalized read counts for each pair of genes. Differentially expressed genes were identified as those with *P* values of <0.05 , determined by a Benjamini and Hochberg correction for the multiple testing results (42, 43), with the false-discovery rate (FDR) set as <0.05 . The normalization effect was evaluated before data analysis by observing the variance among technical and biological repeats after normalization.

Motility comparison between planktonic cells and multicellular aggregates. Cell subpopulations were isolated from *S. Typhimurium* biofilm cultures at TP2 and either tested for motility directly or after homogenization for 30 s in a tissue grinder. One-microliter aliquots (approximately 5×10^6 CFU) of each cell suspension were inoculated into the middle of swim agar (lysogeny broth [Luria] plus 0.25% agar), and the plates were allowed to dry for 20 min. Migration distances from the center of the plate were recorded after incubation at 37°C for 8 h. Five technical replicates of each sample were tested, prepared from three biological replicate cultures. The statistical difference between samples was calculated using unpaired *t* tests.

Invasion assay using polarized Caco-2 cells. Caco-2 cells (obtained from Brett Finlay, University of British Columbia) were grown at 37°C in the presence of 5% CO₂ in complete Dulbecco modified Eagle medium (DMEM) (HyClone; Fisher Scientific) supplemented with 10% fetal bovine serum (Seracare Life Sciences) and 1% nonessential amino acids (Life Technologies). To obtain polarized monolayers, Caco-2 cells were seeded onto Transwell inserts (24-mm diameter; 0.4-μm pore size; product 3470; Corning Life Sciences) for approximately 21 days. The cells were used for invasion assays once the transepithelial resistance (TER) was 700 to 900 Ω cm⁻² (33). Planktonic cells and multicellular aggregates isolated from *S. Typhimurium* cultures were suspended in complete DMEM and applied in 200-μl aliquots to the apical surfaces of the Caco-2 cells; invasion in three replicate wells was assessed for every sample. Wild-type and ΔSPI-1 mutant strains grown to late exponential phase at an OD₆₀₀ of 0.7 in LB broth at 37°C were used as positive and negative invasion controls, respectively. After 1 h exposure to *S. Typhimurium*, Caco-2 cells were washed three times with 200 μl of PBS and incubated for 2 h with complete DMEM containing 1.2 mg ml⁻¹ gentamicin to kill any remaining extracellular bacteria. The Caco-2 cells were washed two times with 200 μl of PBS and lysed by exposure to 1% Triton. Serial dilutions of the *Salmonella*-containing lysate were plated in 4-μl drops on LB agar and incubated

overnight at 37°C. We aimed for a multiplicity of infection (MOI) of 10 based on an estimated 2.5×10^5 polarized Caco-2 cells per well. The true MOI was determined by serial dilution and plating on LB agar, followed by incubation overnight at 37°C.

Gentamicin susceptibility testing. Samples containing approximately 5×10^7 CFU ml⁻¹ of planktonic cells or multicellular aggregates isolated from *S. Typhimurium* 14028 cultures were prepared in cell-free, spent culture medium. One-milliliter aliquots were centrifuged ($8,000 \times g$; 2 min), and the cells were resuspended in 1 ml of complete DMEM containing gentamicin at concentrations ranging from 0.2 to 1.2 mg ml⁻¹. After 2 h exposure, the cells were sedimented, washed, resuspended in PBS, and homogenized for 30 s using a tissue grinder. Serial dilutions of each sample were plated on LB agar and grown overnight at 37°C to determine the number of CFU. Four technical replicates of each sample were tested, and the experiment was repeated three times. The statistical difference between samples was calculated using unpaired *t* tests.

CI infections of mice. Female C57BL/6 mice (6 to 8 weeks old) purchased from Jackson Laboratory (Bar Harbor, ME) were assigned to cage groups using a randomization table prepared in Microsoft Excel, and individual mice were marked with ear notches. Challenge formulations A and B were prepared in 100 mM HEPES, pH 8, at a concentration of $\sim 10^7$ CFU per 100 μl, consisting of an approximate 1:1 ratio of cells isolated from Cm^r or Kn^r *S. Typhimurium* cultures at TP2 (see Fig. S6 in the supplemental material). Animal care technicians assigned challenges A and B to 2 cages each (i.e., 4 groups of 6 mice; *n* = 24) and performed all infections. The cage assignments were revealed to members of the White laboratory only after the final results were analyzed. The infected mice were weighed daily and monitored for clinical signs of infection; mice that had a $>20\%$ drop in weight were euthanized, typically 4 to 7 days postinfection. The spleen, liver, cecum, and mesenteric lymph nodes (MLN) were homogenized in Eppendorf Safe-Lock tubes containing 1 ml of PBS and a 5-mm stainless steel bead (Qiagen; 69989) using a high-speed mixer mill (Retsch; MM400). To determine the number of CFU in the initial challenges or organ homogenates, serial dilutions were plated on LB agar supplemented with 10 μg ml⁻¹ Cm or 50 μg ml⁻¹ Kn. The competitive index (CI) was calculated as follows: (CFU planktonic/CFU biofilm)_{output}/(CFU planktonic/CFU biofilm)_{input}. For statistical analysis, the normality of each set of CI data was assessed using the Shapiro-Wilk normality test, followed by the Wilcoxon signed-rank test to determine if the median CI was significantly different from 1.

Input and output ratios for CI infections. For the initial CI trial, the input ratio for challenge A was 0.76 (7.9×10^6 CFU planktonic cells and 1.0×10^7 CFU aggregates) and that for challenge B was 0.60 (5.9×10^6 CFU planktonic cells and 9.9×10^6 CFU aggregates). To analyze the effect of homogenization, challenge mixtures were homogenized in a tissue grinder for approximately 30 s before oral gavage. The input ratio for challenge A was 1.19 (4.8×10^6 CFU planktonic cells and 4.0×10^6 CFU aggregates), and for challenge B it was 0.85 (5.6×10^6 CFU planktonic cells and 6.6×10^6 CFU aggregates). The input ratios for the trial of wild-type planktonic cells versus Δ*csgD* planktonic cells were 1.07 for challenge A (6.7×10^6 CFU wild type and 6.3×10^6 CFU Δ*csgD* cells) and 1.11 for challenge B (7.7×10^6 CFU wild type and 6.9×10^6 CFU Δ*csgD* cells). For the trial using planktonic cells and multicellular aggregates prepared from ΔSPI-1 cultures, the input ratio for challenge A was 0.27 (4.8×10^6 CFU planktonic cells and 1.8×10^7 CFU aggregates), and for challenge B it was 3.7 (4.4×10^6 CFU planktonic cells and 1.2×10^6 CFU aggregates). For the trial using dehydrated planktonic cells and multicellular aggregates (see below), the input ratio for challenge A was 0.11 (7.2×10^5 CFU planktonic cells and 6.5×10^6 CFU aggregates), and for challenge B it was 0.07 (3.0×10^5 CFU planktonic cells and 4.2×10^6 CFU aggregates).

Desiccation of *S. Typhimurium* challenge formulations. Planktonic cells and multicellular aggregates from the initial CI trial were suspended in spent culture supernatant, and 200-μl aliquots were pipetted into individual wells of 24-well tissue culture plates. The plates were placed in a

FIG 1 Differentiation of cells within an *S. Typhimurium* biofilm culture. (A) Aliquot of *S. Typhimurium* 14028 culture containing multicellular aggregates and planktonic cells after 13 h growth at 28°C, prior to separation of the two subpopulations. (B) Isolated aggregates resuspended in PBS to give an example of their relative sizes. (C) Aggregates isolated from an *S. Typhimurium* culture containing a *csgBAC* promoter-GFP fusion plasmid (25) visualized on a Leica TCS SP5 confocal microscope.

biological safety cabinet, the liquid was allowed to evaporate, and then the plates were covered and left to sit on the laboratory bench. At time zero, 4 weeks, and 5.5 weeks, 500 μ l of PBS was added to individual wells, and the cells were allowed to rehydrate for 30 min prior to homogenization using a tissue grinder and plating to determine the number of surviving CFU from each cell type. The statistical difference between samples was calculated using unpaired *t* tests. For the CI trial performed at 4 weeks, cells were resuspended in a total volume of 3 ml of 100 mM HEPES, pH 8, per 24-well plate.

Ethics statement. All animals were cared for and used in accordance with the Guidelines of the Canadian Council on Animal Care and the Regulations of the University of Saskatchewan Committee on Animal Care and Supply. All mouse experiments were performed under Animal Use Protocol 20110057, which was approved by the University of Saskatchewan's Animal Research Ethics Board.

RNA-seq accession numbers. RNA-seq data are available in the SRA database (study no. [SRP056892](#)) under the following accession numbers: [SRX976427](#), [SRX976344](#), [SRX976343](#), [SRX976341](#), [SRX976337](#), [SRX976336](#), [SRX976335](#), and [SRX974437](#).

RESULTS

We grew *S. Typhimurium* 14028 in liquid culture under biofilm-inducing conditions to examine differentiation within the population. After 13 h growth, multicellular aggregates were clearly visible within the culture (Fig. 1A), ranging in size from 100 to 500 μ m (Fig. 1B). The aggregates had high levels of *csgBAC* (curli) transcription, as measured using a GFP reporter (Fig. 1C). To monitor the dynamics of the differentiation process, we measured the real-time expression of *csgD* in the total population while observing the appearance of multicellular aggregates (Fig. 2A). Four time points were chosen for further analysis: TP1 was considered a preaggregation condition, when overall *csgD* expression was low (Fig. 2A) and the culture appeared homogeneous (data not shown); TP2 had peak *csgD* expression that coincided with the appearance of multicellular aggregates; TP3 had high *csgD* expression, and the culture appeared fully differentiated; and TP4 was

FIG 2 Analysis of multicellular aggregates and planktonic cells isolated from *S. Typhimurium* biofilm cultures. (A) *csgD* expression was measured during growth using a promoter-luciferase (*luxCDABE*) fusion plasmid designed to measure gene expression by light production (CPS, counts per second). The line represents the average CPS of two biological replicate cultures measured at hourly intervals in triplicate; the error bars represent the range of expression. The time points (TP1 to TP4) were chosen to reflect the dynamics of cellular differentiation. (B) Cell fractions were isolated at each time point and analyzed by Western blotting for synthesis of CsgD, the central regulator of *Salmonella* biofilm formation, and CsgA, the major subunit of curli fimbriae. GroEL was detected as a loading control to ensure that equal amounts of protein were loaded into each lane. Agg, multicellular aggregates; Plank, planktonic cells. (C) Cyclic-di-GMP concentrations were calculated as pmol per 10^7 CFU. Each point represents the measurement from one biological replicate culture; c-di-GMP could not be detected for two samples of planktonic cells at TP1. The horizontal bars represent the median values. Statistical significance: *, $P < 0.05$; **, $P < 0.01$.

visually the same as TP3, but *csgD* expression had dropped to near baseline levels. Low-speed centrifugation was used to sediment the multicellular aggregates, while the planktonic cells remained suspended in the medium (see Fig. S1 in the supplemental mate-

TABLE 1 RNA-seq-based transcriptome analysis of planktonic cells and multicellular aggregates isolated from *S. Typhimurium* culture

Characteristic	Value							
	Wild-type planktonic cells				Wild-type aggregates			Δ csgD planktonic (TP2)
	TP1	TP2	TP3	TP4	TP2	TP3	TP4	
Total no. of reads ^a	50,889,232	64,194,848	60,641,580	66,424,412	64,372,950	51,211,612	59,213,696	32,648,668
mRNA and sRNA	5,142,914	9,183,473	6,970,035	4,208,777	7,112,841	4,395,392	4,089,516	3,971,434
Genome coverage ^b	77.7	138.7	105.3	63.6	107.5	66.4	61.8	61.2
Protein-coding genes (%) ^c								
Sense	75.3	74.0	68.9	66.0	74.8	71.6	70.3	73.9
Antisense	5.9	5.4	6.7	8.9	2.8	4.1	4.4	10.4
sRNAs (%) ^d	8.0	10.6	14.2	9.5	14.4	13.9	8.9	6.5
Intergenic regions (%)	10.8	10.0	10.2	15.6	8.0	10.4	16.4	9.1
Plasmid (%)	0.8	0.8	0.7	0.6	0.4	0.4	0.4	0.8

^a Reads of three technical replicates were merged for each time point for each sample. In total, 416 million strand-specific reads of 75-nucleotide length were obtained.

^b The reads mapped to rRNAs and tRNAs were removed prior to statistical and comparative analyses. This yielded an average genome coverage of $89.9 \times (\pm 23.2 \times)$ for each time point of each sample excluded.

^c There are 5,414 putative open reading frames (ORFs) in the *S. Typhimurium* ATCC 14028 genome, including 5,313 chromosome-located and 101 plasmid-borne ORFs.

^d The full set of known sRNAs in *Salmonella* was annotated from reference 40. Approximately $11.5\% \pm 2.8\%$ of the mapped reads represented known sRNAs, and $11.8\% \pm 3.4\%$ of the reads mapped to additional intergenic regions.

rial). The formation of these two cell types was consistent, with a coefficient of variation of less than 10% between biological replicate cultures. The aggregates could be disrupted using a tissue grinder, which generated a primarily single-cell population. Using this technique, we were able to establish a correlation between the wet weight of aggregated cells and the number of CFU (see Fig. S1 in the supplemental material). In addition to the cells in the fluid phase, there were cells that formed an adherent biofilm at the air-liquid interface of the culture flask. These cells differed in terms of oxygen availability (30), and the amount of biofilm formed was highly variable (data not shown); therefore, they were excluded from further analysis.

Western blot analysis of the subpopulations isolated from the fluid phase confirmed the bistable production of CsgD; multicellular aggregates displayed high levels of CsgD at all time points, whereas the planktonic cells had either low (TP2), trace (TP1 and TP3), or undetectable (TP4) levels (Fig. 2B). We also analyzed both subpopulations for production of curli fimbriae, an important biofilm extracellular matrix component and functional bacterial amyloid (29). The major subunit protein of curli, CsgA, was detected in abundance in all aggregate fractions, whereas the planktonic-cell fractions at TP2, TP3, and TP4 were negative (Fig. 2B). These results confirmed that the subpopulations had been cleanly separated by low-speed centrifugation. Trace amounts of CsgA were detected at TP1 (Fig. 2B), indicating that some cells had already begun to differentiate at this early time point.

The bacterial secondary messenger c-di-GMP is known to play a role in regulating *csgD* transcription and CsgD activity (30, 31). High levels of c-di-GMP are associated with biofilm formation, whereas low levels are associated with a single-cell lifestyle (44). To test if these same principles were at work in our system, we measured the intracellular concentrations of c-di-GMP in planktonic cells at TP1 and in multicellular aggregates and planktonic cells at TP2. While planktonic cells at TP1 had nearly undetectable levels of c-di-GMP, there was an order of magnitude increase for planktonic cells at TP2 and another order of magnitude increase for aggregates, which had the highest levels (Fig. 2C). The levels of c-di-GMP correlated with the levels of CsgD in these cell types.

Transcriptome divergence between planktonic and aggregate cell types. To get a comprehensive view of the transcriptional differences, RNA was purified from each cell subpopulation at each time point, and directional RNA-seq was performed (Table 1). In total, 1,856 genes, or $\sim 34\%$ of the genes in the genome, were differentially expressed when comparing planktonic cells to multicellular aggregates at each time point (see Data Set S1 in the supplemental material; $P < 0.05$). The largest differences were identified at TP2, with 1,240 genes being differentially expressed (Fig. 3A). At TP3 and TP4, the numbers of differentially expressed genes were reduced to 778 and 668, respectively; 642 of the differentially expressed genes were shared by at least two time points, and 188 were shared by all three time points. Of the 1,856 genes that were differentially expressed, 784 were expressed at higher levels in multicellular aggregates (Fig. 3B) and 1,072 were more highly expressed in planktonic cells (Fig. 3C). We also performed a comparison between the nonaggregative Δ csgD strain, which represents an entirely single-cell population (13, 21), and the wild-type planktonic cells at TP2. Gene expression in planktonic cells from these two strains was more similar, with only 220 genes being differentially expressed (see Data Set S2 in the supplemental material).

Closer analysis of the activated genes revealed that the planktonic cells had higher expression of numerous pathways involved in virulence, including both SPI-1 and SPI-2 T3SSs, part of the *phoPQ* regulon, motility and chemotaxis, and a high proportion ($>40\%$) of function unknown (FUN) genes (Fig. 3D). In contrast, multicellular aggregates had higher expression of genes associated with extracellular matrix production in biofilms (i.e., *csgDEFG*, *csgBAC*, and *yaiC* [*adrA*]), changes in metabolism (i.e., amino acid, carbohydrates, lipids, and nucleotides), and pathways associated with stress response (i.e., osmoprotection) (Fig. 3D). The nature of the transcriptional differences between planktonic cells and multicellular aggregates suggested that they represented specialized cell types. Many of the genes listed in Data Sets S1 and S2 in the supplemental material were distributed into operons predicted from RNA-seq through a combination of transcriptional start and termination sites, intergenic distance, and coexpression information (91).

FIG 3 RNA-seq-based transcriptome comparison between multicellular-aggregate and planktonic-cell subpopulations. (A) Venn diagram showing the total number of differentially expressed genes ($P < 0.05$) between planktonic cells and multicellular aggregates at each time point. (B and C) The total was subdivided into genes that were more highly expressed in aggregates (B) and genes that were more highly expressed in planktonic cells (C). (D) Differentially expressed genes in aggregates versus planktonic cells were categorized using the KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/kegg/>) pathway database and are listed in Data Set S1 in the supplemental material.

Expression of c-di-GMP enzymes and other genetic factors contributing to CsgD bistability. We identified several differentially expressed genes associated with the complex c-di-GMP signaling system that contributes to CsgD regulation (Table 2). Two diguanylate cyclase (DGC) (i.e., c-di-GMP-generating enzymes) genes, STM1987 (45) and *adrA* (46), were more highly expressed in aggregates at TP2, which correlated with higher levels of c-di-GMP in these cells. *yhjH*, which encodes a phosphodiesterase (PDE) (i.e., a c-di-GMP-degrading enzyme) with a critical role in controlling motility (46, 47), was more highly expressed in planktonic cells, and this correlated with smaller amounts of c-di-GMP. Genes encoding three diguanylate cyclases characterized as having positive effects on invasion (*yeaJ*) (48), motility (STM2503) (49), or motility and virulence (STM4551) (49) were more highly expressed in planktonic cells at TP2, which correlated with the predicted phenotype of these cells. *nlpI*, encoding a putative membrane protein with a repressive effect on CsgD synthesis (50), was also more highly expressed in planktonic cells, which matched the lower levels of CsgD in these cells. In contrast, high expression of *ycgR* and *ydiV* in planktonic cells at TP2 did not match the predicted phenotypes. YcgR contains a PilZ domain for binding c-di-GMP and represses motility by acting as a type of flagellar brake (51, 52). Based on the published function, we would have expected *ycgR* to be more highly expressed in multicellular aggregates and to function at higher c-di-GMP levels inside the cell. YdiV re-

presses expression of *yhjH* and also negatively regulates motility (53). It is possible that the synthesis of YcgR and YdiV results in localized effects within the cell, but the overall c-di-GMP pool is predominantly influenced by the increased expression of *yhjH* (46, 47). In general, the RNA-seq findings were consistent with the levels of c-di-GMP detected and the predicted phenotypes of planktonic cells and multicellular aggregates.

Bistable gene expression in bacteria is usually the result of positive autoregulation of a master regulatory gene (54, 55). A feed-forward loop has been proposed for *csgD* in *E. coli*, but it has not been proven in *S. Typhimurium* (30). CsgD is proposed to activate the expression and synthesis of IraP, which inhibits the degradation of RpoS, thus leading to greater *csgD* transcription (56). RNA-seq analysis revealed that multicellular aggregates had elevated transcription of *rpoS* at TP2 and *csgD* and *iraP* (*yaiB*) at TP3 and TP4, potentially confirming this regulatory loop (see Fig. S2 in the supplemental material).

SPI-1 T3SS expression, synthesis, and motility are increased in planktonic cells. The SPI-1 T3SS is a 3.5-MDa translocation apparatus that is required for injection of *Salmonella* effector proteins into the cytoplasm of host cells. These effector proteins stimulate *Salmonella* invasion and dissemination, as well as host inflammation (6). The current dogma is that SPI-1 T3SS synthesis is activated by the body temperatures of warm-blooded hosts (57). This is primarily based on the finding that the nucleoid-associated

TABLE 2 Differentially expressed genes in the c-di-GMP signaling network

Gene name ^a	Enzyme type ^b	Function ^c	Fold change ^d	Time point(s) ^e
<i>STM14_5467</i> (STM4551)	DGC	Positive regulator of motility and virulence by both c-di-GMP-dependent and -independent mechanisms (49); an additional report states that the function is the exact opposite (48)	4.05	TP2, TP3, TP4
<i>yeaJ</i> (STM1283)	DGC	Stimulates invasion (48)	3.18	TP2, TP3
<i>yhjH</i> (STM3611)	PDE	Negative regulator of rdar morphotype; positive regulator of motility (46)	4.47	TP2
<i>STM14_3068</i> (STM2503)	DGC	Positive effect on motility (49)	3.13	TP2
<i>ycgR</i>	C-di-GMP biosensor	Binds c-di-GMP through a high-affinity PilZ domain; represses motility by binding directly to FlgG (51, 52)	4.07	TP2
<i>ydiV</i> (STM1344)	EAL-like protein	Positive regulator of <i>csgD</i> transcription and negative regulator of motility, but does not have a direct role in c-di-GMP turnover; represses expression of <i>yhjH</i> (53)	3.58	TP2
<i>nlpI</i>	Outer membrane protein	NlpI can negatively influence the expression of <i>yjcC</i> , which codes for a DGC affecting CsgD expression (50)	3.89	TP2, TP3, TP4
<i>adrA</i> (<i>yaiC</i>)	DGC	Positive regulator of cellulose production (46)	0.34	TP2, TP3, TP4
<i>STM14_2408</i> (STM1987)	DGC	Positive effect on rdar morphotype, CsgD expression, and cellulose (45)	0.54	TP2

^a Gene names are listed as they appear in the *S. Typhimurium* 14028 genome (39); the *S. Typhimurium* LT2 gene names are listed in parentheses.

^b An enzyme that generates c-di-GMP is called a DGC; an enzyme that breaks down c-di-GMP is called a PDE. PDE enzymes contain EAL domains.

^c Functions are listed in the context of c-di-GMP regulation and turnover, CsgD expression, motility, and virulence.

^d Fold change represents the expression ratios of planktonic cells/aggregates at TP2; genes with values of >1 have higher expression in planktonic cells, whereas genes with values of <1 have higher expression in multicellular aggregates.

^e Time points where the expression difference between planktonic cells and multicellular aggregates was statistically significantly ($P < 0.05$) are listed.

protein H-NS represses transcription of key SPI-1 regulators at lower growth temperatures, such as 25°C (58). Biofilm growth conditions (i.e., low osmolarity, 28°C, and stationary phase) were traditionally thought to be non-SPI-1 inducing because they are so different from classical SPI-1-inducing conditions (i.e., high osmolarity, 37°C, and late exponential phase) (57, 59, 60).

RNA-seq analysis revealed that 41 of 44 SPI-1 T3SS-related genes were more highly expressed in planktonic cells grown under biofilm-inducing conditions (see Data Set S1 in the supplemental material). These genes included pivotal transcriptional regulators (Fig. 4A), as well as apparatus, chaperone, and effector genes (Fig. 4B). HilA activates transcription of SPI-1 apparatus genes distributed in the *inv-spa* and *prg-org* operons (57). InvF is encoded as part of the *inv-spa* operon and, together with SicA, promotes transcription of the *sic-sip* operon encoding the translocon, as well as numerous effector genes. Temporal expression analysis of the SPI-1 genes followed a pattern consistent with the establishment of secretion-competent organelles in planktonic cells; apparatus genes had the highest expression at TP1, while translocon and numerous effector genes had peak expression at TP2 (see Fig. S3 in the supplemental material). In contrast, the expression levels of all SPI-1 genes were reduced in multicellular aggregates (see Fig. S3 in the supplemental material). Comparison between wild-type planktonic cells and Δ *csgD* planktonic cells at TP2 revealed that *sipD* and *sopE2* were more highly expressed in the wild-type cells (see Data Set S2 in the supplemental material). In addition, several other SPI-1 genes were more highly expressed in wild-type cells but had *P* values just above the cutoff for significance (data not shown). These findings suggested that there could be a potential regulatory link between CsgD and the SPI-1 T3SS.

To determine if SPI-1 transcriptional differences were reflected at the protein level, whole-cell lysates of either cell type were analyzed by Western blotting. InvG, the outer membrane (OM) secretin (61), was detected at high levels in planktonic cells at all time points and was nearly undetectable in the multicellular-aggregate samples (Fig. 4C). The on/off pattern of synthesis for this

critical structural component indicated that planktonic cells had the capacity to form a greater number of SPI-1 T3SS organelles than aggregated cells. SipD, the translocon tip protein that interacts with host cell membranes (62), was also present at high levels in planktonic cells at all time points (Fig. 4C). SopB and SopE2, two of the earliest expressed effectors that stimulate host cell cytoskeleton rearrangements (63), were also synthesized at higher levels in planktonic cells at TP2 and TP3, with the difference decreasing by TP4 (Fig. 4C). SPI-1 protein levels were also analyzed in Δ *csgD* cells at TP2. InvG and SipD levels were noticeably lower in Δ *csgD* cells than in wild-type planktonic cells (Fig. 4D), whereas SopB and SopE2 had no detectable difference between the two strains (data not shown).

Recent evidence suggests that the SPI-1 T3SS and flagellum-mediated motility are required for targeting of permissive invasion sites on host cells (64) and that these systems are often co-regulated (65). In our RNA-seq experiment, 28 genes coding for flagellar assembly and chemotaxis proteins were identified as being more highly expressed in planktonic cells (see Data Set S1 in the supplemental material). We performed a swim assay, which confirmed that planktonic cells had enhanced motility compared to multicellular aggregates but were not significantly different from Δ *csgD* planktonic cells (see Fig. S4 in the supplemental material).

Transcriptional priming of the SPI-2 T3SS in planktonic cells. SPI-2 encodes a second T3SS that has an important role once *Salmonella* has invaded host cells. The SPI-2 T3SS is required for modification of the phagolysosome to make it conducive for *Salmonella* replication, particularly inside macrophages (6). Thirty-seven of 42 genes encoding the SPI-2 T3SS were more highly expressed in planktonic cells than in the multicellular aggregates (see Data Set S1 in the supplemental material). However, unlike SPI-1, we were unable to detect SPI-2 protein synthesis, such as that of SsaC, the equivalent of InvG, in any cell fractions (data not shown). This suggested that functional SPI-2 needles were not present on the surfaces of planktonic cells and supported the pos-

FIG 4 Expression and synthesis of the SPI-1 T3SS in *S. Typhimurium* planktonic cells. (A) Schematic of a bacterial cell showing the regulatory map and main transcriptional activators (green) and repressors (red) for SPI-1 T3SS expression (6). The fold change values for the regulators shown are displayed as red bars on the accompanying graph, calculated from RNA-seq data at TP2. (B) The SPI-1 T3SS translocation apparatus (left) (90) was divided into five component groups, including proteins embedded in the bacterial inner (IM) or outer (OM) membrane or host cell membrane (HCM). Each point on the accompanying graph represents the fold change value at TP2 for the genes for effectors (*sopE2*, *sopB*, *sipA*, *sopD*, *gtgE*, *sopA*, *avrA*, *sptP*, and *gogB*), chaperones (*sicA*, *sicP*, *invB*, and *sigE* [*pipC*]), ATPase platform (*invC*, *spaO*, *orgA*, and *orgB*), export apparatus (*invG*, *spaR*, *prgH*, *invI*, *invA*, *invE*, *prgK*, *spaP*, *spaQ*, *invJ*, *spaS*, and *invH*), needle (*prgI* and *prgJ*), and translocon (*sipB*, *sipC*, and *sipD*) (6). The dashed line at 1 represents no difference in gene expression; the vertical lines represent the mean fold changes for the groups. (C) Whole-cell lysates of planktonic cells and multicellular aggregates were analyzed by Western blotting to detect the SPI-1 T3SS proteins. Plank, planktonic cells; Agg, multicellular aggregates. (D) InvG and SipD were detected in Δ csgD planktonic cells at TP2, and the relative levels were compared to those of aggregates and planktonic cells isolated from wild-type cultures.

FIG 5 Invasion of polarized Caco-2 cells by different *S. Typhimurium* subpopulations. Planktonic cells and multicellular aggregates isolated from *S. Typhimurium* 14028 biofilm cultures at TP2 and TP4 were used to infect polarized Caco-2 cells. Aggregates that were homogenized using a tissue grinder were also tested, along with planktonic cells prepared from $\Delta csgD$ cultures. Wild-type (WT) and $\Delta SPI-1$ cells grown in LB broth at 37°C were used as positive and negative invasion controls, respectively. Each point represents the average value from invasion of three replicate wells of Caco-2 cells; the horizontal bars represent the mean values. The average MOI was 17, based on an estimate of 2.5×10^5 polarized Caco-2 cells per well. Statistical significance: ***, $P < 0.001$; *, $P < 0.05$; ns, $P > 0.05$.

sibility that transcriptional priming was occurring, as has been observed prior to host cell invasion (66, 67).

Planktonic cells display enhanced invasion of a human intestinal cell line. To our knowledge, SPI-1 T3SS synthesis has never been observed before under biofilm-inducing conditions but is usually correlated with increased invasion and virulence (68). Therefore, we tested the invasion by different *S. Typhimurium* cell types of polarized Caco-2 cells, a human intestinal cell line. Planktonic cells isolated at TP2 displayed the same invasiveness as planktonic cells grown under classical SPI-1-inducing conditions (Fig. 5), confirming that the SPI-1 organelles synthesized under biofilm-inducing conditions were functional. Planktonic cells isolated at later time points (i.e., TP4) still retained efficient invasion capability, suggesting that this phenotype was stable. In contrast, the multicellular aggregates were significantly less invasive, presumably because of reduced type three secretion synthesis. Surprisingly, $\Delta csgD$ cells, which had reduced InvG and SipD levels, showed only a slight drop in invasion that was not statistically different than that of wild-type planktonic cells. For the aggregates, we reasoned that reduced invasion could be influenced by the presence of extracellular matrix polymers blocking access to the Caco-2 cell surface. To disrupt the matrix, we homogenized

the aggregates prior to repeating the assay, but it did not lead to increased invasion (Fig. 5, homogenized aggregates). It was noted, however, that the aggregated cells invaded significantly better than a $\Delta SPI-1$ deletion strain, suggesting that they would retain a capacity for virulence.

As part of the Caco-2 cell invasion assays, the aminoglycoside antibiotic gentamicin was added to kill any extracellular bacteria that had not invaded. Prior to performing the assays, we tested the susceptibilities of multicellular aggregates and planktonic cells to gentamicin. While the planktonic cells were completely killed (below the limit of detection) at standard concentrations of 200 to 400 $\mu\text{g ml}^{-1}$ (33), the multicellular aggregates displayed enhanced resistance and were depleted only once the concentration reached 1,200 $\mu\text{g ml}^{-1}$ (see Fig. S5 in the supplemental material). This confirmed that the multicellular aggregates represented a more resistant subpopulation of cells.

Planktonic cells display a significant virulence advantage in murine infection. Based on the expression data and Caco-2 invasion results, we hypothesized that the planktonic cells were more virulent, whereas the multicellular aggregates were likely to have enhanced persistence. To test the colonization efficiency and virulence of each cell type, we performed formally randomized, blinded coinfections of mice with 1:1 ratios of multicellular aggregates and planktonic cells isolated from kanamycin- or chloramphenicol-resistant strains of *S. Typhimurium* (see Fig. S6 in the supplemental material). For the initial CI trial, we wanted to keep the cell subpopulations in their native physical states; therefore, we minimized the handling of the cells and used a wide-bore gavage needle to prevent shearing of aggregates during oral delivery. Under these conditions, the planktonic cells exhibited a significant virulence advantage, as demonstrated by their proportionally increased recovery from spleen, liver, cecum, and mesenteric lymph nodes in the majority of infected mice (Fig. 6A, red triangles). As predicted, the multicellular aggregates did retain a capacity for virulence and were recovered in larger proportions in several mice (Fig. 6A, blue triangles). We reasoned that, despite being comprised of hundreds to thousands of cells, each aggregate might function as only a single infectious unit. Therefore, we homogenized the aggregates to generate a single-cell suspension prior to repeating the CI trial. The CI values were slightly reduced, but the overall trend remained the same (Fig. 6B), indicating that the increased virulence of planktonic cells was not due to a difference in the multiplicity of infection. We also competed wild-type planktonic cells and $\Delta csgD$ planktonic cells, and again, the wild-type planktonic cells displayed a strong virulence advantage (Fig. 6C). This was surprising, because the invasion phenotypes of the two strains were similar; however, there could be other impairments related to the $\Delta csgD$ mutation (69). To assess the importance of SPI-1 in the colonization advantage displayed by planktonic cells, we repeated a CI trial with planktonic cells and multicellular aggregates prepared from a $\Delta SPI-1$ mutant strain. For each organ, the median CI values dropped approximately 10-fold and were near 1 (Fig. 6D), which indicated that the SPI-1 T3SS was the primary factor responsible for increased colonization of planktonic cells. However, small differences were still detected for the spleen, liver, and cecum (Fig. 6D), indicating that the planktonic cells had other adaptations (e.g., increased motility) that provided them with a colonization advantage.

Persistence of multicellular aggregates reduces the competitive advantage of planktonic cells. To assess how the multicellu-

FIG 6 Competitive infections between multicellular aggregates and planktonic cells prepared from wild-type, $\Delta SPI-1$, and $\Delta csgD$ cultures. The CI was determined by oral coinfection of C57BL/6 mice with a 1:1 ratio of different cell types. (A) Trial between planktonic cells and multicellular aggregates, using a wide-bore gavage needle to prevent shearing of aggregates. (B) Trial between planktonic cells and multicellular aggregates after homogenization of the aggregates to generate a single-cell population. (C) Trial between wild-type planktonic cells and $\Delta csgD$ planktonic cells. (D) Trial between planktonic cells and multicellular aggregates isolated from a $\Delta SPI-1$ biofilm culture. Each point represents the CI value calculated from one organ from a single mouse, with either cell type (red or blue triangles) winning the competition. The solid lines represent the median of all CI values from a particular organ. The dashed line represents a CI value of 1, which indicates no difference in virulence. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, $P > 0.05$.

lar aggregates and planktonic cells responded to a potential environmental stress, aliquots of the challenge inocula from the first CI experiment were desiccated and stored in 24-well plates at room temperature. Viability assays performed on the desiccated challenge inocula revealed that the multicellular aggregates survived significantly better than the planktonic cells (Fig. 7A). At the 4-week time point, there were approximately 10 times more aggregated cells alive (Fig. 7A, dashed line). We performed another CI trial at this time point, after rehydration and resuspension of the cells. The values for each organ indicated no significant advantage for either subpopulation, with aggregates or planktonic cells winning the competition in approximately equal numbers of mice (Fig. 7B). This indicated that upon exposure to environmental stress, the multicellular aggregates had a fitness advantage. If these results were extrapolated into the natural world, one could envision a scenario where, after extended periods of environmental exposure, only multicellular aggregates would be alive and able to infect new hosts.

DISCUSSION

In this study, we isolated and analyzed two distinct subpopulations of cells within a clonal population of *S. Typhimurium*. Based on genetic, biochemical, and functional characterization, we hypothesize that the planktonic cells and multicellular aggregates formed under biofilm-inducing conditions represent specialized

cell types that are adapted for virulence and persistence, respectively. Due to the presence of functional SPI-1 T3SS organelles, we predict that the planktonic cells are proficient at direct host-to-host transmission, whereas the resistant multicellular aggregates can survive in the environment and cause infections at later times. The connection of virulence and persistence within an individual population represents an elegantly simple strategy that would prepare *S. Typhimurium* for unpredictability and improve the chances for transmission each time the bacteria leave an infected host. This scenario also implies that the host immune system may respond differently to these specialized cell types. If hosts routinely encounter multicellular aggregates, it could explain why curli fimbriae are such potent stimulators of the innate immune system (29, 70, 71), even though the organelles do not appear to play a role in *Salmonella* infections (25).

The concept of bistability was described by Novick and Weiner over 50 years ago as the existence of different subpopulations of enzyme-producing cells within a genetically identical population of *E. coli*, controlled by a feed-forward loop (72). CsgD lies at the heart of a feed-forward loop in *S. Typhimurium* when cells are grown under biofilm-inducing conditions. CsgD is synthesized at high levels in specific cells, leading to production of extracellular matrix polymers and the formation of multicellular aggregates (25, 30). The regulation of *csgD* transcription and CsgD synthesis

FIG 7 Enhanced persistence of *S. Typhimurium* multicellular aggregates. (A) Multicellular aggregates and planktonic cells used for the trial in Fig. 6A were desiccated and stored in 24-well plates. Survival of each cell type was determined after storage for the times displayed on the x axis. Statistical significance: **, $P < 0.01$; ****, $P < 0.0001$. The dashed line represents the time at which cells were resuspended and a CI trial was performed. (B) Each point represents the CI value calculated from one organ from a single mouse, and the solid lines represent the median of all CI values from a particular organ. The dashed line represents a CI value of 1, or no difference in virulence; none of the organs displayed CI values that were significantly different from 1 (ns, $P > 0.05$).

is extremely complex (reviewed in reference 31). Numerous transcription factors are involved, including OmpR, MlrA, and CpxR and the nonspecific DNA-binding proteins H-NS and IHF, in addition to the feed-forward loop involving *rpoS* and the factors that regulate RpoS activity, including IraP and Crl. In addition, at least five sRNAs can bind to the 5' untranslated region of the *csgD* mRNA and regulate CsgD synthesis (73). Robust control of CsgD production is clearly important, given that >30% of the genes in the genome were differentially expressed between the *S. Typhimurium* multicellular aggregates and planktonic cells. The large number of differentially expressed genes represents a tremendous amount of nonheritable genetic change, on the order of a developmental program in bacteria (74). Since only 20 CsgD-specific targets were identified in a recent *E. coli* chromatin immunoprecipitation sequencing (ChIP-seq) study (75), we hypothesize that CsgD initiates the aggregation process and that a cascade of gene expression changes follows as cells produce an extracellular matrix

(35). Ultimately, this cascade would be the cumulative result of numerous connected signaling pathways, including c-di-GMP (44).

Recent reports have shown that bistable gene expression can occur within bacterial virulence gene networks. In *Vibrio cholerae*, bimodal expression of ToxT results in virulent and nonvirulent cell subpopulations (76). Bistable production of the SPI-1 T3SS in *S. Typhimurium* has also recently been described (77–79). The authors observed that nearly 100% of *S. Typhimurium* cells interacting with intestinal tissues were positive for SPI-1 expression while only 15% of cells in the intestinal lumen were positive (77). The presence of both SPI-1⁺ and SPI-1[−] cells seems to be important for stabilizing the virulence traits of *S. Typhimurium* (78). At the moment, it is unclear how these findings are connected to what we have observed. Our finding that SPI-1 T3SS synthesis occurs in planktonic cells under biofilm-inducing conditions may indicate that an alternative regulatory pathway exists for SPI-1 activation. It is also possible that the planktonic population consists of both SPI-1⁺ and SPI-1[−] cells. Alternatively, the subpopulation of cells that form multicellular aggregates under biofilm-inducing conditions could be related to the SPI-1[−] cells described by Hardt and colleagues (e.g., similar genetic pathways activated or repressed) (77–79). One of our initial goals was to determine if the differentiation of cells was more than an *in vitro* phenomenon. The fact that the planktonic cells were more virulent than multicellular aggregates, even after the aggregates were homogenized into a single-cell suspension, suggested that differentiation was retained until the cells reached the murine intestine. The importance of SPI-1 in this process was confirmed, since planktonic cells and multicellular aggregates from a Δ SPI-1 mutant strain had nearly equal colonization efficiencies. Furthermore, the reduction in virulence that we observed for the Δ csgD strain, together with recent connections between c-di-GMP, virulence (28), and motility (52), suggest that the master biofilm regulator, CsgD, can modulate the virulence capacity of *S. Typhimurium*.

Bet hedging is characterized as a risk-spreading strategy displayed by clonal populations, where each phenotype performs more or less well at any time, depending on the selection pressures present (80). Although we cannot be certain of the evolutionary selections that led to bistable CsgD expression, the passage of *Salmonella* isolates between host and nonhost environments represents a model of unpredictability (7). Aggregation is known to have many evolutionary benefits to ensure the survival of populations, but rather than being interpreted as altruistic behavior, it may be driven by harsh penalties associated with an individual not being part of the group (81). For pathogens like *S. Typhimurium*, the maintenance of a subset of cells primed for invasion but not ideally suited for environmental survival would impose a penalty unless a host is encountered. The energy commitment required to make SPI-1 T3SS organelles (82) or extracellular matrix polymers (83, 84) in different subpopulations indicates that it might be a strategy for *S. Typhimurium* to prepare for unpredictability. A proportion of cells could survive exposure to either the host or nonhost environment, with the ultimate goal being preservation of the shared genome for the next generation (54, 80). We speculate that signaling can occur between the host immune system, the microbiota, and the invading pathogen, with the distinct possibility that *S. Typhimurium*, and presumably other NTS isolates, can modulate its virulence (85) or persistence (7) as a result of these interactions.

As pathogens evolve, there is thought to be continual selection pressure for them to adapt to their hosts (86). Host adaptation has the benefit of reducing the variation and unpredictability that a bacterial pathogen is exposed to but makes strains more dependent on a direct means of transmission (4). Differences in transmission could potentially explain the correlation between an invasive, host-adapted way of life and loss of the rdar morphotype in *Salmonella* and *E. coli* (17, 26). In *E. coli*, bistable expression of CsgD does occur in specific pathogenic strains (87); however, human-adapted enteroinvasive *E. coli* and *Shigella* isolates have lost the ability to aggregate (27). Similarly, the evolution of *S. Typhi* has been strongly influenced by the human carrier state (5), and nearly all isolates are negative for the rdar morphotype (25, 26). We hypothesize that host-adapted or host-restricted isolates favor a single-cell mode of growth, because shedding from chronic carriers makes them less dependent on long-term survival to complete the transmission cycle. *S. Typhi* is capable of other forms of biofilm growth that are required for persistence inside human carriers, such as attachment to the surfaces of gallstones (88), and recent evidence indicates that *S. Typhi* can be transmitted indirectly via contaminated water (89). This suggests that there are other factors to consider when correlating a loss of persistence with host adaptation. Nevertheless, identifying the molecular mechanisms that connect virulence and persistence may hold the key for understanding transmission and ultimately reducing the spread of these human pathogens. We predict that the bet-hedging program described here for *S. Typhimurium* is a common strategy for survival, especially for other enteric pathogens that spend a significant portion of their life cycle in the environment.

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