Salmonella Extracellular Matrix Components Influence Biofilm Formation and Gallbladder Colonization

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Salmonella enterica serovar Typhi, the causative agent of typhoid fever in humans, forms biofilms encapsulated by an extracellular matrix (ECM). Biofilms facilitate colonization and persistent infection in gallbladders of humans and mouse models of chronic carriage. Individual roles of matrix components have not been completely elucidated in vitro or in vivo. To examine individual functions, strains of Salmonella enterica serovar Typhimurium, the murine model of S. Typhi, in which various ECM genes were deleted or added, were created to examine biofilm formation, colonization, and persistence in the gallbladder. Studies show that curli contributes most significantly to biofilm formation. Expression of Vi antigen decreased biofilm formation in vitro and virulence and bacterial survival in vivo without altering the examined gallbladder pro- or anti-inflammatory cytokines. Oppositely, loss of all ECM components (ΔwcaM ΔcsgA ΔyihO ΔbcsE) increased virulence and bacterial survival in vivo and reduced gallbladder interleukin-10 (IL-10) levels. Colanic acid and curli mutants had the largest defects in biofilm-forming ability and contributed most significantly to the virulence increase of the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant strain. While the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant was not altered in resistance to complement or growth in macrophages, it attached and invaded macrophages better than the wild-type (WT) strain. These data suggest that ECM components have various levels of importance in biofilm formation and gallbladder colonization and that the ECM diminishes disseminated disease in our model, perhaps by reducing cell attachment/invasion and dampening inflammation by maintaining/inducing IL-10 production. Understanding how ECM components aid acute disease and persistence could lead to improvements in therapeutic treatment of typhoid fever patients.

Salmonella enterica serovar Typhi, a human-specific pathogen and the etiologic agent of typhoid or enteric fever, is a globally rampant disease agent reported to cause over 20 million infections and 200,000 deaths annually (1, 2). Infection by S. Typhi is most commonly caused by consumption of contaminated food or water. Once ingested, S. Typhi crosses the intestinal epithelial barrier, where it is phagocytosed by macrophages. This allows the bacteria to spread systemically to common sites of infection: bone marrow, spleen, liver, and pancreas. From the liver, the bacterium is able to transit into the gallbladder, where it can either induce inflammation (cholecystitis) and an acute infection or persist chronically, creating a carrier state (3, 4). Chronic carriers are a threat to public health as they are able to live asymptomatically while shedding bacteria in their feces, thereby maintaining the pathogen within the population.

Bacterial biofilms are of high medical significance, as they are responsible for 60 to 80% of human infections (5). Bacterial cells within a biofilm are encased in a mucoid substance known as an extracellular matrix (ECM), comprised of polysaccharides, proteins, and nucleic acids. The primary function of the matrix in human infections is protection of the bacterial community against the hazards of external (e.g., antibiotics) and internal (e.g., innate immune system) factors (6). While these general functions are associated with most microbial biofilms, the individual ECM components often possess unique properties for the bacterial community and with regard to the host (7).

An important factor in the development of chronic gallbladder carriage is cholesterol gallstones, the presence of which correlates with 80 to 90% of chronic carriers (8). Without gallbladder removal as a means of bacterial eradication, these patients become a critical reservoir for continued spread of disease (9, 10). We have demonstrated that biofilm formation is observed on the surface of gallstones, both in humans and in a mouse model of carriage (11, 12). In this model, 129×1/SvJ mice are fed a lithogenic diet to induce gallstone development, mimicking the condition of the majority of human carriers. This facilitates colonization of the gallbladder and increases shedding. Additionally, biofilm formation on and invasion of the gallbladder epithelium aid in the establishment and maintenance of carriage (11). Salmonella biofilm ECM components have been determined and include proteins (curli fimbriae and BapA), polysaccharides (extracellular DNA, cellulose, O-antigen capsule, colanic acid [in nontyphoidal serovars], and Vi antigen [ViAg] [in serovars Typhi, Dublin, and Paratyphi C]) (7, 13–17). Individual mutations in these ECM factors have been tested by various groups for their roles in biofilm formation, and some have also been examined for phenotypes in vertebrate models of infection (18–24). However, combinations of mutations and direct comparisons in vitro or in vivo have not been made.

In the present study, we further examine the role of selected ECM components (both individual and combined mutants) in...
biofilm formation in vitro and gallbladder colonization in our gallstone carriage mouse model. Our results indicate that, under the conditions studied, the ECM components play various individual roles both in vitro and in vivo that impact host colonization and virulence.

**MATERIALS AND METHODS**

**Ethics statement.** Mice were housed and cared for in accordance with determined guidelines established by the Ohio State University (OSU) Institutional Animal Care and Use Committee (IACUC). All work was approved by OSU IACUC. The Ohio State University Animal Care and Use Program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Research activities conform to the statutes of the Animal Welfare Act and guidelines of the Public Health Service as required in the Guide for the Care and Use of Laboratory Animals (25).

**Bacterial strains and growth conditions.** Wild-type (WT) *Salmonella enterica* serovar Typhimurium ATCC 14028 (JSG210) and its derivatives were used in these studies (Table 1). Cultures were grown in either Luria-Bertani (LB) broth or tryptic soy broth (TSB). When grown in the presence of bile, sodium cholate from ox bile (MP Biomedicals, LLC, Solon, OH) was included at 0.1% in 1:20 TSB. Antibiotics, when needed, were used at the following concentrations: chloramphenicol, 25 g/ml; ampicillin, 100 or 200 µg/ml; kanamycin, 45 µg/ml; streptomycin, 100 µg/ml.

**Generation of mutants and recombination procedures.** ECM mutants were created by the λ-Red mutagenesis method (26) with specific primers designed for the gene of interest (Table 2). Marked gene deletions were transduced into the WT or a mutant strain with bacteriophage P22HTint to create double, triple, and quadruple mutants starting with ΔwaM, followed by ΔcsgA, ΔyihO, and finally ΔbcsE. Mutants were verified throughout the process via PCR and electrophoretic gel analysis, while the final mutant was confirmed with sequencing through the OSU Plant-Microbe Genomics Facility. Plasmid pTH122 containing the entire Vi antigen (viaB locus) (21) was electroporated into S. Typhimurium and was verified Vi antigen positive by serum agglutination.

**Biofilm growth and crystal violet assays.** Biofilm attachment was tested on polypropylene microtiter wells coated by evaporation with 5 µg of cholesterol (diluted in 1:1 ethanol-isopropanol [Sigma-Aldrich, St. Louis, MO]). Microtiter wells were inoculated with a 1:10 dilution of 2 × 10² bacteria in 1:20 TSB with bile and incubated for 24 h at 30°C. Attached biofilms were then washed twice in double-distilled water (ddH₂O), heat fixed for 1 h at 60°C, and stained with 0.33% crystal violet for 5 min. After two subsequent washes in ddH₂O, the dye was released using 33% acetic acid, and the optical density at 570 nm (OD₅₇₀) was measured in a SpectraMax spectrophotometer with SoftMax Pro software (Molecular Devices, Sunnyvale, CA) to determine the amount of dye retained, which correlates to the amount of biofilm present. All biofilm experiments were performed in triplicate.

**Mouse infections.** Because *S. Typhi* is a human-restricted pathogen, in vitro studies of *S. Typhi* pathogenesis are performed using a mouse model with infection by *S. Typhimurium*. Naturally resistant NRAMP1−/− (SLC11A1−/−) 129/SvJ mice (n = 128) (Jackson Laboratory, ME) were fed a lithogenic diet (1% cholesterol and 0.5% cholic acid [Envigo/ Harlan Laboratory, IN]) to induce gallstone formation. After 8 weeks on the diet, mice were infected intraperitoneally with 10⁴, 10⁵, or 10⁶ S. Typhimurium bacteria and sacrificed at 7 or 8 days postinfection (p.i.). The gallbladder, bile, and gallstones were collected, homogenized, and/or diluted to enumerate the bacteria using LB plates with or without antibiotics. For bacterial CFU enumeration of *Salmonella* in competition experiments, mice were given intraperitoneal injections containing 10⁶ CFU of an equal mixture of WT cells marked with streptomycin resistance and cells of a mutant strain marked with either kanamycin or chloramphenicol resistance and sacrificed at 7 or 8 days postinfection. Gallbladder, bile, and gallstones were collected, homogenized, and/or diluted to enumerate the bacteria using LB plates with appropriate antibiotics. The competitive index was calculated by determining the output (mutant divided by the WT CFU) and dividing that value by the input (initial dose in CFU).

**IL-10 and TNF-α binding ELISA.** Tissue lysates from the euthanized mice were prepared as described earlier (OptEIA set protocol [BD Biosciences, San Diego, CA]). Briefly, Maxisorp 96-well plates were coated with purified anti-IL-10 and anti-TNF-α capture antibody (100 µl/well) in 0.2 M sodium phosphate buffer, pH 7.6. Lysates were added to the plates, and the amount of IL-10 or TNF-α bound to the antigen was determined using ELISA. The plates were then incubated with 100 µl of a 1:100 dilution of anti-IL-10 or anti-TNF-α antibody conjugated to horseradish peroxidase (HRP) (BD OptEIA set protocol [BD Biosciences, San Diego, CA]). Finally, plates were washed, and color was developed with 3,3',5,5'-tetramethylbenzidine (TMB) (Molecular Devices). The absorbance at 450 nm was measured in a SpectraMax spectrophotometer with SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

**Table 1: Strains used in this study**

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<tr>
<th>Strain</th>
<th>Genotype or relevant phenotype</th>
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<td>JSG210</td>
<td>Wild-type S. Typhimurium ATCC 14028</td>
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<tr>
<td>JSG3934</td>
<td>JSG210 StrEp</td>
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*a* Cam, chloramphenicol; Kan, kanamycin; StrEp, streptomycin resistant.

**Table 2: Oligonucleotide primers used in this study**

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<td>5′-GTTCGCTCAATAGGCGCACCA-3′</td>
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<td>5′-CCATATTACGGCATGGACGCCG-3′</td>
<td>Forward csgA</td>
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<td>5′-GCTGGTTTTGCCAGCATGTAATTCTCG-3′</td>
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<td>λ-Red deletion of reverse bcsE</td>
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errors of the means.

one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison test correction was used to determine significant differences between mutant strains and the WT. *, *P<0.05; **, *P<0.01. The error bars indicate standard errors of the means.

(pH 6.5) and incubated overnight at 4°C. The plates were blocked for 1 h at room temperature (RT) with 200 μl of blocking buffer (phosphate-buffered saline [PBS] with 10% fetal bovine serum [FBS]) and then washed four times with PBS plus 0.05% Tween 20 (PBST). Samples and respective recombinant standards for IL-10 (1,000 pg/ml to 31.25 pg/ml) and TNF-α (1,000 pg/ml to 15.62 pg/ml) were added (100 μl/well) in duplicate, and the plates were incubated for 2 h at RT. Plates were washed with PBST, 100 μl of biotinylated detection antibody with streptavidin-horseradish peroxidase (working detector) was added to each well, and the mixture was incubated for 1 h at RT. Plates were washed and further developed using substrate solution (tetramethyl benzidine [TMB] and hydrogen peroxide) followed by adding 50 μl of 1 M phosphoric acid (H3PO4) to stop the reaction. The optical density of the reaction at 450 nm (OD450) was measured with a microplate reader SpectraMax spectrophotometer with SoftMax Pro software (Molecular Devices) within 30 min with a λ correction at 570 nm.

Intramacrophage survival assays. J774.1 cells were seeded at 2 × 105 cells/well in a 24-well plate format for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were then infected with either the WT or mutant (multiplicity of infection [MOI] of 20). Infection was synchronized by centrifugation at 233 × g for 5 min at RT. The plates were incubated for 1 h at 37°C with 5% CO2. The extracellular bacteria were removed by adding gentamicin (50 μg/ml) for 45 min followed by three subsequent washes with warm medium. The cells were lysed using 0.1% Triton X-100 (Sigma-Aldrich). The intracellular bacteria at different time points were enumerated by serial plating. To obtain information on attachment and invasion, wells were also deprived of gentamicin, washed three times, and enumerated alongside bacteria immediately after exposure to gentamicin for 45 min as described above. Attached bacteria were calculated by subtracting the total bacteria recovered in the absence of gentamicin from those with gentamicin.

Serum sensitivity. Serum sensitivity was assessed by following the protocol described by Wilson et al. (19). Bacterial cells (1 × 107) were incubated in 10% human serum complement (Quidel, San Diego, CA) diluted in 1× PBS. At the indicated time points, survival was quantified by enumeration of serial dilutions on LB agar plates.

RESULTS
Salmonella ECM components impact biofilm formation. In order to assess the involvement of the ECM components in biofilm formation, we performed a biofilm assay on cholesterol-coated polystyrene plates in the presence of 0.1% bile for 24 h. These conditions mimic the environment within the gallbladder, where biofilm formation by Salmonella is biologically significant. The strains examined include WT S. Typhimurium, a WT strain expressing S. Typhi Vi antigen from a plasmid (WT+ViAg), mutants with single-gene deletions of colanic acid (∆wcaM), curli (∆csgA), O-antigen capsule (∆yihO), and cellulose (∆bcsE), as well as double (∆wcaM ∆csgA), triple (∆wcaM ∆csgA ∆yihO), and quadruple (∆wcaM ∆csgA ∆yihO ∆bcsE) ECM mutants (Fig. 1). Interestingly, we observed a reduction in biofilm formation for the WT+ViAg strain, although this did not reach statistical significance. All of the individual mutants showed a decrease in biofilm formation, with the ∆csgA strain demonstrating a significant reduction of 45%. The double, triple, and quadruple mutants containing the ∆csgA mutation were at a level similar to that of the ∆csgA single mutant alone, suggesting a major contribution of curli fimbriae and a minor contribution of the other ECM components to biofilms in this in vitro model.

ViAg expression in S. Typhimurium results in decreased virulence and gallbladder colonization. Since the addition of ViAg to WT S. Typhimurium resulted in decreased biofilm formation and because ViAg has been demonstrated to mediate anti-inflammatory effects (18, 19, 21), we tested whether it had an effect on virulence using a mouse model of infection. 129X1/SvJ mice fed a laboratory diet (to induce gallstone development) for 8 weeks were infected with the WT and WT+ViAg strains at a dose of 1 × 108, 2 logs higher than typical in the gallstone mouse model. Mice given the WT+ViAg strain showed 100% survival at day 7 postinfection, while the WT strain-infected mice demonstrated only 45% survival (Fig. 2). At the typical dose of 1 × 106, mice given the WT+ViAg or WT strain had 100% survival. Commensurate with decreased virulence, those infected with WT+ViAg had signifi-
that without these extracellular components, bacteria are hyper-postinfection compared to mice infected with the WT, suggesting /H9004 bcsE WT strain significantly outcompeted WT /H11001 ViAg strains. As hy-/H9004 curli, O-antigen capsule, and cellulose (wcaM strain missing four major ECM biofilm contributors: colanic acid, 1/SvJ mice were coinfected with /H11003 tition assay was performed. 129 /H11003 this /H9004 ence in levels of proinflammatory (TNF- /H11001 were examined for cytokine levels, there was no significant differ-/H9004 ence in cytokine expression levels in the gallbladder (Fig. 4). However, competitive infection experiments were performed between the ΔwcaM ΔcsgA ΔyihO ΔbcsE and WT strains (Fig. 5D). Consistent with the single-mutant strain virulence and CFU results, the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant outcompeted the WT strain in the gallbladder.

Loss of ECM components results in reduced levels of IL-10. To determine whether cytokine expression levels in the gallbladder differ in response to infection with the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant and WT, IL-10 and TNF-α levels were measured at days 7 to 8 postinfection. The levels of the proinflammatatory cytokine TNF-α were significantly lower in bile but did not statistically differ between the strains in gallbladder tissue. However, the anti-inflammatory cytokine IL-10 was both significantly lower in the bile and 2-fold reduced in gallbladder tissue from those mice infected with the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant (Fig. 6).

Loss of ECM components results in enhanced macrophage attachment/invasion but not intracellular survival or complement resistance. To determine if the ECM components contribute to cell attachment, invasion, intracellular survival, or resistance to innate immune factors, in vitro assays were performed with the ΔwcaM ΔcsgA ΔyihO ΔbcsE strain versus the WT. Assays with J774.1 macrophages demonstrated the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant to have a 47% enhancement in attachment and a 40% increase in invasion versus the WT strain. However, once in the macrophage, there was no difference in growth of the bacteria over a 24-h period (Fig. 7A).

To examine the potential role of the ECM components in innate immune resistance, complement resistance assays were performed. In 10% human serum, the WT and ΔwcaM ΔcsgA ΔyihO ΔbcsE strains demonstrated no significant difference in survival (Fig. 7B). The control of Escherichia coli DH5α was efficiently killed by the serum, demonstrating its potency.

Competition experiments reveal that colanic acid and curli fimbriae contribute most significantly to the ΔwcaM ΔcsgA ΔyihO ΔbcsE virulence phenotype. To investigate the individual extracellular components and their roles in the observed ability of increasing trend of persistence upon the deletion of each additional gene, culminating in a 2- to 3-log increase in the quadruple mutant versus the WT (Fig. 5C). Growth curves in both rich and minimal media verified that these increases in virulence and bacterial load were likely not due to enhanced growth rates but rather represent a true increase in persistence or immune evasion (see Fig. S2 in the supplemental material). Furthermore, competitive infection experiments were performed between the ΔwcaM ΔcsgA ΔyihO ΔbcsE and WT strains (Fig. 5D). Consistent with the single-mutant strain virulence and CFU results, the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant outcompeted the WT strain in the gallbladder.

Loss of ECM components enhances virulence. In order to observe the importance of the ECM components on virulence in the gallstone mouse model, 129 x 1/SvJ mice were infected with a /H9004 mutant to have a 47% enhancement in attachment and a 40% increase in invasion versus the WT strain. However, once in the macrophage, there was no difference in growth of the bacteria over a 24-h period (Fig. 7A).

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the ΔwcaM ΔcsgA ΔyihO ΔbcsE strain to outcompete the WT, 129×1/Svj mice were infected with an equal mixture of WT and differentially marked individual mutant strains (ΔwcaM, colanic acid; ΔcsgA, curli fimbriae; ΔyihO, O-antigen capsule; or ΔbcsE, cellulose), and the recovered bacterial enumerations were compared (Fig. 8). Bacterial loads recovered from the ΔwcaM and ΔcsgA mutants versus WT competitions showed a slight but significant outcompetition favoring the mutant in the gallbladder for both the ΔwcaM and ΔcsgA mutants and in bile and on gallstones for the ΔcsgA mutant, which is consistent with the ΔwcaM ΔcsgA ΔyihO ΔbcsE strain competition results (Fig. 5D). Contrary to those assays, the ΔyihO and ΔbcsE mutants were unable to outcompete the WT strain, with the ΔbcsE mutant equally competitive to and the ΔyihO mutant less competitive than the WT. Thus, the absence of either curli fimbriae or colanic acid resulted in the gain of a competitive advantage similar to that of the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant.

**DISCUSSION**

Microbial biofilm formation is an important component of persistent/chronic infections. S. Typhi biofilm formation on gallstone surfaces aids the development and establishment of gallbladder chronic carriage. The key component of mature biofilms is the ECM that encases and protects the bacteria from microenvironmental insults. The specific components of the ECM in *Salmonella* spp. include curli fimbriae, cellulose, O-antigen capsule, colanic acid, and Vi antigen (ViAg). Many of these components are intimately related in both regulation and expression, and some are only present in certain serovars. How these components are intimately related in both regulation and expression, and some are only present in certain serovars. How they interact and their roles are still not fully understood. In an effort to elucidate their roles, we hypothesized that one or more of these components influence biofilm formation, gallbladder colonization, and subsequent disease persistence.

In this study, we first examined the role of each ECM component in biofilm formation *in vitro*. Independent disruption of curli fimbriae (ΔcsgA) alone resulted in significantly impaired biofilm formation. Deletion of cellulose, O-antigen capsule, and colanic acid also each decreased biofilm development, but not to the same extent as deletion of curli. As filamentous appendages, curli fimbriae have highly adhesive properties and are an important component in the ECM of many Enterobacte-
riaceae. These fibers, along with cellulose expression, have been previously shown to be critical in initial adhesion of the bacterial cells to surfaces, cell aggregation, pellicle formation, and biofilm formation (27–31). Combinations of mutations resulted in only modest further decreases in biofilm formation, except when combined with ΔcsgA, which reduced biofilms to the level of the ΔcsgA mutant alone. These data suggest that while all components contribute to biofilm formation, curli fimbriae are clearly the most important under our in vitro conditions simulating the gallbladder environment. In fact, curli is a key element in community structure as it forms a highly hydrophobic matrix containing cells tightly aligned in parallel formation, an important method of construction for the tolerance of biofilms to environmental insults (30).

ViAg is expressed in S. Typhi but not S. Typhimurium. It has been demonstrated that ViAg dampens several aspects of host immunity, allowing S. Typhi to disseminate systemically (32–34). Heterologous expression of ViAg in S. Typhimurium enables this strain to also suppress immunity in both a bovine ligated ileal loop and the streptomycin-pretreated mouse model (35). We demonstrated that expression of ViAg in S. Typhimurium reduced biofilms in vitro and decreased virulence and gallbladder colonization in the gallstone mouse model. While a growth defect (tested in rich and minimal medium) was likely not responsible for the observed phenotype, the biofilm defect may be due to altered ECM interactions (with each other or the surface) caused by the addition of a new polysaccharide on the bacterium. The virulence phenotype may be related to the defect in biofilm formation allowing immune or mechanical clearance of bacteria from the gallbladder to occur. No differences in tested pro- or anti-inflammatory cytokines was noted, thus making altered immune responses in the gallbladder less likely as an explanation of the observed colonization and virulence defect of the S. Typhimurium ViAg-positive strain. Additional immunological and gene expression experiments are under way and will shed more light on the mechanism(s) at play.

Opposely, the strain missing all four ECM components under study showed increased gallbladder colonization and virulence. The observed phenotype increased in a stepwise pattern as additional mutations were added, with the largest increase in recovered CFU occurring after the edition of the csgA mutation to the wcaM mutant. This double mutant also showed the biggest defect in biofilm formation. It is perhaps counterintuitive to attribute increased colonization of the gallbladder to decreased biofilm formation, but immune alterations were also noted. In general, in response to the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant, IL-10 production was decreased in infected tissue. E. coli (○) was used as a positive control to ensure complement killing, and E. coli with heat-inactivated serum (△) served as a negative control. The limit of detection was 100 CFU/mL. Experiments were repeated 3 times. An unpaired t test with Welch’s correction was used to determine significant differences between mutant strain and WT (ns, no significance).

FIG 6 (A) IL-10 and (B) TNF-α levels in gallbladder tissue or bile from mice infected with either WT (gray bars) or ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant (white bars) cells. The data from gallbladder tissue or bile are from 10 separate samples. An unpaired t test was used to determine significant differences between the mutant strain and WT (*, P < 0.05). Error bars indicate standard errors.

FIG 7 (A) Macrophage cell line J774.1 was infected with either WT (●) or ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant (■) cells. Numbers of bacteria per cell (both inside and membrane associated) were recovered at 1 h postinfection and after 45 min of gentamicin treatment. An unpaired t test was used to determine significant differences between mutant strains and the WT in each organ (ns, not significant). Bars indicate means. (B) Human serum sensitivity assays were performed on WT (●) and ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant (■) cells. E. coli (●) was used as a positive control to ensure complement killing, and E. coli with heat-inactivated serum (△) served as a negative control. The limit of detection was 100 CFU/mL. Experiments were repeated 3 times. An unpaired t test with Welch’s correction was used to determine significant differences between mutant strain and WT (ns, no significance).
and on gallstones, while the ΔwcaM strain only outcompeted the WT in the gallbladder tissue. All other single mutants showed equal or defective survival versus the WT strain. Thus, consistent with biofilm formation in vitro and CFU data in vivo, curli fimbriae, in association with colanic acid, affect colonization and persistence in the gallbladder. In fact, curli fimbriae, recognized by Toll-like receptor 1 (TLR1)/TLR2, induce IL-10 production (36). Consistent with this finding, as mentioned above, we observed decreased IL-10 in the gallbladder in vivo in response to the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant, which is likely attributed to the lack of curli fimbriae. Perhaps the lack of sufficient IL-10 alters the immune balance in the gallbladder that favors ECM mutant survival and persistence.

In addition to the cytokine alterations that were observed, the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant demonstrated increased adherence and invasion into macrophages. This may also hold true for other cell types, including gallbladder epithelial cells. There was no significant difference in intracellular survival rates in macrophages or difference in levels of resistance to another innate immune factor, complement. Thus, in addition to cytokine alterations of the local environment, increased numbers of intracellular salmonellae may also help account for the observed enhanced presence of the ΔwcaM ΔcsgA ΔyihO ΔbcsE mutant in the gallbladder in the gallstone mouse model.

The role of the ECM components in altering host gallbladder colonization and persistence remains an active area of investigation. It is important to understand the relative abundance of ECM components in biofilms in the gallbladder, and we recently developed a gallstone sectioning technique to allow for ECM detection by microscopy (37). Knowing the major ECM constituents in vivo coupled with the above-described data regarding their functions in vitro and in biofilms in the gallbladder will allow unique insight into this biofilm-mediated infection. Based on their roles established here and elsewhere, future work will focus on immunomodulatory properties of curli and ViAg and their participation in gallbladder colonization and persistent gallbladder infection.

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FIG 8 Bacterial CFU enumeration of Salmonella in competition experiments at 8 days postinfection in the presence of gallstones. Mice were given intraperitoneal injections containing 10^7 CFU that represent an equal mixture of WT cells marked with streptomycin resistance and (A) ΔwcaM::Kan, (B) ΔcsgA::Kan, (C) ΔyihO::Cam, or (D) ΔbcsE::Cam mutant cells. The limit of detection was 100 CFU/ml. The dotted line represents a completely neutral competitive index. Wilcoxon’s signed rank test was used to determine significant differences between mutant strains and the WT (*, P < 0.05; **, P < 0.01; *** , P < 0.001). The bars indicate the means of the groups. Positive values represent the mutant outcompeting the WT.


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