

## Thin Aggregative Fimbriae and Cellulose Enhance Long-Term Survival and Persistence of *Salmonella*

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*Salmonella* spp. are environmentally persistent pathogens that have served as one of the important models for understanding how bacteria adapt to stressful conditions. However, it remains poorly understood how they survive extreme conditions encountered outside their hosts. Here we show that the rdar morphotype, a multicellular phenotype characterized by fimbria- and cellulose-mediated colony pattern formation, enhances the resistance of *Salmonella* to desiccation. When colonies were stored on plastic for several months in the absence of exogenous nutrients, survival of wild-type cells was increased compared to mutants deficient in fimbriae and/or cellulose production. Differences between strains were further highlighted upon exposure to sodium hypochlorite, as cellulose-deficient strains were 1,000-fold more susceptible. Measurements of gene expression using luciferase reporters indicated that production of thin aggregative fimbriae (Tafi) may initiate formation of colony surface patterns characteristic of the rdar morphotype. We hypothesize that Tafi play a role in the organization of different components of the extracellular matrix. Conservation of the rdar morphotype among pathogenic *S. enterica* isolates and the survival advantages that it provides collectively suggest that this phenotype could play a role in the transmission of *Salmonella* between hosts.

The genus *Salmonella* comprises an ancient and diverse group of organisms that causes disease in, and can be commonly isolated from, a large number of animal hosts. In humans, *Salmonella* is the etiological agent of gastroenteritis and typhoid fever. The worldwide estimates of annual infection are 1.3 billion cases of gastroenteritis and/or diarrhea due to nontyphoidal salmonellosis, with 3 million deaths, and 16.6 million cases of typhoid fever, with nearly 600,000 deaths (24). One common feature among all *Salmonella* spp. is that they display enhanced survival in non-host environments, including soil and water (42). This fits into the cyclic lifestyle that has been proposed for *Salmonella* spp., consisting of passage through a host into the environment and back into a new host (42).

Most *Salmonella* isolates possess the ability to form patterned, aggregative colonies when grown on surfaces (2, 26). This multicellular phenomenon has been termed the rdar morphotype (colonies are “red, dry, and rough” when grown on media containing Congo red [9, 29]). The rdar morphotype is primarily controlled by expression of the divergent *agfDEFG* and *agfBAC* (*csgDEFG/BAC*) operons encoding thin aggregative fimbriae (Tafi or curli) (7, 16). AgfD is a positive regulator of Tafi production by activating transcription of *agfB* (16, 47). AgfD also stimulates cellulose production by transcriptional activation of *adrA* (47). AdrA contains the GGDEF domain involved in the synthesis of cyclic di-GMP, a signaling molecule required for activation of cellulose synthesis (14, 27, 34). Tafi and cellulose, along with additional exopolysaccharides (11, 41), form a recalcitrant extracellular matrix that serves to phys-

ically link cells together within a colony. These extracellular factors are produced by *Salmonella* under nutrient-limiting conditions at low osmolarity and growth temperatures below 30°C (9, 29).

We and others have hypothesized that the rdar morphotype could be important for environmental survival due to the following qualities: the recalcitrant nature of extracellular matrix components (9, 35, 41, 47), characteristic growth conditions (29), and increased resistance to antimicrobial stresses (2, 32, 35). Here, we show that the rdar morphotype enhances *Salmonella* survival during desiccation in the absence of nutrients. After storage on plastic for 9 months, wild-type cells of *S. enterica* serovar Typhimurium ATCC 14028 had 10 to 30 times increased survival compared to mutants deficient in Tafi and cellulose production. Increased resistance to desiccation would undoubtedly aid in long-term survival of *Salmonella* in the environment and promote passage to future hosts.

### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *S. enterica* serovar Typhimurium strain ATCC 14028 was used as the reference strain in this study. Strains from *Salmonella* reference collection B (SARB) have been described previously (5). Strains were routinely grown for 20 h at 37°C with agitation in 1% tryptone, pH 7.2 (T), or Miller's Luria-Bertani broth (1.0% salt), supplemented with 50 µg/ml kanamycin (Kan), if necessary, before performing additional experiments. For growth of cells on agar, cultures were diluted to an  $A_{600}$  of 1.0, and 1 µl was spotted onto solid T media containing 1.5% Difco agar. For strain-mixing experiments, equal volumes of *agfA*, *agfD*, or *bcsA* culture were mixed before spotting on agar. Cells were grown at room temperature (RT; ~22°C) or 28°C as indicated. To visualize the production of cellulose, cells were grown on T agar containing 200 µg/ml calcofluor white (fluorescent brightener 28; Sigma-Aldrich Canada). Colony pictures were taken with a Kodak Image Station 2,000MM camera system. For bioluminescence assays, overnight cultures were diluted 1:600 in T broth supplemented with 50 µg/ml Kan to a final volume of 150 µl in 96-well clear-bottom black plates (9520 Costar; Corning Inc.). The culture in each well was overlaid with 50 µl mineral oil prior to starting the assays. Cultures

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were assayed for luminescence (0.1 s) and absorbance (620 nm, 0.1 s) every 30 min during growth at 28°C with agitation in a Wallac Victor<sup>2</sup> (Perkin-Elmer Life Sciences, Boston, Mass.).

**Recombinant DNA techniques and construction of *lux* reporters.** Genomic DNA was prepared following the method of Walsh et al. (38). Promoter-containing regions for *agfD*, *agfB*, and *adrA* were PCR amplified from ATCC 14028 using primers *agfD*1 (GTGCTCGAGGGACTTCATTAACATGATG [a XhoI site is underlined]) and *agfD*2 (GCCGGATCCTGTTTTTCATGCTGTAC [a BamHI site is underlined]), *adrA*1 (GACCTCGAGAAGGCGATGACCGTAG CAAG [a XhoI site is underlined]) and *adrA*2 (GACGGATCCCGCTGTTCACCGCTTTT [a BamHI site is underlined]). PCR products were purified (QIAGEN Inc.), sequentially digested with XhoI and BamHI (Invitrogen Canada Inc.), and ligated using T4 DNA ligase (Invitrogen Canada Inc.) into pCS26-Pac (XhoI-BamHI) or pU220 (BamHI-XhoI) reporter vectors containing the *luxC-DABE* operon from *Photobacterium luminescens* (4). The majority of pCS26 and pU220 cloning was performed in *Escherichia coli* DH10B (Invitrogen Canada Inc.). All plasmids were transformed into *Salmonella* strains by electroporation (Bio-Rad Laboratories Inc.). Promoter:*lux* plasmids were purified from *Salmonella* (QIAGEN Inc.), and DNA sequencing was performed by Macrogen (Seoul, South Korea) using primers pZE05 and pZE06 (4).

**Construction of a sig38H4 reporter designed to measure RpoS activity.** A synthetic, 58-nucleotide, RpoS ( $\sigma^{38}$ )-responsive promoter (CTCGAGATAATTCCATGCGGTTTTGCTAAAATCATGTATACTTATTATCAATTGGATCC; -35 and -10 promoter regions are underlined, XhoI and BamHI restriction sites are in boldface) was generated using overlapping oligonucleotides. Primer clone (GCGCGCTCGAGAATAATTCCATGCGGTTTTGCTAAAATCA) and sig38-reverse (GCGCGGATCCAATTGATAATAANTATANATGATTTTACGAAA) were mixed, denatured at 94°C for 10 min, and allowed to anneal on ice before ligation into XhoI- and BamHI-digested pCS26. Primer sequences were designed based on consensus promoter sequences from *E. coli* genes that are RpoS dependent (3). The -35 promoter region was designed to be divergent from the RpoD ( $\sigma^{70}$ ) consensus sequence and therefore reduce binding of RpoD and subsequent transcription. Luminescence of the sig38H4 reporter in an  $\Delta rpoS$  mutant strain of *S. enterica* serovar Typhimurium ATCC 14028 remained near background levels (<8,000 cps) during growth in 1% tryptone (data not shown).

**Generation of serovar Typhimurium ATCC 14028 mutant strains.** Deletion constructs for *agfA* and *bcsA* generated from serovar Enteritidis 27655-3b DNA and cloned into pHSG415 have been described previously (39, 41). An in-frame deletion removing 612 bp in *agfD* (encoding amino acids 6 to 210 in AgfD) was generated using PCR primers *agfD*-A (GACGAATCCGTGTGTTATGCCGCATGGG [an EcoRI site is underlined]), *agfD*-B (GGACTGCAGTAAACATGATG [a PstI site is underlined]), *agfD*-C (GCCCTGCAGCAAACGATAATCTCAGCGCG [a PstI site is underlined]), and *agfD*-D (GCCAAGCTTTGTCCTGTGACGTTGAGCTGG [a HindIII site is underlined]). The two PCR fragments generated were directionally cloned into EcoRI- and HindIII-digested pTZ18R (Amersham Biosciences) and subcloned into pHSG415 (17). Each mutation was introduced into the chromosome of ATCC 14028 by following established procedures (40) with modifications (A. P. White, E. Ambrose, B. W. Jones, R. DeVinney, W. W. Kay, and M. G. Surette, unpublished data). Screening for strains containing the truncated *agfD* gene was accomplished using primers *agfD*ko1 (CACCTTGCTTTAAGATTTGTAATGGC) and *agfD*ko2 (ATTCGCTTCCCATTGTGTCG).

**Long-term survival experiments and treatment with sodium hypochlorite.** Cells from 1  $\mu$ l of ATCC 14028,  $\Delta agfD$ ,  $\Delta agfA$ , and  $\Delta bcsA$  overnight cultures were inoculated onto T agar and grown for 6 days at RT. Colonies were removed from the agar surface and tested immediately (initial numbers) or stored in 24-well tissue culture plates (one colony per well) at RT for 3 or 9 months. After rehydration of colonies in 500  $\mu$ l phosphate-buffered saline (PBS), pH 7.4, for 1 h at RT, 100  $\mu$ l of sodium hypochlorite solution (to a final concentration of 30 to 300 ppm) or PBS (control) was added and mixed continuously using a rotating microtube mixer for 20 min at RT. Cells and colony materials were centrifuged (7,000  $\times$  g, 2 min), and supernatant was removed. Fresh PBS (500  $\mu$ l) was added, and colony slurries were mixed in a tissue homogenizer until uniform turbidity was reached (~20 s). For planktonic-grown cells, 20-h cultures in 1% tryptone were normalized to an optical density of 1 at 600 nm, and 1-ml aliquots of cells were pelleted (7,000  $\times$  g, 2 min) prior to the treatment. The homogenization step was omitted. To determine the number of viable cells remaining in each sample, cell mixtures were serially diluted in triplicate and plated in duplicate in 5- $\mu$ l drops.

TABLE 1. Prevalence of multicellular pattern formation (rdar morphotype) in *Salmonella enterica* subgroup I strains (SARB) (5)

Serovar <sup>a</sup>	No. of strains tested	No. of isolates with indicated phenotype <sup>b</sup>			
		Pattern (rdar)	Incomplete pattern	No pattern	Calcofluor binding <sup>c</sup>
Agona	1	1			1
Anatum	1	1			1
Brandenburg	1	1			1
Choleraesuis	4	0		4	0
Decatur	1	1			1
Derby	3	2	1		3
Dublin	3	3			3
Duisburg	1	1			1
Enteritidis	4	3	1		4
Emek	1	1			1
Gallinarum	1	1			1
Haifa	1	1			1
Heidelberg	2	2			2
Indiana	1	1			1
Infantis	2	2			2
Miami	2	2			2
Montevideo	2	2			2
Muenchen	4	3		1	3
Newport	3	2		1	2
Panama	3	3			3
Paratyphi A	1	1			1
Paratyphi B	5	1	3	1	4
Paratyphi C	3	2		1	2
Pullorum	2	2			2
Reading	1	1			1
Rubislaw	1	1			1
Saintpaul	3	3			3
Schwarzengrund	1	1			1
Sendai	1	1			1
Senftenberg	1	1			1
Stanley	1	0		1	0
Stanleyville	1	1			1
Typhi	2	2			2
Typhimurium	4	4			4
Typhisuis	2	2			2
Wien	2	2			2
Total	72	58	5	9	63

<sup>a</sup> Strains are described in Boyd et al. (5).

<sup>b</sup> Colony morphology and associated phenotypes were recorded after growth on T agar at 28°C for up to 10 days: pattern (rdar), colonies had complete surface patterns and could be lifted intact from the agar surface; incomplete pattern, colonies could not be lifted from the agar surface; no pattern, colonies were smooth and mucoid.

<sup>c</sup> Fluorescence of colonies grown on T agar supplemented with 200  $\mu$ g/ml calcofluor was observed under a 366-nm UV light source and compared to ATCC 14028 positive control and  $\Delta bcsA$  negative control strains.

## RESULTS

**Prevalence of the rdar morphotype in *Salmonella enterica* subgroup I strains.** *S. enterica* subgroup I contains 99% of *Salmonella* serovars and all major human pathogenic types. To determine the prevalence of the rdar morphotype within *S. enterica* subgroup I, we analyzed isolates from *Salmonella* reference collection B (SARB) (5). Although the rdar morphotype was initially described during growth on media containing Congo red, this phenotype can be visualized during growth on standard agar by the formation of colony surface patterns (2). In addition, production of cellulose can be visualized by growing *Salmonella* on agar containing calcofluor (35). Three dis-

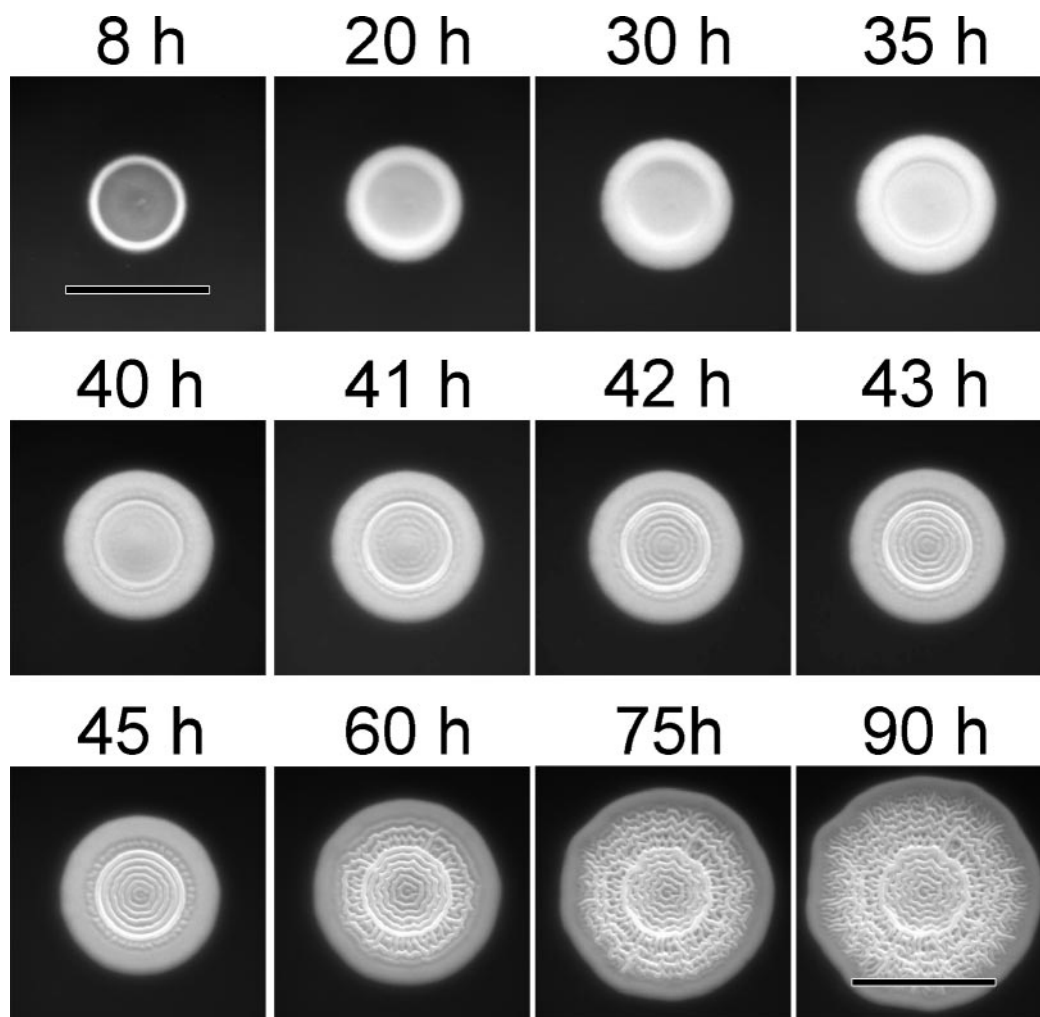


FIG. 1. Multicellular pattern formation during growth of *Salmonella* in colonies of the rdar morphotype. *S. enterica* serovar Montevideo (SARB 30) cells were inoculated on T agar and incubated at room temperature for 90 h. Colony images were recorded every 30 min. Bars represent 5 mm. The entire sequence of 170 pictures can be viewed as a movie ([www.med.ucalgary.ca/webs/bprg/Surette/Surette.html](http://www.med.ucalgary.ca/webs/bprg/Surette/Surette.html)).

tinct phenotypic classes were observed within SARB: 80.5% of isolates formed complete patterns (rdar morphotype), 7% formed incomplete patterns, and 12.5% had no surface patterns (Table 1). For all complete pattern strains, the entire colony could be lifted off the agar surface, indicating that a strong Tafi and cellulose network had formed. Members of this group included host-restricted serovars such as Typhi and Pullorum (Table 1). Colonies from isolates in the incomplete pattern class could not be removed from the agar surface in one piece. Each of these strains produced cellulose, suggesting that production of Tafi or other extracellular matrix components was impaired. Strains without colony surface patterns were not clustered into any particular groups, except for serovar Choleraesuis (four out of four strains) (Table 1). Overall, the majority of *S. enterica* subgroup I isolates were able to form the rdar morphotype.

**Real-time pattern formation during formation of the rdar morphotype.** During analysis of SARB colony morphologies, we observed unusual concentric ring morphologies in colonies from several isolates. To determine if this was an in-

termediate stage in the formation of a complete surface pattern, we examined the growth of *S. enterica* serovar Montevideo (SARB30) by time-lapse photography (Fig. 1 and supplementary movie 1 at [www.med.ucalgary.ca/webs/bprg/Surette/Surette.html](http://www.med.ucalgary.ca/webs/bprg/Surette/Surette.html)). The surface of the colony was smooth for the initial 35 h of growth (Fig. 1, top panels). Between 40 and 41 h of growth, there was a rapid transition in morphology resulting from the formation of regular, concentric rings in the center of the colony (Fig. 1, middle panels). For the remainder of the growth period, new rings were formed between the colony center and colony edge until the entire surface was covered with a symmetrical pattern (Fig. 1, bottom panels). Similar growth patterns were observed for most SARB isolates and serovar Typhimurium ATCC 14028, although there was some variability in the observed pattern morphology (data not shown). The rapidity and uniformity of initial pattern formation suggested that individual cells within the colony were organized. This demonstrated that pattern formation in the rdar morphotype was a coordinated multicellular behavior,

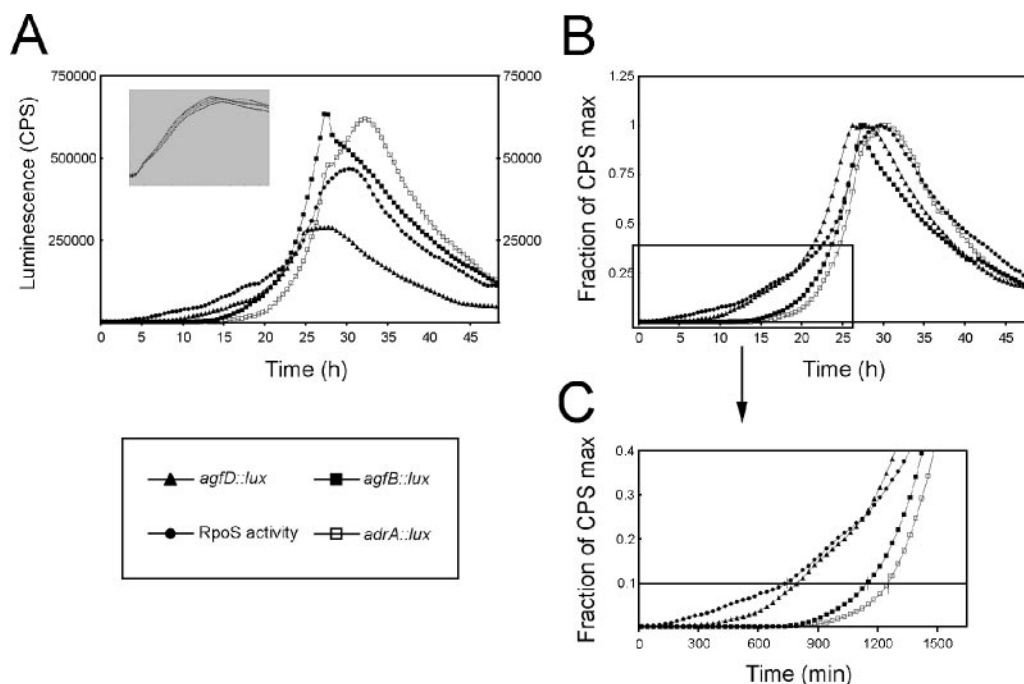


FIG. 2. Temporal expression of genes required for formation of the rdar morphotype in serovar Typhimurium. Expression of *agfD*, *agfB*, *adrA*, or RpoS ( $\sigma^{38}$ )-responsive promoter::*lux* fusions during ATCC 14028 growth in 1% tryptone at 28°C with agitation. A. Light production as a function of time. Luminescence (counts per second [cps]) measurements for *agfD* and *agfB*::*lux* fusions are on the left axis; measurements for *adrA* and RpoS-responsive *lux* fusions are on the right axis. Representative expression curves from one experiment are shown. The inset shows cell density measurements ( $A_{620}$ ) for each reporter strain as a function of time. B. Light production of *lux* reporter strains as a function of time, normalized by the maximum cps of each strain. To ensure accurate timing of expression during growth, each assay was repeated at least six times and individual growth curves were matched. The expression curves represent the average from these experiments. C. Expression curves from panel B were enlarged to highlight the temporal resolution of gene expression. The line is representative of the 10% gene activation level.

consistent with a developmental shift in the growing colony (33).

**Ordering the expression of genes required for *Salmonella* multicellular aggregation.** Expression of key gene promoters involved in *Salmonella* multicellular aggregation was monitored during growth in 96-well plates (Fig. 2). This growth state is different than *Salmonella* growth on surfaces. However, when liquid cultures are overlaid with mineral oil, a microaerophilic environment is created (C. M. Southward and M. G. Surette, unpublished data), and this was shown to be one of the main requirements for *agfD* expression in liquid culture (15). Transcription of *agfB* and *adrA*, leading to the production of Tafi and cellulose, respectively, is known to be dependent on AgfD. Expression of *agfD* is dependent on activity of the stationary-phase sigma factor, RpoS (25). To examine the timing of expression of operons involved in this pathway, *agfD*, *agfB*, and *adrA* promoters were cloned in front of a promoterless *lux* reporter (4, 45). In order to monitor RpoS activity during growth, we constructed a synthetic RpoS ( $\sigma^{38}$ )-responsive promoter::*lux* fusion.

Light production from each of the reporter strains was measured during growth of ATCC 14028 (Fig. 2A). The strength of the promoters varied over 10-fold; *agfD* and *agfB* were the strongest with maximal light production of 285,000 cps and 650,000 cps, respectively. *adrA* had weaker expression with a maximal light production of 60,000 cps. Despite magnitude differences, the peak activity of these three reporters appeared

to be synchronized (Fig. 2A). To better resolve temporal differences, expression curves were normalized by the maximum light production from each reporter (Fig. 2B). Peak expression occurred after 25 h, indicating that all promoters were transcriptionally active during the stationary phase of growth. Furthermore, activation of each gene corresponded with increased RpoS activity in the cell as measured by our synthetic promoter (Fig. 2B).

We compared normalized promoter expression profiles at the 10% activation level to determine the relative order of transcription (Fig. 2C) (20, 45). An increase in RpoS activity from basal levels was measured between 8 and 12 h of growth. This increase was followed immediately by activation of *agfD* transcription. There was a 5- to 6-h lag period before activation of *agfB* and *adrA* reporters, both of which had steep activation profiles. *agfB* was activated 90 to 120 min before *adrA*, on average (Fig. 2C). This suggests that fimbrial biosynthesis may be an initiating event in formation of the *Salmonella* extracellular matrix. The sharp expression peaks observed for *agfB* and *adrA* correspond to the sharp transition in pattern formation during colony growth (Fig. 1, 40 to 42 h).

**Extracellular complementation of colony surface patterns.** ATCC 14028 forms large, patterned colonies (Fig. 3A) that can be peeled off the agar surface intact (29). An isogenic  $\Delta$ *agfD* strain lacking both Tafi and cellulose production formed smaller colonies that remained smooth and mucoid (Fig. 3A) (28, 47). Strains lacking either Tafi production ( $\Delta$ *agfA*) (8, 25)

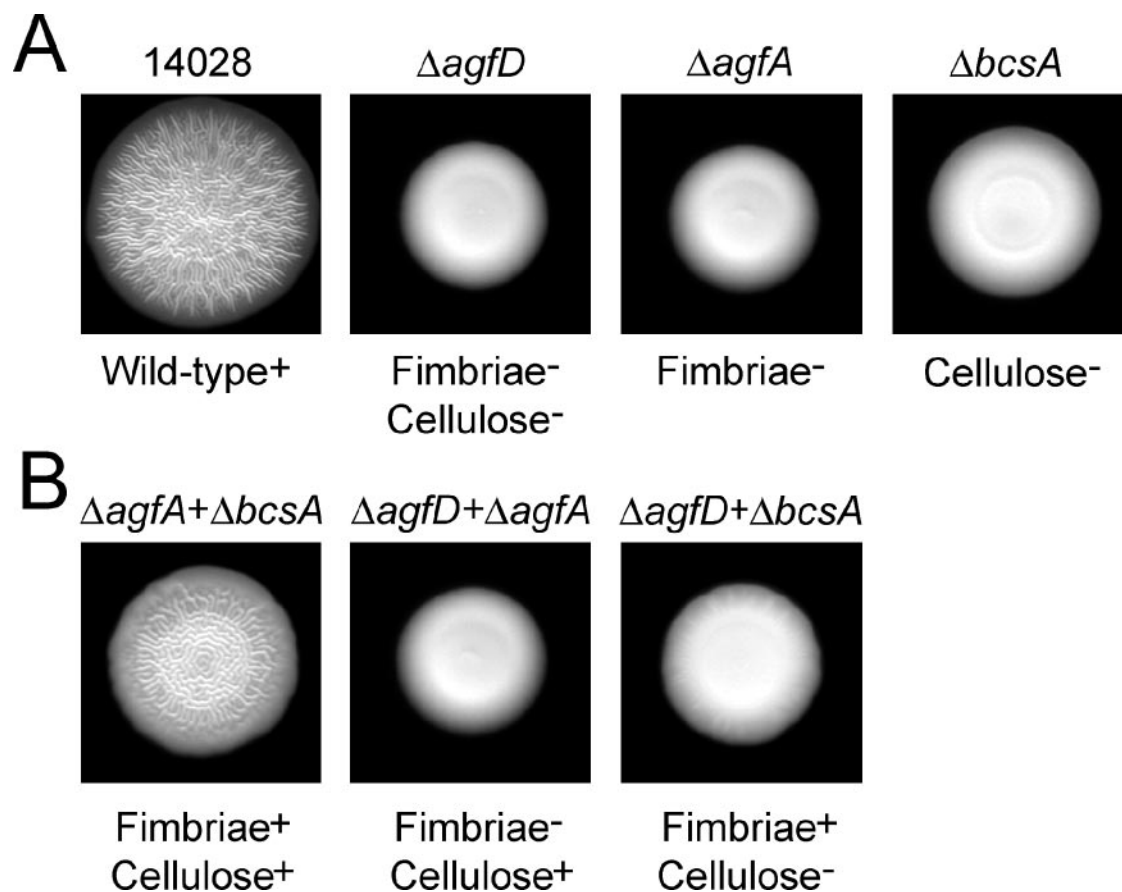


FIG. 3. Tafi and cellulose interact extracellularly to form surface patterns in *Salmonella* rdar morphotype colonies. A. Colony morphologies of ATCC 14028 and *agfD*, *agfA*, and *bcsA* deletion strains grown on 1% tryptone (T) agar at 28°C for 48 h. B. Colony morphologies of mixtures of equal numbers of  $\Delta agfD$ ,  $\Delta agfA$ , or  $\Delta bcsA$  cells after growth on T agar at 28°C for 48 h.

or cellulose production ( $\Delta bcsA$ ) (47) also formed smooth colonies (Fig. 3A), but these colonies were not entirely mucoid; Tafi provide short-range cell-cell interactions that yield an adhesive texture, and cellulose provides long-range cell-cell interactions that yield a sticky texture (28). To highlight the extracellular interactions leading to pattern formation, mutant strains lacking Tafi, cellulose, or both components ( $\Delta agfA$ ,  $\Delta bcsA$ , or  $\Delta agfD$ ) were mixed prior to spotting on T agar. When  $\Delta agfA$  and  $\Delta bcsA$  cells were combined, the Tafi and cellulose polymers produced from different cells were able to restore pattern formation on the colony surface (Fig. 3B). These colonies had an adhesive texture, indicating that a partial multicellular network had formed. In contrast, mixtures of cells lacking either Tafi or cellulose formed colonies that remained smooth and partially mucoid (Fig. 3B). Thus, extracellular interactions between individual cells are responsible for the formation of colony surface patterns in the rdar morphotype of *Salmonella*.

**Long-term survival of *Salmonella* in colonies of the rdar morphotype.** Preliminary experiments with rdar morphotype colonies of ATCC 14028 indicated that cells were viable after several weeks to months of growth at RT. Since RpoS is known to control the rdar morphotype (25) along with the general stress response (18), we monitored RpoS activity during rdar colony growth. Peak RpoS activity was observed between 3 and

7 days (Fig. 4A). After this time, luciferase production was gradually reduced but remained above detectable levels even after 47 days (Fig. 4A). This was in contrast to a synthetic RpoD ( $\sigma^{70}$ )-responsive promoter that had peak expression at day 1 and dropped below detectable limits after 7 days (data not shown). These data indicated that cells were alive and respiring after nearly 2 months. This led us to investigate the fate of cells in colonies that were peeled off from the agar surface and dried out in the plastic wells of a multiwell plate (Fig. 4B). Surprisingly, a large number of cells remained viable even after several months of storage. We hypothesized from these initial experiments that the rdar morphotype may contribute to the long-term survival of *Salmonella*.

**Survival of *Salmonella* during long-term desiccation.** To determine if Tafi, cellulose, and other matrix components were contributing to long-term survival, colonies of ATCC 14028 and isogenic *agfD*-, *agfA*-, and *bcsA*-deleted strains were compared directly after 3 and 9 months of storage on plastic. Despite the difference in colony sizes (Fig. 3A), each colony type had similar numbers of viable cells before storage on plastic (Table 2). After 3 months of storage, the ATCC 14028 reference strain, capable of producing Tafi and cellulose, had the highest rate of survival at 68% (Table 2). In contrast, cell numbers for  $\Delta agfD$ ,  $\Delta agfA$ , and  $\Delta bcsA$  colonies were significantly reduced to 6.2%, 11%, and 21% of initial numbers,

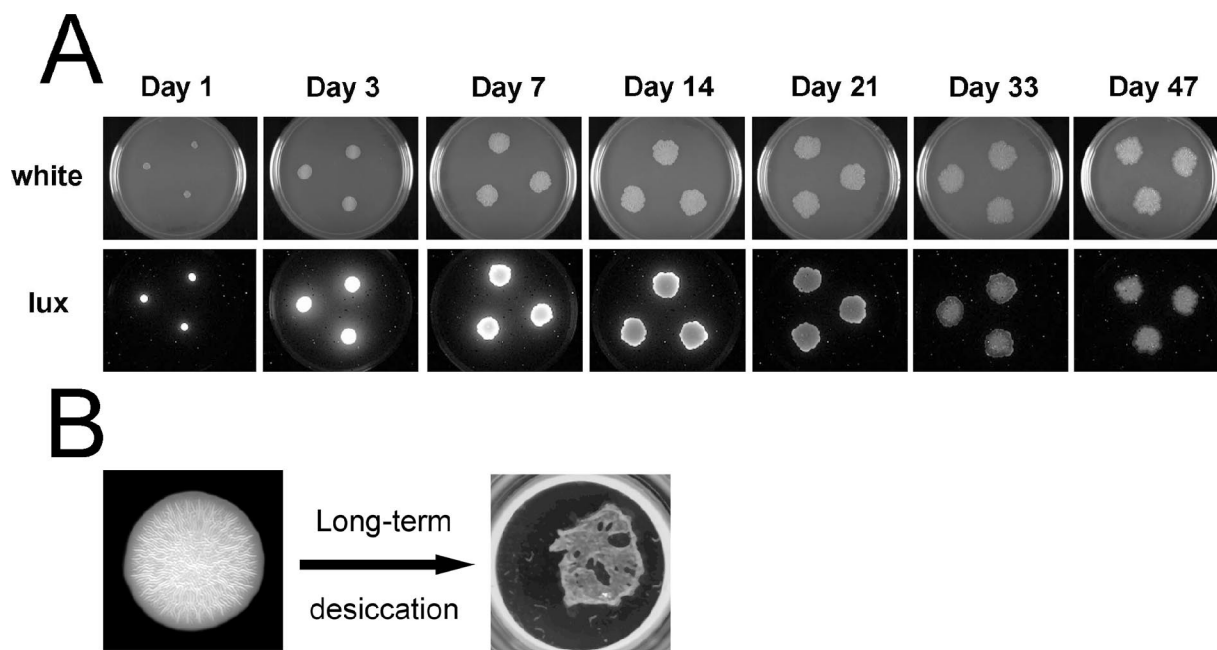


FIG. 4. Long-term survival of serovar Typhimurium in colonies of the rdar morphotype. A. RpoS ( $\sigma^{38}$ ) activity in growing cells was monitored using the sig38H4 promoter::lux fusion. Colonies were inoculated with  $10^5$  cells from an overnight culture and were grown at RT ( $\sim 22^\circ\text{C}$ ) for 50 days. Pictures were taken at the times indicated to record colony morphology (white) or luminescence production (lux). B. Individual rdar colonies peeled off the surface of T agar were stored for 3 to 9 months at RT in plastic wells of a 24-well plate in the absence of nutrients prior to testing survival and resistance to bleach treatment.

respectively (Table 1). After 9 months of storage, survival of ATCC 14028 cells was nearly 10%, whereas cell numbers for  $\Delta agfD$  and  $\Delta agfA$  colonies were further reduced to 0.27% and 1.2%, respectively (Table 1). Survival of  $\Delta bcsA$  cells was also reduced compared to ATCC 14028, but the difference was not statistically significant (Table 1). These data demonstrated that

TABLE 2. Survival of *Salmonella* after months of desiccation in the absence of exogenous nutrients

Strain (phenotype)	CFU/colony <sup>a</sup>	% Survival <sup>b</sup>
ATCC 14028 (wild type <sup>+</sup> )		
Initial colony	$2.3 \times 10^9 \pm 0.14 \times 10^9$	
3 mo desiccation	$1.6 \times 10^9 \pm 0.15 \times 10^9$	68
9 mo desiccation	$2.2 \times 10^8 \pm 0.19 \times 10^8$	9.7
$\Delta agfD$ (fimbriae <sup>-</sup> , cellulose <sup>-</sup> )		
Initial colony	$3.3 \times 10^9 \pm 0.14 \times 10^9$	
3 mo desiccation	$2.0 \times 10^8 \pm 0.28 \times 10^8$	6.2
9 mo desiccation	$8.7 \times 10^6 \pm 1.4 \times 10^6$	0.27
$\Delta agfA$ (fimbriae <sup>-</sup> )		
Initial colony	$3.2 \times 10^9 \pm 0.64 \times 10^8$	
3 mo desiccation	$3.4 \times 10^8 \pm 0.12 \times 10^8$	11
9 mo desiccation	$3.8 \times 10^7 \pm 0.34 \times 10^7$	1.2
$\Delta bcsA$ (cellulose <sup>-</sup> )		
Initial colony	$2.3 \times 10^9 \pm 0.18 \times 10^9$	
3 mo desiccation	$4.8 \times 10^8 \pm 0.37 \times 10^8$	21
9 mo desiccation	$1.6 \times 10^8 \pm 0.17 \times 10^7$	6.8

<sup>a</sup> Average number of viable cells (CFU) per dried colony plus or minus the standard error. At least four individual colonies were tested.

<sup>b</sup> Percent survival of cells in desiccated colonies determined relative to values from initial colonies.

production of Tafi and cellulose enhanced long-term survival of *Salmonella* under these conditions. The  $\Delta bcsA$  and  $\Delta agfA$  mutants had different survival characteristics, demonstrating that these two extracellular polymers collectively enhance survival, with cellulose being slightly less important.

**Resistance of cells in rdar morphotype colonies to treatment with sodium hypochlorite.** Survival in a desiccated environment without exogenous nutrients would aid in the persistence of *Salmonella* in non-host environments. However, this is just one aspect of survival. To examine how cells respond to additional stresses, ATCC 14028 rdar colonies that had been stored for 3 months were rehydrated and exposed to sodium hypochlorite for 20 min (Fig. 5A). No reductions in CFU were measured using a concentration of 30 ppm (Fig. 5A). At 300 ppm, a concentration in excess of normal sanitization levels (30), only a 3-log reduction in survival was observed (0.1% survival). At 1,000 ppm or 0.1% sodium hypochlorite, all cells were killed (data not shown).

To determine if Tafi, cellulose, and other extracellular matrix components conferred protection to sodium hypochlorite, ATCC 14028,  $\Delta agfD$ ,  $\Delta agfA$ , and  $\Delta bcsA$  cells were compared after growth in conditions when Tafi and cellulose are not produced (i.e., stationary-phase planktonic cells at  $37^\circ\text{C}$ ) versus rdar colonies that had been stored for 3 months (Fig. 5B). Planktonic cells from all strains were susceptible to 60 ppm sodium hypochlorite and showed greater than 6-log reductions in CFU after treatment (Fig. 5B, gray bars). In contrast, cells in dried colonies of the  $\Delta agfA$  strain were resistant with less than 1-log reduction in CFU after treatment. This level of resistance was similar to that of rdar colonies from ATCC 14028 (Fig. 5A). Cells in dried colonies from the cellulose-

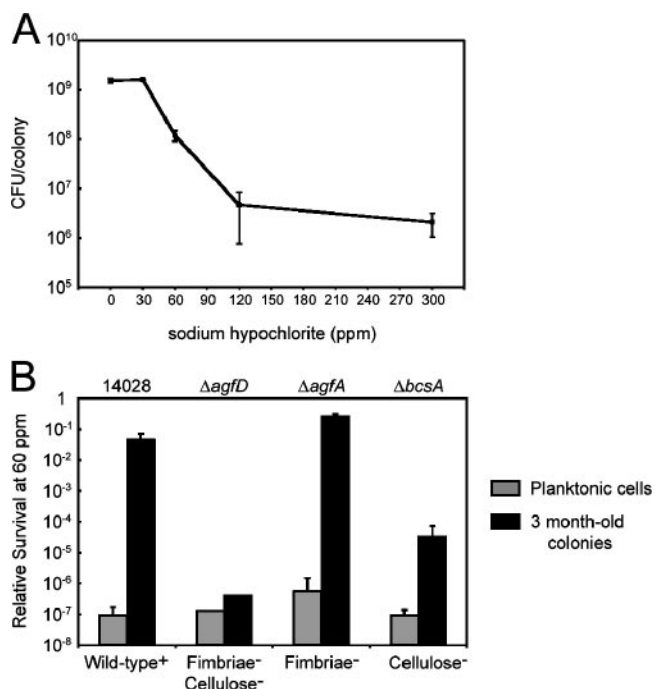


FIG. 5. Survival of serovar Typhimurium cells in colonies of the rdar morphotype following a 20-min exposure to sodium hypochlorite. A. ATCC 14028 rdar colonies stored for 3 months on plastic were rehydrated and treated with different concentrations of sodium hypochlorite. Each point represents the average number of surviving cells (CFU) per colony and standard errors corresponding to at least two individual colonies. B. Survival of cells from 3-month-old colonies of ATCC 14028,  $\Delta agfD$ ,  $\Delta agfA$ , and  $\Delta bcsA$  was compared to that of planktonic cells grown at 37°C for 20 h (stationary phase) after treatment with 60 ppm sodium hypochlorite. Bars represent the average relative survival (log CFU after treatment/log CFU before treatment) and standard errors from at least four individual colonies or aliquots of planktonic cells. CFUs for colonies before treatment are displayed in Table 1; for planktonic cells, the initial values ranged from  $4.2 \times 10^8$  to  $5.5 \times 10^8$  CFU.

deficient  $\Delta bcsA$  strain were susceptible and showed greater than a 4-log reduction in CFU ( $<0.01\%$  survival; Fig. 5B). Mucoid colonies of the  $\Delta agfD$  strain were most susceptible; survival was reduced to levels similar to that of planktonic-grown cells (Fig. 5B). These results demonstrated that cellulose and additional components regulated by *AgfD* confer protection to sodium hypochlorite.

When equal numbers of  $\Delta bcsA$  and  $\Delta agfA$  cells were combined prior to growth,  $\Delta bcsA$  cells in the hybrid rdar colonies (Fig. 3B) showed increased resistance to sodium hypochlorite (data not shown). The degree of protection correlated with the number of cellulose-positive ( $\Delta agfA$ ) cells present, indicating that survival was dependent on the overall amount of cellulose present in the colony. This reinforced the correlation between the extracellular interactions leading to the rdar morphotype and the inherent resistance of this multicellular state.

## DISCUSSION

*Salmonella* has evolved specialized mechanisms for exploiting many host environments, yet these pathogens also have a remarkable ability to persist in extreme environments outside

the host (42). Here we have demonstrated that coordinated multicellular behavior leading to the rdar morphotype can be linked to the long-term survival of *Salmonella*. The uniformity of pattern formation in colonies of the rdar morphotype indicates a high level of cell-cell organization. We hypothesize that the rdar morphotype is an example of a microbial developmental process (33). Presumably, colony surface patterns arise because *Salmonella* senses its environment and triggers production of extracellular matrix components, such as Tafi, cellulose, and additional exopolysaccharides (41). Under laboratory conditions, the environmental triggers for this phenotype appear to mimic life outside a host (i.e., limiting nutrients, low temperatures, and low osmolarity).

The rdar morphotype was formed by 58 of 72 (80.5%) natural isolates in the SARB collection. Previous studies to determine the prevalence of the rdar morphotype within *S. enterica* subgroup I have primarily focused on human-associated serovars Enteritidis and Typhimurium (26, 35). More recent work has focused on a variety of serovars isolated from clinical and food sources (36). In each of these studies, rdar morphotype formation was detected in greater than 70% of isolates. Given that the SARB represents a comprehensive strain collection, we conclude that the ability to form the rdar morphotype is prevalent throughout *S. enterica* subgroup I. Serovars Choleraesuis and Typhi appear to be exceptions, since all 36 isolates tested by Romling et al. (26) did not express the rdar morphotype. However, both serovar Typhi isolates tested in this study were capable of forming rdar colonies. The reason for this discrepancy is unclear but is presumed to represent diversity within serovar Typhi.

Our experiments demonstrate an important role for the rdar morphotype in the enhanced long-term survival and persistence of *Salmonella*. The enhanced survival under conditions of desiccation would be advantageous in many natural environments and may also contribute to the contamination of dried food products (19). The finding that almost 10% of ATCC 14028 cells were alive after 9 months of storage illustrates the remarkable survival properties of *Salmonella*. The increased resistance of 3-month-old rdar colonies to sodium hypochlorite has obvious implications for the sanitization of contaminated surfaces (i.e., in the agricultural and food-processing industries). Our observations were comparable to those of a recent study performed on cells forming pellicles at the air-liquid interface in standing liquid culture (32). This was not surprising, since the same ATCC 14028 strain was analyzed and Tafi and cellulose are required for pellicle formation (14). These and other studies (2, 21, 31) stress the differences between studying planktonic cells grown in typical laboratory conditions and cells growing in more resistant physiological states.

Individual extracellular matrix components had different contributions to long-term survival and persistence. The Tafi-deficient strain ( $\Delta agfA$ ) had a surprising reduction in survival of desiccation compared to the cellulose-deficient strain ( $\Delta bcsA$ ). Our gene expression analyses indicated that Tafi production may be an initiating event during the formation of the rdar morphotype. The temporal difference between *agfB* (Tafi production) and *adrA* (cellulose production) activation was approximately 90 to 120 min. Overexpression of *adrA* from a multicopy plasmid resulted in a more rapid appearance of the

rdar phenotype, although the colony morphology was visibly altered (data not shown). Therefore, the significance of the temporal difference between *agfB* and *adrA* is unknown. Tafi production is required for the increased surface spreading observed in rdar colonies;  $\Delta bcsA$  (Cellulose<sup>-</sup>, Tafi<sup>+</sup>) colonies are consistently larger than  $\Delta agfA$  (Cellulose<sup>+</sup>, Tafi<sup>-</sup>) colonies (Fig. 3A). From these different observations, we hypothesize that Tafi plays a critical role in organizing the extracellular matrix, perhaps leading to an optimal spatial arrangement of cells (47) and increased survival. Cellulose, on the other hand, was not as important for survival but conferred protection against sodium hypochlorite. Cellulose-positive strains (wild type and  $\Delta agfA$ ) had greater than 4 orders of magnitude increased survival compared to that of cellulose-deficient strains ( $\Delta bcsA$  and  $\Delta agfD$ ). This confirmed previous sodium hypochlorite tests with broth-grown cells of *Salmonella* (35). The protective nature of cellulose could apply to other reactive compounds, such as hydrogen peroxide (2). Cellulose polymers may directly remove or neutralize reactive species, as suggested by Scher et al. (32). The presence of cellulose may also trap additional *Salmonella* polysaccharides on the cell surface (41) to enhance protection.

Long-term survival during desiccation and resistance to sodium hypochlorite was most reduced in the *agfD* mutant strain lacking Tafi and cellulose production (47). The absence of these two polymers did not entirely account for the reduction measured, however. Survival of  $\Delta agfD$  was four times less than that of the Tafi-deficient ( $\Delta agfA$ ) strain after 9 months of storage and 2 logs less than that of the cellulose-deficient strain ( $\Delta bcsA$ ) after treatment with sodium hypochlorite. AgfD is a global transcriptional regulator known to regulate the expression of several genes related to biofilm formation (6) as well as newly identified components of the extracellular matrix, such as capsular polysaccharides (D. L. Gibson, A. P. White, S. D. Snyder, C. Heiss, P. Azadi, M. G. Surette, and W. W. Kay, unpublished) and BapA, a large cell surface protein containing repeated sequence motifs (23). It is hypothesized that  $\Delta agfD$  cells have increased susceptibility to desiccation stress and sodium hypochlorite due to lack of these additional extracellular components.

The relationship between the *Salmonella* rdar morphotype and survival described here may be just one example of a generalized survival strategy used by many other microorganisms, both pathogens and nonpathogens. The genes for Tafi (*agf* or *csg*) and cellulose (*bcs* or *yhj*) are conserved throughout the salmonellae (<http://globin.cse.psu.edu/enterix/enteric/enteric.html>) (12), and these polymers are expressed by the vast majority of isolates. Curli and cellulose can also be produced by environmental *E. coli* isolates (10) and organisms commonly isolated from the human digestive tract (46). In addition, cellulose is produced by numerous soilborne organisms and plant pathogens, such as *Agrobacterium tumefaciens* (1), *Pseudomonas fluorescens* (37), and *Erwinia chrysanthemi* (43), and contributes to the fitness of these organisms in their natural environments. *P. aeruginosa* (13), *Candida* spp. (22), and *Vibrio cholerae* (44) all display coordinated multicellular behavior when grown on solid media. Unlike *Salmonella* spp., none of these microorganisms are known to produce Tafi or cellulose. However, in each case, protein and sugar polymers are involved in the formation of an extracellular matrix that encases individual cells and leads to

aggregation. This is likely the principle function of Tafi and cellulose in *Salmonella*, to enable individual cells to stick together on surfaces and withstand stresses in the environment. This would allow for the smooth transition of *Salmonella* between hosts.

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