Aggregation via the Red, Dry, and Rough Morphotype Is Not a Virulence Adaptation in *Salmonella enterica* Serovar Typhimurium[▽]

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The *Salmonella* rdar (red, dry, and rough) morphotype is an aggregative and resistant physiology that has been linked to survival in nutrient-limited environments. Growth of *Salmonella enterica* serovar Typhimurium was analyzed in a variety of nutrient-limiting conditions to determine whether aggregation would occur at low cell densities and whether the rdar morphotype was involved in this process. The resulting cultures consisted of two populations of cells, aggregated and nonaggregated, with the aggregated cells preferentially displaying rdar morphotype gene expression. The two groups of cells could be separated based on the principle that aggregated cells were producing greater amounts of thin aggregative fimbriae (Tafi or curli). In addition, the aggregated cells retained some physiological characteristics of the rdar morphotype, such as increased resistance to sodium hypochlorite. Competitive infection experiments in mice showed that nonaggregative $\Delta agfA$ cells outcompeted rdar-positive wild-type cells in all tissues analyzed, indicating that aggregation via the rdar morphotype was not a virulence adaptation in *Salmonella enterica* serovar Typhimurium. Furthermore, in vivo imaging experiments showed that Tafi genes were not expressed during infection but were expressed once *Salmonella* was passed out of the mice into the feces. We hypothesize that the primary role of the rdar morphotype is to enhance *Salmonella* survival outside the host, thereby aiding in transmission.

Thin aggregative fimbriae (Tafi), also known as curli fimbriae, mediate cell-cell aggregation in *Salmonella* spp. These cell surface organelles are produced together with cellulose (58, 63), as well as additional polysaccharides (19) and proteins (BapA [26]), to form an extracellular matrix that links individual cells together. The phenotype associated with matrix production has been termed the rdar morphotype, characterized by the formation of colonies with red, dry, and rough morphology when cells are grown on agar media containing Congo red (11, 42). When *Salmonella* bacteria are grown statically in liquid media, cells can aggregate through similar physiology to form pellicles at the air-liquid interface (10, 42, 50, 60). Collectively, these phenotypes have been referred to as multicellular behaviors (41, 42).

Regulatory control of the rdar morphotype has been the subject of numerous recent studies (47, 55, 60). Genetic control is primarily focused at the level of expression of AgfD (CsgD), a transcriptional regulatory protein. AgfD activates the production of Tafi and cellulose polymers, as well as additional extracellular matrix components, such as O-antigen (O-Ag) capsule and BapA. The *agfD* promoter has low basal

activity, relying on several accessory proteins to activate gene expression (16, 18, 60). This enables cells to respond quickly to environmental cues. The underlying patterns of gene expression are conserved in most *Salmonella* isolates (40, 50, 51, 60) as well as other enterobacterial species (62). One of the key signaling molecules involved in this process is cyclic diguanosine monophosphate (c-di-GMP). Kader et al. and Simm et al. have recently shown that AgfD expression and subsequent Tafi production are regulated by c-di-GMP (22, 47), through the action of several proteins containing c-di-GMP-generating, diguanylate cyclase (GGDEF) domains (43) and/or c-di-GMP-degrading, phosphodiesterase (EAL) domains (45). AdrA, a GGDEF domain-containing protein whose expression is dependent on AgfD, is required for cellulose production (41, 63). Additional GGDEF-EAL proteins may also regulate cellulose production (15), contributing to an AgfD-independent pathway (12). Some of the effects of c-di-GMP in control of the rdar morphotype may be specific to *Salmonella*. However, the association between high intracellular levels of c-di-GMP and sessile modes of growth is common in many bacterial species (9, 21, 25, 30, 48).

In most *Salmonella* isolates, the rdar morphotype is expressed under conditions of low osmolarity, nutrient limitation, and temperatures below 30°C (11, 17). This fits with current hypotheses that the rdar morphotype is important for persistence and survival of *Salmonella* in nonhost environments (44, 59). Cells of the rdar morphotype have enhanced resistance to desiccation as well as resistance to various antimicrobial agents (1, 19, 44, 59). Many studies have also implicated roles for Tafi,

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and presumably the rdar morphotype, during pathogenesis (reviewed in reference 3). Tafi have been shown to bind human extracellular matrix proteins (2, 10, 33), contact-phase proteins (4, 20, 34), plasminogen (49), and major histocompatibility complex class I (35) and appear to be recognized by Toll-like receptor 2 (TLR2) (53). In addition, *Salmonella* isolates can express Tafi at 37°C under specific conditions, such as iron limitation, or as the result of acquired *agfD* promoter mutations (42, 60).

The apparent polyfunctional nature of Tafi led us to question the role of the rdar morphotype in the life cycle of *Salmonella*. Since nonhost environments are generally assumed to represent nutrient-poor conditions (61), we investigated whether aggregation would occur at low cell densities and whether the rdar morphotype was involved in this process. In addition, we tested whether aggregation would provide a competitive advantage for infection in mice. We discovered that cultures grown under nutrient-limiting conditions consisted of two populations of cells, aggregated and nonaggregated, with the aggregated cells possessing resistant properties that were characteristic of the rdar morphotype. Competition experiments between aggregative wild-type (wt) and Tafi-negative, nonaggregative $\Delta agfA$ strains showed that aggregation was a disadvantage for murine infection. Moreover, the *agfB* promoter controlling Tafi expression was not activated during murine infection as determined by in vivo imaging. However, *agfD* and *yihU* (O-Ag capsule) were activated in vivo, revealing that some components of the rdar morphotype could be involved in virulence.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Salmonella enterica* serovar Typhimurium strain ATCC 14028 (American Type Culture Collection, Manassas, VA) was used as the wild-type strain in this study. The isogenic $\Delta agfA$ strain has been described previously (59). Strains were routinely grown for 16 to 20 h at 37°C with agitation in Miller's Luria broth (Miller's LB) (1.0% salt), supplemented with 50 μ g/ml kanamycin (Kan) if necessary, before additional experiments were performed. To induce microcolony formation, overnight cultures were inoculated at a dilution of 1 in 500 into 50 ml of 1/10 LB without salt (LBns) supplemented with 40 μ M 2,2'-dipyridyl (Dp) (1/10 LBns-Dp) and 50 μ g/ml Kan, if necessary, and grown for 2 to 5 days at 28°C without agitation. For mixed reporter experiments, overnight cultures were normalized to an A_{600} of 1.0, and equal volumes were mixed prior to inoculation of the final cultures. For *lux* assays, overnight cultures were diluted 1 in 600 in LBns, 1% tryptone, minimal medium containing morpholinepropanesulfonic acid (MOPS) (28, 31), M9 minimal medium (Difco), or EPS medium (0.05% yeast extract, 10 mM Na_2HPO_4 , 0.1% NH_4Cl , and 0.3% KH_2PO_4) (19) supplemented with 50 μ g/ml Kan to a final volume of 150 μ l in 96-well clear-bottom black plates (9520 Costar; Corning Inc.). The culture in each well was overlaid with 50 μ l mineral oil before the assays were started (assays performed at 28°C).

Preparation of fibronectin-coated magnetic beads (FN-beads). Tosyl-activated magnetic beads (4.5 μ m) (M450; Invitrogen Canada Inc.) were coated with human plasma fibronectin (catalog no. 33016-015; Invitrogen Canada Inc.) following the procedures outlined in reference 29, adding 50 μ g of fibronectin to 1 ml of bead solution. Final bead solutions contained approximately 3×10^9 beads per ml.

Magnetic bead-based fractionation of *S. enterica* serovar Typhimurium strain ATCC 14028 cultures. Ten microliters of FN-beads was added to each 1-ml aliquot of 1/10 LBns-Dp culture in 1.5-ml microcentrifuge tubes and mixed continuously at 28°C for 30 to 60 min. FN-beads and attached cells were bound to the tube edge using a Dynal MPC-96 magnet, and the supernatant was carefully removed into a new tube. Each FN-bead fraction was resuspended in 1 ml of spent medium. Spent medium was prepared from 1/10 LBns-Dp cultures by sedimentation of cells by centrifugation ($6,000 \times g$, 15 min) and filtration of the culture supernatant (0.22- μ m-pore-size filter; Millipore). For all experiments, five replicate 1-ml aliquots were analyzed.

Luminescence, total DNA, and CFU measurements. Promoter-luciferase fusions for *agfB*, *agfD*, *adrA*, and *yihU* in pCS26-Pac or pU220 reporter vectors (5) have been described previously (19, 59). Luminescence (counts per second [cps]) from three 200- μ l aliquots from each 1-ml sample of final culture, FN-bead, or supernatant fraction was measured in a 96-well clear-bottom black plate (9520 Costar; Corning Inc.) using a Wallac Victor² (Perkin-Elmer Life Sciences, Boston, MA). After measurement, cells were recovered from the wells and combined with initial samples. Total DNA levels were measured by the method of Kim and Surette (24). Briefly, 500 μ l of cells from the initial samples was sedimented by centrifugation ($8,000 \times g$, 2 min), resuspended in 150 μ l of filter-sterilized water, boiled for 4 min, and cooled on ice. One hundred microliters of this cell slurry was added to a 96-well plate, and 10-fold serial dilutions were performed to a final volume of 90 μ l per well. Ten microliters of a 3.4 μ M solution of Syto-9 (Molecular Probes, Invitrogen Canada Inc.) was added to each well, and the plate was incubated for 1 h at room temperature in the dark with agitation. Fluorescence measurements (1 s per well; 485-nm excitation, 525-nm emission) were performed in a Fusion microplate reader (Perkin-Elmer). Normalized expression values were compared by two-way analysis of variance with the sample type and number of days of growth representing independent variables; *P* values of <0.05 or <0.01 were considered significant.

Sodium hypochlorite experiments. FN-bead and supernatant fractions from 15 1-ml aliquots of 48-h 1/10 LBns-Dp cultures were combined prior to analysis. Aliquots (500 μ l) of FN-bead or supernatant fractions were combined with 100 μ l of sterile water (pH 7) (Invitrogen Canada Inc.) or sodium hypochlorite solutions (bleach; to a final concentration of 20, 40, 60, or 100 ppm) and mixed continuously for 10 min at room temperature. Cells were sedimented by centrifugation ($7,000 \times g$, 2 min), the supernatant was removed, and 500 μ l of phosphate-buffered saline (PBS), pH 7.4, was added. For CFU determination, each individual sample was homogenized in a tissue grinder for 15 to 20 s, serially diluted in triplicate in PBS, and plated in duplicate in 5- μ l drops onto LB agar and incubated at 28°C overnight. The initial CFU values were $4.8 \times 10^5 \pm 1.8 \times 10^5$ CFU for FN-beads and $1.7 \times 10^7 \pm 0.1 \times 10^7$ CFU for supernatants. The survival values (log CFU after treatment/log CFU before treatment) at each concentration of sodium hypochlorite were compared by unpaired *t* tests; *P* values of <0.05 were considered significant.

Fluorescence microscopy. Fluorescence reporters for *agfB*, *adrA*, and *yihU* were generated from the corresponding pCS26 or pU220 vector by replacing the NotI-NotI fragment containing *luxCDABE* with NotI-NotI fragments containing either *gfp* from pCS21 (5) or eCherry PCR amplified from pFFUNR (M. Elowitz) using primers eCherryF (CTGATTGCGGCCGCGTTTAACITTAAGAA GGAGCACCTG) and eCherryR (CTGATTGCGGCCGCGTTATTATTATTT GTACAGCTCATCCATG) (NotI restriction sites are underlined). For phase-contrast microscopy, differential interference contrast microscopy (DICM), or fluorescence microscopy (FM), 30- μ l aliquots of culture, FN-bead, or supernatant fractions were pipetted onto glass slides, covered with 9- by 9-mm glass coverslips pretreated with poly-L-lysine (Sigma) following established procedures (14), and sealed with Mowiol glue (EMD Biosciences Inc.). Cells and reporters were visualized with Leica DM IRE2 and DM RXA2 fluorescence microscopes using 470-nm excitation and 525-nm emission for green fluorescent protein (GFP) and 575-nm excitation and 640-nm emission for eCherry.

Competitive infection of mice. Female C57BL/6 (*Salmonella*-susceptible) mice (6 to 10 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in the animal facilities at the University of British Columbia in direct accordance with guidelines drafted by the University of British Columbia's Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Groups of five mice were infected orally with approximately 2×10^6 CFU total of a mixed inoculum in 100 μ l of 100 mM HEPES containing an approximate 1:1 ratio of strain ATCC 14028 and $\Delta agfA$ strain. Strains used in the inoculum were grown in either nutrient-rich (NR) conditions (Miller's LB at 37°C for 16 h; A_{600} of 3.0 to 3.1) or nutrient-limiting (NL) conditions (1/10 LBns-Dp at 28°C for 48 h; A_{600} of 0.25 to 0.29). The appropriate dilutions for each strain under each growth condition were determined by total DNA measurements, and the doses administered were determined by serial dilution and plating. The ratios of strains administered (CFU of $\Delta agfA$ strain/CFU of strain ATCC 14028) were 1.10 for the mice under NR conditions and 1.24 for the mice under NL conditions. Infected mice were humanely euthanized 96 h postinfection, prior to homogenization of the spleen, mesenteric lymph nodes (MLN), small intestine, and colon in PBS using a high-speed mixer mill (MM301; Retsch, Newtown, PA). Organ homogenates were serially diluted in PBS and plated on XLD agar (Oxoid, United Kingdom) for determination of total *Salmonella* CFU. Colonies were replica plated onto 1% tryptone agar containing 100 μ g/ml Congo red, and cells were grown at 28°C to differentiate between wt and $\Delta agfA$ bacteria. The competitive index (CI) was calculated as [CFU mutant/CFU wild-type]_{output}/

FIG. 1. *agfB* expression versus cell density during growth of *S. enterica* serovar Typhimurium strain ATCC 14028 in nutrient-limiting conditions. Expression of an *agfB* promoter-luciferase fusion was measured during growth of strain ATCC 14028 at 28°C in different media. The strain was grown in different media as follows: bar 1, LB without salt (LBns); bar 2, 1/4 LBns; bar 3, 1/10 LBns; bar 4, M9 minimal medium alone; bars 5 to 7, M9 minimal medium supplemented with 0.5% Casamino Acids (bar 5), 0.05% glycerol (bar 6), or 0.05% glucose (bar 7); bar 8, M9 minimal medium without sulfate; bars 9 and 10, minimal medium containing MOPS supplemented with 0.72% glucose (bar 9) or 0.036% glucose (bar 10); bars 11 to 13, minimal medium containing MOPS without bicarbonate (bar 11), nitrogen (bar 12), or phosphate (bar 13); and bar 14, EPS medium (bar 14). Bars correspond to the average maximum optical density measurements during the 48-h growth period; white circles represent the average maximum luminescence values (counts per second [CPS]). Error bars represent the 95% confidence intervals determined from three replicate samples.

[CFU mutant/CFU wild-type]_{input}. CI data were analyzed using the Mann-Whitney U test; *P* values of <0.05 were considered significant.

Bioluminescence imaging of *Salmonella* reporter strains during murine infection. Female C57BL/6 mice were infected orally with ~10⁷ CFU of strain ATCC 14028 *agfD*, *agfB*, *yihU*, *sig70_16*, or *sig38H4* luciferase reporter strains grown for 16 h in Miller's LB at 37°C. *sig70_16* and *sig38H4* reporters contain control promoters dependent on RpoD (σ^{70}) and RpoS (σ^s), respectively, and have been described previously (23, 59). Infected mice were euthanized, and their gastrointestinal tracts and organs were removed and imaged for 4 min using an IVIS 100 (Xenogen, Alameda, CA). Grayscale reference images were overlaid with images capturing the emission of photons from the ATCC 14028 reporter strains using LIVING IMAGE software (Xenogen) and Igor (Wavemetrics, Seattle, WA). To determine the total number (CFU) of bioluminescent *Salmonella*, the spleen, liver, MLN, ileum, cecum, and colon were homogenized in PBS using a high-speed mixer mill (MM301; Retsch, Newtown, PA), diluted, and plated on LB agar supplemented with 50 μ g/ml Kan. To determine the prevalence of plasmid loss, ground tissue mixtures were grown on XLD agar (Oxoid, United Kingdom), and the colonies were imaged for bioluminescence production.

RESULTS

***Salmonella* aggregation occurs at low cell densities in NL liquid media.** Although the *rdar* morphotype is easily studied during *Salmonella* growth in rich media, such as LB, problems arise when trying to compare aggregative strains that form large, recalcitrant cell aggregates to nonaggregative strains where the cells can be easily dispersed. If starvation could be induced at low cell densities, we hypothesized that cell aggregates formed would remain small enough to facilitate comparisons between strains and allow us to evaluate the role of aggregation in *Salmonella* virulence. Furthermore, we reasoned that starvation at low cell densities would represent a more natural growth state for *Salmonella*.

We investigated growth of *S. enterica* serovar Typhimurium ATCC 14028 in a variety of NL conditions. The level of aggregation was estimated by monitoring *agfB* expression from a promoter-luciferase reporter. As expected, cell density and *agfB* expression reached the highest level in LBns (Fig. 1, bar

FIG. 2. Temporal expression of *rdar* morphotype genes during growth of strain ATCC 14028 in 1/10 LB without salt at 28°C. Expression of *agfD*, *agfB*, *adrA*, or *yihU* promoter-luciferase fusions was measured during growth of strain ATCC 14028 in 1/10 LBns (open symbols) or 1/10 LBns supplemented with 40 μ M 2,2'-dipyridyl (closed symbols) at 28°C with agitation. Curves represent the average luminescence (counts per second [CPS]) from three biological replicates of each reporter strain as a function of time. Measurements for *yihU* and *adrA* are on the right *x* axis. The broken line represents *agfB* expression during growth of strain ATCC 14028 in LBns at 28°C with agitation. Inset in upper left represents cell density of cultures (A_{620}) as a function of time.

1), which was used as a nutrient-rich control medium. However, when cells were grown in dilute LBns (Fig. 1, bars 2 and 3), cell density was lower ($A_{600} < 0.3$), but *agfB* expression remained relatively high. In M9 minimal medium, both cell density and *agfB* expression measurements were generally low (Fig. 1, bars 4, 5, 6, and 7). Only in M9 minimal medium without sulfate did *agfB* expression reach higher levels (Fig. 1, bar 8). In minimal medium containing MOPS supplemented with 0.72% glucose, cell density was relatively high, while *agfB* expression was low (Fig. 1, bar 9). However, in minimal medium containing MOPS supplemented with 0.036% glucose or containing MOPS without bicarbonate, nitrogen, or phosphate (Fig. 1, bars 10, 11, 12, and 13), *agfB* expression was increased, and cell density was low. Cells grown on EPS medium had intermediate cell density and *agfB* expression levels (Fig. 1, bar 14). The addition of 5 mM c-di-GMP to dilute LBns or minimal medium containing MOPS had very little effect on *agfB* expression (data not shown). Further analysis by light microscopy revealed that cultures grown in 1/10 LBns (Fig. 1, bar 3) contained the highest proportion of aggregated cells. Therefore, this medium was chosen for use in subsequent experiments.

***rdar* morphotype genes are expressed during growth in NL conditions.** Expression of *rdar* morphotype genes *agfD*, *agfB*, *adrA*, and *yihU*, a gene required for biosynthesis of O-antigen capsule (19), was monitored during growth of strain ATCC 14028 in 1/10 LBns at 28°C. Activation of *yihU* occurred before the other genes, within the first 5 h of growth (Fig. 2). Expression of *agfD*, *agfB*, and *adrA* occurred approximately 5 h later and was synchronized (Fig. 2) as previously reported (55, 59). *agfB* expression was activated at least 10 h earlier in 1/10 LBns than in nutrient-rich LBns medium (Fig. 2, broken line); this same trend was observed for *agfD*, *adrA*, and *yihU* (data not

FIG. 3. Cell-cell aggregation in strain ATCC 14028 and $\Delta agfA$ cultures. *S. enterica* serovar Typhimurium strain ATCC 14028 (ST 14028) and $\Delta agfA$ cultures were analyzed by differential interference contrast microscopy after growth in LBns for 6 h (A) or in 1/10 LBns-Dp for 48 h (B) at 28°C without agitation. Pictures in the top and bottom panels in panel B were taken from the same cultures. A_{600} values were 0.208 (A) and 0.229 (B) for strain ATCC 14028 cultures and 0.288 (A and B) for $\Delta agfA$ cultures. Each picture was magnified 40,000 $\times$. Dp, 2,2'-dipyridyl.

shown). Since iron limitation is known to stimulate *agfD* expression (41, 42), gene expression was measured in media supplemented with 40 μ M Dp, a well-characterized iron chelator (5). Overall growth was unaffected by iron depletion under these conditions (Fig. 2, inset), but the reporter strains had altered gene expression. For each time point between 25 h and 40 h, expression levels for all four reporter strains were significantly higher in the iron-depleted media ($P < 0.05$; unpaired t tests). Maximum expression was significantly increased only for *agfD* ($P < 0.05$; unpaired t test). These experiments indicated that Tafi, cellulose, and O-Ag capsule were being produced under NL conditions.

Tafi are essential for cell-cell aggregation. To investigate the importance of Tafi in the aggregation process, strain ATCC 14028 and $\Delta agfA$ cultures were analyzed by DICM. After 6 h of growth in LBns at 28°C, cells in both wt and $\Delta agfA$ cultures were dispersed as individual, planktonic cells (Fig. 3A). This result was expected, since 6 h corresponds to a time period when *agfD* and *agfB* expression are at background levels (Fig. 2). After 48 h growth in 1/10 LBns-Dp, DICM analysis revealed that strain ATCC 14028 cultures consisted of small clumps of cells (Fig. 3B, ST 14028, top panel), large aggregates of cells or “microcolonies” (Fig. 3B, ST 14028, bottom panel) and individual, planktonic cells. In contrast, $\Delta agfA$ cultures consisted entirely of planktonic cells and were devoid of cell clumps (Fig. 3B). This demonstrated that Tafi were essential for cell-cell aggregation under these growth conditions.

Aggregated cells can be isolated from strain ATCC 14028 cultures based on the presence of Tafi. To visualize gene expression in individual cells, cultures of strain ATCC 14028 containing an *agfB::gfp* reporter were analyzed by FM. After 48 h of growth in 1/10 LBns-Dp at 28°C, GFP expression was primarily localized to the aggregated cell clumps, whereas individual, planktonic cells had little or no expression (Fig. 4A). This indicated that the aggregated cells were producing greater amounts of Tafi than the planktonic cells. Based on the principle that purified Tafi are able to bind fibronectin (K_D of ~ 74 μ M [10]), fibronectin-coated magnetic beads (FN-beads) were used to fractionate the cultures. The FN-bead fraction consisted of large “microaggregates” greater than 75 μ m in diameter (Fig. 4B) and smaller clumps of ~ 20 to 50 cells (Fig. 4C), with all cells in the aggregates displaying uniform GFP expression. In contrast, cells that did not bind to the FN-beads and remained in the supernatant fraction had little or no GFP expression (Fig. 4D). Recovery of cells after culture fractionation was not 100% efficient; cells in the FN-bead fraction represented $10.4\% \pm 7.1\%$ of the total cells in the culture based on CFU and $6.5\% \pm 3.3\%$ based on total DNA measurements, whereas cells in supernatant fraction represented $73.6\% \pm 21.5\%$ based on CFU and $88.1\% \pm 8.2\%$ based on total DNA measurements.

Expression differences between the aggregated and planktonic cell fractions were confirmed using strain ATCC 14028 *agfB::lux* cultures grown for 2, 3, 4, or 5 days. On day 2, *agfB* expression was approximately 20 times higher in cells from the FN-bead fraction compared to cells in the supernatant fraction (Fig. 5). Expression in the FN-bead fractions remained high on day 3 but dropped to only three times higher than the supernatant fractions by day 5 (Fig. 5). During this time period, there were no visible changes in the cultures and A_{600} measurements remained constant (data not shown). *agfB* expression in cells from the supernatant fractions was consistently low ($< 1,000$ relative light units) for the duration of the experiment (Fig. 5). Expression levels did fluctuate for the unfractionated cultures but always were intermediate between the FN-bead and supernatant fractions (Fig. 5).

Strain ATCC 14028 microaggregates are not clonal. To determine whether strain ATCC 14028 microaggregates were producing cellulose, *adrA* expression was monitored using a fluorescent eCherry reporter. Cultures were fractionated with FN-beads, and the majority of large cell clumps were positive for eCherry expression (data not shown). This indicated that the cell aggregates were producing cellulose. Some individual cells were eCherry positive, but these were quite rare (data not shown). These results enabled us to perform dual-labeling experiments to test whether the cell aggregates were clonal. When strain ATCC 14028 *agfB::gfp* and *adrA::eCherry* reporter strains were grown together and cultures were fractionated with FN-beads, each one of the > 50 large cell aggregates examined displayed both GFP and eCherry expression (Fig. 6A and B). GFP expression was often localized to specific clumps of cells in the aggregate, whereas eCherry expression appeared to be spread throughout (Fig. 6A and B). Several regions of the cell aggregates were yellow, with overlapping GFP and eCherry expression; this was due to cells present in different focal planes of the three-dimensional clumps. These experiments showed that the larger cell aggregates were not clonal,

FIG. 4. *agfB* expression in individual cells from different cell fractions of strain ATCC 14028 cultures. Strain ATCC 14028 *agfB::gfp* reporter strain cultures grown in 1/10 LBns-Dp for 72 h at 28°C were visualized by light microscopy (images on left) and fluorescence microscopy (images on right) either directly (A) or after fractionation with fibronectin-coated magnetic beads (B, C, and D). Representative cells from bead fractions (B and C) and supernatant fractions (D) are shown; arrowheads indicate beads that are bound to cells. Scale bars are shown at bottom right of the light microscopy images.

indicating that aggregation occurred between adjacent cells during growth.

Strain ATCC 14028 microaggregates are more resistant to sodium hypochlorite than planktonic cells. From the observation that strain ATCC 14028 microaggregates were expressing Tafi and cellulose, we hypothesized that aggregated and planktonic cells would have measurable physiological differences. Cells in FN-bead or supernatant fractions from strain ATCC 14028 cultures were exposed to sodium hypochlorite. Cells from both fractions survived equally well when exposed to low

concentrations (Fig. 7; 20 ppm) and were killed when exposed to high concentrations (Fig. 7, 100 ppm). However, at 40 ppm, cells in the supernatant fraction had a decrease in survival of 1 $\log_{10}$ unit compared to FN-bead cells, and at 60 ppm, the difference was statistically significant at greater than 4 $\log_{10}$ units (Fig. 7). Therefore, the aggregated cells in FN-bead fractions represented a more resistant physiological state than planktonic cells in the supernatant fractions.

Competitive index infections in mice. We reasoned that the rdar morphotype might be a common physiological form of

FIG. 5. Quantification of *agfB* expression in strain ATCC 14028 cultures fractionated with fibronectin-coated magnetic beads. The ATCC 14028 *agfB::lux* reporter strain was grown in 1/10 LBns-Dp for 48 to 120 h at 28°C without agitation prior to fractionation with fibronectin-coated magnetic beads (FN-beads). Expression (cps) from cells in unfractionated culture, FN-bead, and supernatant fractions was normalized to the amount of DNA in each sample and is represented as relative light units (RLU). Bars represent the average RLU values $\pm$ standard deviations (error bars) from five independent samples measured in triplicate. Statistically significant differences between FN-beads, supernatant, and culture samples are noted by asterisks (**, $P < 0.01$; *, $P < 0.05$) as determined by two-way analysis of variance.

Salmonella encountered by susceptible hosts, an idea strengthened by the finding that Tafi are recognized by TLR-2 (53). This theory led us to investigate whether cell aggregates expressing the rdar morphotype would have an advantage over planktonic cells for passage through the murine intestinal tract. The test group of C57BL/6 mice (susceptible) were infected orally with an equal mixture of strain ATCC 14028 and $\Delta agfA$ cells grown in NL conditions at 28°C, conditions where the wt

FIG. 6. Expression of *adrA* and *agfB* in strain ATCC 14028 cell aggregates bound to fibronectin-coated magnetic beads. ATCC 14028 *agfB::gfp* and *adrA::eCherry* reporter strains were grown together in 1/10 LBns-Dp for 72 h at 28°C without agitation prior to fractionation with FN-beads and analysis by differential interference contrast microscopy and fluorescence microscopy. (A and B) DICM images of representative cell aggregates with scale bars shown; (C and D) DICM and FM images that have been merged.

FIG. 7. Survival of cells in different strain ATCC 14028 culture fractions following exposure to sodium hypochlorite. Strain ATCC 14028 cells were grown in 1/10 LBns-Dp for 48 h at 28°C without agitation prior to fractionation with FN-beads. Cells in the FN-bead and supernatant fractions were exposed to different levels of sodium hypochlorite for 10 min. Each value represents the average relative survival from at least four samples; the error bars reflect the 95% confidence intervals. Statistically significant differences ($P < 0.05$) between the FN-bead and supernatant fractions at different hypochlorite concentrations are noted by an asterisk.

culture was a mixture of cell aggregates and planktonic cells, whereas the $\Delta agfA$ culture consisted of planktonic cells only. Control mice were infected orally with an equal mixture of strain ATCC 14028 and $\Delta agfA$ cells grown in NR conditions at 37°C, conditions where both wt and $\Delta agfA$ cultures consisted entirely of planktonic cells. Organ colonization levels and CI ratios were determined at 4 days postinfection.

The numbers of colonizing *Salmonella* ranged from 10^1 to 10^5 CFU in spleen and MLN and 10^1 to 10^6 CFU in the colon and small intestine (Fig. 8A). In general, mice infected with *Salmonella* grown in nutrient-limiting conditions had higher CFU loads in these organs than mice infected with cells grown in nutrient-rich conditions (Fig. 8A), although the differences were not statistically significant. The average CI for $\Delta agfA$ cells was greater than 1 in each of the organs of the mice grown in NL condition (Fig. 8B), indicating that the nonaggregative $\Delta agfA$ cells were able to outcompete aggregation-positive wt cells during colonization and infection. In contrast, the CI values measured in organs of the mice grown in NR conditions were close to a value of 1 (Fig. 8B), indicating there was no advantage for the $\Delta agfA$ strain compared to the wt when both strains were planktonic. The difference between the NL and NR groups of mice was statistically significant in the spleen (Fig. 8B).

Bioluminescence imaging of rdar morphotype gene expression during murine infection. To visualize rdar morphotype gene expression during infection, mice were infected orally with strain ATCC 14028 containing *agfD*, *agfB*, or *yihU* luciferase reporter. Control groups of mice were infected with strain ATCC 14028 containing σ^{70} (RpoD)- or RpoS-dependent luciferase reporters. All strains were grown in NR conditions prior to infection. *agfD* expression was detected from the small intestine, liver, spleen, and MLN (Fig. 9A). Expression of *yihU* was also detected from the ileum, spleen, and liver (Fig. 9A). In contrast, *agfB* expression was not detected in any of the infected mice, indicating that Tafi were not produced during murine infection. As expected, bioluminescence corresponding

FIG. 8. Competitive index infections of $\Delta agfA$ strain and strain ATCC 14028 in C57BL/6 mice. Groups of mice were infected with a mixed inoculum of $\Delta agfA$ strain and strain ATCC 14028 cells that were grown in nutrient-rich conditions at 37°C (NR) or nutrient-limiting conditions at 28°C (NL). The total numbers of colonizing *Salmonella* (A) and CI for the $\Delta agfA$ strain (B) were determined in the spleen, MLN, colon, and small intestine (Sm Int.). Each data point represents one animal, and horizontal bars indicate means. The broken line in panel A represents the limit of detection (10 CFU), and the broken line in panel B represents a CI value of 1, which would result if there was no competitive difference between the $\Delta agfA$ strain and strain ATCC 14028. P values are listed above CI results from each organ. The asterisk represents statistical significance (P < 0.05).

to σ^{70} activity was detected in all mice, from the spleen, liver, and MLN, as well as throughout the gastrointestinal tract (Fig. 9A). RpoS activity was also detected but was primarily localized to the ileum, liver, and spleen (Fig. 9A). Colonization levels for the *Salmonella* reporter strains in C57BL/6 mice ranged from 10^5 to 10^9 CFU in the spleen, 10^5 to 10^8 CFU in the liver, 10^5 to 10^7 CFU in the MLN, 10^7 to 10^8 CFU in the small intestine, 10^5 to 10^9 CFU in the cecum, and 10^6 to 10^9 CFU in the colon. The percentage of plasmids carrying *Salmonella* recovered from the different tissues ranged between 100 and 10; generally, bacteria isolated from the cecum and colon had more plasmid loss than bacteria isolated from the spleen, liver, and MLN. When plasmid loss values from all tissues were averaged, each ATCC 14028 reporter strain was determined to have plasmid loss of less than 40% with no significant differences between reporters (data not shown). Bioluminescence from all reporters, including *agfB*, was detected in fecal pellets from the infected mice (Fig. 9B). Shedding of bioluminescent *Salmonella* occurred as early as 15 h

postinfection (data not shown). The strongest signals were detected from *agfD* and the control σ^{70} reporter (Fig. 9B).

DISCUSSION

In this study, we analyzed growth of *S. enterica* serovar Typhimurium under nutrient-limiting conditions, conditions predicted to occur in natural environments, to examine the role of the *rdar* morphotype in nature. We observed that the formation of microaggregates occurred at low cell densities. The *rdar* morphotype was directly linked to the aggregation observed; *agfD*, *agfB*, *adrA*, and *yihU* genes associated with production of Tafi, cellulose, and O-Ag capsule were activated early in growth. Iron depletion caused an increase in *agfD* expression as reported previously (42), as well as prolonged elevated expression for downstream components *agfB*, *adrA*, and *yihU*. Since the timing of gene expression was not changed by iron depletion, we hypothesized that cells were aggregating in response to the depletion of additional nutrients. We also observed that *Salmonella* aggregation was associated with a lack of phosphate, bicarbonate, and sulfate. It is assumed that each of these starvation responses converges through the activation of RpoS (36). The addition of exogenous c-di-GMP did not affect *agfB* gene expression, although this may not accurately mimic production of this signaling molecule by GGDEF domain proteins inside the cell.

At low cell densities, *Salmonella* cultures consisted of two distinct populations of cells, aggregated and nonaggregated, with the aggregated cells contributing the majority of *rdar* morphotype gene expression. This is in contrast to *Salmonella* aggregation during growth in rich media, where the cell population behaves as a multicellular unit (42, 47, 59, 63). We confirmed a difference between aggregated and nonaggregated cells using fluorescence microscopy with GFP and eCherry reporters, isolation of aggregated cells through binding to fibronectin-coated magnetic beads, and quantitation of expression differences using an *agfB* luciferase reporter. It was determined that large cell aggregates in the wt cultures were formed from clusters of cells that aggregated followed by growth within the aggregates. Many cells in the wt cultures remained planktonic; therefore, aggregation was assumed to be an active process without “trapping” of planktonic cells, which may occur in the formation of large pellicles at the air-liquid interface in mixed cultures (26, 44). We were unable to determine whether smaller cell clumps (i.e., <20 cells) were clonal due to the difficulty in visualizing *adrA*::eCherry expression in individual cells. This difficulty is most likely explained, because *adrA* is a weaker promoter than *agfB* (Fig. 2) (59) and eCherry is not as bright as GFP (46). However, it is possible that *adrA* expression is activated only after large cell aggregates are formed.

Physiological differences were detected between aggregated cells and nonaggregated cells isolated from strain ATCC 14028 cultures. The aggregated cells had an enhanced resistance to 60 ppm sodium hypochlorite of up to 4 log₁₀ units compared to the nonaggregated cells. Since cellulose is strongly associated with resistance to sodium hypochlorite (44, 50, 59), these results confirmed indirectly that aggregated clumps of cells were producing cellulose. The aggregated cells did not have enhanced resistance to desiccation compared to nonaggregated

FIG. 9. Bioluminescence imaging of *Salmonella* rdar morphotype gene expression in mice infected by oral gavage. (A) In vivo bioluminescence of the gastrointestinal tracts and organs of C57BL/6 mice following infection with strain ATCC 14028 *agfD*, *agfB*, *yihU*, *sig70_16* (σ^{70}), and *sig38H4* (RpoS) luciferase reporter strains. Images show the relative signal intensity visualized at a given anatomical location within tissue (small intestine [Sm Int], mesenteric lymph nodes [MLN], spleen [Sp], liver [Li], and colon [Co]). In the color bars displayed on the right of each image, red corresponds to the highest signal intensity and blue corresponds to the lowest signal intensity in units of light measurement (photons/second/cm²/steradian). Representative images are shown from 3 (*yihU* and RpoS) or 13 (*agfD*, *agfB*, and σ^{70}) mice. (B) Bioluminescent images of fecal pellets from mice infected with strain ATCC 14028 reporter strains. Pellets were collected between 60 h and 84 h postinfection. The scale of signal intensity for each image is located on the right.

cells (A. P. White and M. G. Surette, unpublished data). We were unable to determine whether aggregated cells were producing O-Ag capsular polysaccharide, which is of primary importance for desiccation resistance (19), because the *yihU* promoter was not strong enough to visualize with either the GFP or eCherry reporter (data not shown). Nevertheless, the aggregated cells exhibited some of the same physiological properties as those of the rdar morphotype colonies and pellicles (1, 44, 59), suggesting that the formation of small cell aggregates could enhance *Salmonella* survival in natural environments.

Competitive infection experiments between wt strain ATCC 14028 and Δ *agfA* strains indicated that aggregation is a disadvantage for murine infection. This result contradicts previous hypotheses that resistant rdar morphotype components would aid in passage through the stomach and intestinal tract (11, 63). The inclusion of 100 mM HEPES in our inoculums would have buffered the stomach acid, reducing any survival differences between aggregated and planktonic cells (1). This is

potentially significant, since tests with human volunteers have demonstrated that buffering of the stomach pH reduces the infectious dose of *Salmonella* (6). Determination of the CI ratios at the 96-h time point may be more a measure of colonization efficiency and may not accurately assess passage through the stomach; therefore, it is possible that the ratio of strains was different at earlier time points. Despite these potential caveats, the Δ *agfA* strain was consistently detected at higher levels in the internal organs of the infected mice. If formation of microaggregates provided wt cells an advantage over Δ *agfA* planktonic cells in passage through the stomach, it is assumed that this would have been detected. One possibility is that phagocytosis is inhibited by the presence of the extracellular matrix and, therefore, fewer *Salmonella* go systemic. Planktonic cells may also have increased access to host receptors required for systemic spread. The overall conclusion from these CI experiments was that aggregation is not a virulence adaptation in *Salmonella*.

Analysis of rdar morphotype gene expression during infection revealed unexpected complexities. Although *agfD* expression was consistently detected inside the infected mice, *agfB* expression was not detected. This result was unexpected because *agfD* and *agfB* expression has been tightly coupled in all culture conditions examined (17, 38, 39, 41, 59) (White and Surette, unpublished). *agfD* expression in vivo may be below a threshold level required for *agfB* activation (60). Alternatively, the uncoupling of *agfD* and *agfB* expression may indicate the absence of a signaling event or presence of an inhibitory condition or factor that is preventing *agfB* expression inside the host. *agfB* was expressed by *Salmonella* as the bacteria were shed in the feces, however, which indicates that this environment was conducive for Tafi production. The change in *agfB* expression may be partially due to a drop in temperature, since maximal expression in vitro is achieved at temperatures below 30°C (42, 55).

The finding that Tafi were not expressed during murine infection explains why Δ *agfA* strains are not attenuated for virulence as reported previously (56). In addition, this result calls into question previous reports on the ability of Tafi to bind various host proteins and associated factors (3). These interactions could conceivably occur if Tafi are present in the initial stages of infection and cells are engulfed and passed into the bloodstream (54) where Tafi could persist for longer time periods. On the other hand, the “sticky” nature of native preparations of Tafi or curli fimbriae, which are mixtures of aggregative protein and polysaccharides (19, 58, 63), could easily cause in vitro binding artifacts. *adrA* was not included in our in vivo bioluminescence experiments, but it has been shown that cellulose production is also not required for *Salmonella* virulence in the murine model of infection (50). It is important to consider whether it is suitable to extrapolate results from the systemic infection caused by serovar Typhimurium in mice to the self-limiting gastroenteritis infection that occurs in humans. We hypothesize that the principles of survival and passage through the stomach and intestine are similar and that Tafi and cellulose are not important for *Salmonella* virulence under infection conditions similar to those reported here.

Expression of *agfD* inside the host suggested that AgfD could be functioning in a regulatory capacity during murine infection. AgfD is known to regulate *yihU* (19), *bapA* (26), and many additional genes in culture (7, 8, 18). The possibility that *Salmonella* produces an O-Ag capsule during infection is significant. Polysaccharide capsules can mediate resistance to important host defense mechanisms, including phagocytosis and complement-mediated killing, and are important for virulence of diverse pathogenic bacteria, such as *S. enterica* serovar Typhi (reviewed in reference 37), *Streptococcus* spp. (27, 32), *Haemophilus influenzae* (52), *Francisella tularensis* (57), and *Burkholderia mallei* (13). AgfD may also be responsible for *bapA* expression in vivo. BapA was recently shown to be important for initial colonization in mice, and presumably as a result, *bapA* mutants were attenuated for virulence (26). These results indicate that the rdar morphotype could have a complex role in the life cycle of *Salmonella*.

In our competitive infection experiments, there was no difference in colonization by cells grown in nutrient-rich versus nutrient-limiting conditions, suggesting that *Salmonella* infection will occur if enough cells reach the small intestine. Ex-

pression of the rdar morphotype would ensure that more cells survive outside the host to be able to cause infection, which may explain why this phenotype has been conserved through evolution (60). The finding that all rdar morphotype genes analyzed were expressed in fecal pellets seems to be consistent with this hypothesis. The few *S. enterica* serovars that have lost the ability to aggregate via the rdar morphotype, such as serovars Typhi and Choleraesuis (40; A. P. White, S. L. Stocki, K. E. Sanderson, and M. G. Surette, unpublished observations), share an ability to cause systemic disease. It is possible that the loss of the rdar morphotype in these groups of strains represents a biological trade-off for increased virulence or host colonization at the expense of long-term survival in the environment. If the primary role of aggregation in the *Salmonella* life cycle is for survival in nutrient-limiting conditions, loss of the rdar morphotype could reflect differences in the modes of transmission for different serovars.

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