AEM
Journals.ASM.org

Biofilm Formation Protects *Escherichia coli* against Killing by *Caenorhabditis elegans* and *Myxococcus xanthus*

William H. DePas,[a] Adnan K. Syed,[b] Margarita Sifuentes,[b] John S. Lee,[b] David Warshaw,[b] Vinay Saggar,[b] Györgyi Csankovszki,[b] Blaise R. Boles,[c] Matthew R. Chapman[b]

Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan, USA[a]; Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan, USA[b]; Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA[c]

Enteric bacteria, such as *Escherichia coli*, are exposed to a variety of stresses in the nonhost environment. The development of biofilms provides *E. coli* with resistance to environmental insults, such as desiccation and bleach. We found that biofilm formation, specifically production of the matrix components curli and cellulose, protected *E. coli* against killing by the soil-dwelling nematode *Caenorhabditis elegans* and the predatory bacterium *Myxococcus xanthus*. Additionally, matrix-encased bacteria at the air-biofilm interface exhibited ~40-fold-increased survival after *C. elegans* and *M. xanthus* killing compared to the non-matrix-encased cells that populate the interior of the biofilm. To determine if nonhost *Enterobacteriaceae* reservoirs supported biofilm formation, we grew *E. coli* on media composed of pig dung or commonly contaminated foods, such as beef, chicken, and spinach. Each of these medium types provided a nutritional environment that supported matrix production and biofilm formation. Altogether, we showed that common, nonhost reservoirs of *E. coli* supported the formation of biofilms that subsequently protected *E. coli* against predation.

Escherichia coli is a rod-shaped facultative anaerobe that lives as a commensal in the mammalian gut. Through acquisition of various virulence factors, particular *E. coli* strains have gained the ability to colonize and cause disease at specific host sites. Enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are common causative agents of gastroenteritis (1–3). Extraintestinal pathogenic *E. coli* (ExPEC) can infect host niches other than the intestinal tract and causes various diseases, such as sepsis, neonatal meningitis, and urinary tract infections (UTIs). Uropathogenic *E. coli* (UPEC) accounts for approximately 80% of the acute UTIs reported in the United States (3–5). ExPEC is becoming increasingly problematic due to a recent rise in antibiotic resistance (5, 6).

Intestinal pathogenic *E. coli* (IPEC) is spread through the fecal-oral route. A common mechanism of host-to-host transmission is shedding of bacteria in the feces of pathogen-bearing farm animals (7–9). Indeed, contact with animal feces is a risk factor for sporadic infection with EHEC (10). Although UPEC is adapted to infect the bladder, it can also colonize the gut with no apparent fitness defect (11). The intestine may serve as a reservoir for UPEC in patients with recurrent UTIs, and it is likely that UPEC from the gastrointestinal tract is able to infect and colonize the urethra (12–14). UPEC outbreaks have been reported, with a likely cause being UPEC contamination of food, indicating that ExPEC is also transmitted host-to-host via the fecal-oral route (15–19).

Compared to the host or lab setting, the physiology of *E. coli* in environmental reservoirs is poorly understood. A detailed understanding of the mechanisms involved in nonhost persistence is paramount to developing effective strategies to prevent contamination of food products by *E. coli* and other pathogenic *Enterobacteriaceae*. One important aspect of *E. coli* nonhost persistence and survival is biofilm formation (20). CsgD is a transcriptional regulator in *E. coli* and *Salmonella enterica* serovar Typhimurium that controls biofilm development (21–23). The CsgD regulon includes genes involved in the production of curli fibers and the polysaccharide cellulose (21, 24, 25). Curli fibers are functional amyloids composed largely of CsgA subunits (24). Depolymerizing of amyloids such as curli requires pretreatment with a strong denaturant, such as hexafluoroisopropanol (HFIP) (26). CsgD directly induces the curli subunit operon, while cellulose is activated via CsgD induction of the diguanylate cyclase gene *adrA* (25, 27). AdrA produces the second messenger cyclic-di-GMP, which activates the cellulose synthase BcsA (25, 28).

E. coli biofilm formation can be monitored by the development of wrinkled or rugose colonies on agar plates. Rugose colonies are indicative of curli and cellulose expression in a variety of *Enterobacteriaceae* species (27, 29, 30). UTI89 develops at least two distinct populations within rugose biofilms (29). A population of matrix-encased bacteria lines the air-biofilm interface (termed the "matrix fraction"), while a distinct population of non-matrix-encased cells lines the biofilm interior (termed the "washout fraction"). These two populations can be separated using a washout assay, which involves suspension of the washout fraction bacteria in buffer (29). The washout and matrix fractions demonstrate different susceptibilities to hydrogen peroxide stress (29).

In the environment, curli and cellulose production is correlated with increased resistance to desiccation and tolerance to disinfectants (31–33). Furthermore, matrix production increases EHEC attachment to commonly contaminated foods and to abiotic surfaces (34, 35). While curli and cellulose have various roles during enteric pathogenesis (20, 36), an expression study found

Received 25 July 2014 **Accepted** 3 September 2014
Published ahead of print 5 September 2014
Editor: H. Nojiri
Address correspondence to Matthew R. Chapman, chapmanm@umich.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02464-14.

doi:10.1128/AEM.02464-14

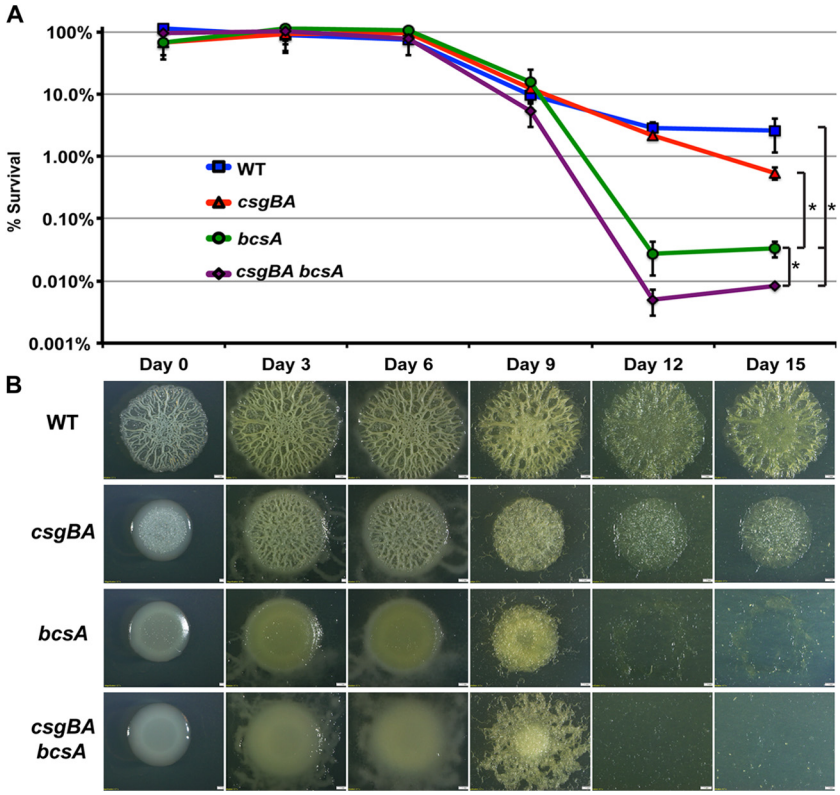

FIG 1 Biofilm formation protects *E. coli* against *C. elegans* predation. Approximately 25 L1- to L2-stage *C. elegans* worms were moved to the center of an agar plate on which four UTI89 strains had grown for 2 days under biofilm-inducing conditions. At 3-day intervals, plates to which *C. elegans* had been added and *C. elegans*-free control plates were harvested for CFU counts, and the percentage of survival was calculated (A). Each data point is an average of biological triplicates, and error bars represent standard deviations. A Student's *t* test was employed to determine significant differences between strains at day 15, and asterisks represent a *P* value of <0.05. An image was taken of each strain at each time point from a representative *C. elegans*-positive plate that was harvested at day 15 (B).

that the curli promoter is relatively inactive during *S. enterica* serovar Typhimurium passage through a mouse host. However, curli expression is immediately induced once *S. enterica* serovar Typhimurium is excreted in stool (32).

Outside the host, bacteria are exposed to a variety of predators. Biofilm-associated *Vibrio cholerae* and *Pseudomonas aeruginosa* survive protozoan grazing better than planktonic cells (37–39). Biofilm formation by *Yersinia pestis* and *Staphylococcus epidermidis*, respectively, blocks food intake and prevents efficient digestion by the nematode *Caenorhabditis elegans* (40, 41). Additionally, *C. elegans* is less efficient at feeding on *Myxococcus xanthus* colonies that produce a more robust biofilm matrix (42). In this study, we sought to determine whether *E. coli* biofilms confer protection against two ubiquitous soil predators, nematodes and myxobacteria, that feed on bacterial prey by divergent mechanisms (43, 44). Nematodes such as *C. elegans* rely on mechanical suction to feed on bacteria, while myxobacteria such as *M. xanthus* employ an array of secondary metabolites to lyse and kill target cells (44–46). In this study, we tested whether *E. coli* biofilm formation outside the host protects against *C. elegans* and *M. xanthus* killing, and we assessed how relevant environmental conditions influence *E. coli* biofilm formation.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and media. All *Enterobacteriaceae* strains used in this study were routinely passaged at 37°C in LB media. All UTI89 strains as well as *S. enterica* serovar Typhimurium and *Citrobacter koseri* have been previously described (29). *M. xanthus* DK1622 was a kind gift from Lawrence Shimkets and was routinely grown at 30°C on Casitone yeast extract (CYE) medium (10 mM morpholinepropanesulfonic acid [MOPS], pH 7.6, 10 g/liter Casitone, 5 g/liter yeast extract, 8 mM $MgSO_4$) in shaking culture or on 1.5% agar plates. Dung from grass-fed cows and pigs was collected in sterile conical tubes from a farm in southeast Michigan. Ground beef, chicken breasts, and spinach were purchased from a local grocery store. Dung and food products were blended using a Waring commercial blender and then centrifuged in 50-ml conical tubes for 10 min at 7,500 rpm. Supernatants were serially filtered using a 0.5-μm-pore pre-filter followed by a 0.2-μm-pore filter. Fecal extract was diluted 1:3 into sterile water with 1.5% noble agar, while food extracts were diluted 1:10. *E. coli* strains WADS1 and WADS2 were isolated by streaking pig dung on MacConkey agar plates and incubating them at 37°C for 24 h. Pink colonies were restreaked and verified as *E. coli* by sequencing of the 16S gene. WADS1 and WADS2 were the only *E. coli* strains isolated.

***C. elegans* predation assay.** *C. elegans* wild-type (WT) strain Bristol N2 worms as well as *C. elegans myo-2*::*rfp ceh22*::*gfp* worms were routinely grown at room temperature on *E. coli* OP50 on nematode growth medium (NGM) agar plates (3 g/liter NaCl, 2.5 g/liter peptone, 17 g/liter agar, supplemented with 25 ml of 1 M KPO_4 [pH 6.0] and 1 ml each of 1 M $CaCl_2$, 1 M $MgSO_4$, and 5 mg/ml cholesterol in ethanol [EtOH]). For predation assays (Fig. 1), WT UTI89 and *csgBA*, *bcsA*, and *csgBA bcsA* mutants were grown in LB broth overnight (ON), diluted to an optical density at 600 nm (OD_{600}) of 1.0 in yeast extract-Casamino Acids (YESCA) medium (10 g/liter Casamino Acids, 1 g/liter yeast extract). Two

microliters of diluted overnight cultures was spotted at the cardinal direction points of 60-mm-by-15-mm YESCA agar plates (2% agar) that had been supplemented with 5 μg/ml cholesterol. The plates were incubated at 26°C for 48 h. Twenty to 30 L1- to L2-stage *C. elegans* worms grown on OP50 were then transferred into the center of the *C. elegans* plus plates, while no worms were added to *C. elegans* negative-control plates (designated day 0). Plates were stored in humidity chambers at 20°C for the 15-day experimental time course. At day 0 and at 3-day intervals thereafter, three plates with worms and three plates without worms were harvested. Briefly, agar slabs containing each colony were cut from the agar plate. Agar slabs containing a whole colony were placed in a 24-well polypropylene plate. One milliliter of potassium phosphate buffer, pH 7.2 (kPi) was added to each well, and the plate was rotated on an orbital shaker for 5 to 10 min until all cells had gone into suspension or the biofilm had lifted off the agar surface. For the WT strain and the *csgBA* and *bcsA* mutants, the entire sample, including biomass and the kPi that had been added to the well, was moved into a 1.5-ml Eppendorf tube. These samples were tissue homogenized for 15 s at high speed. The *csgBA bcsA* mutant went into suspension without homogenization. Suspensions were serially diluted in kPi, and then 100 μl of an appropriate dilution was plated on an LB plate and grown ON at 37°C for CFU quantitation. Each plate with *C. elegans* was randomly paired with a plate without *C. elegans*, and the percentage of survival was calculated as CFU from the plate with *C. elegans* divided by the CFU from the control plate without *C. elegans*. At each time point, pictures of each strain were taken from a representative nematode-positive plate that was harvested at day 15. All colony pictures were taken on an Olympus DP72 camera mounted on an Olympus SZX16 research stereomicroscope.

For the noncompetition assay (see Fig. S2 in the supplemental material), a single agar plate was inoculated with either 6 WT UTI89 dots or 6 UTI89 *csgD* dots. After 48 h of incubation at 26°C, 20 to 30 L1- to L2-stage *C. elegans* worms grown on OP50 were then transferred into the center of the *C. elegans*-positive plates, while no worms were added to the *C. elegans*-negative plates (designated day 0). At day 0 and at 3-day intervals, a single dot was cut out of the agar of each *C. elegans*-positive plate and each *C. elegans*-negative plate and harvested for CFU counts.

***C. elegans* tracking assay.** Overnight bacterial cultures were diluted to an OD_{600} of 1.0. Two microliters of WT UTI89 was spotted near the top of a 60- by 15-mm agar plate containing YESCA plus 5 μg/ml cholesterol, and 2 μl of UTI89 *csgBA bcsA* was spotted near the bottom of the same plate. The plate was then incubated at 26°C for 48 h to allow biofilm development. A small piece of agar containing *C. elegans myo-2*::*rfp ceh22*::*gfp* worms from all growth stages was moved to the center of each UTI89 plate. At 1-, 6-, and 24-h intervals, images of each colony were taken on an Olympus DP72 camera mounted on an Olympus SZX16 research stereomicroscope using bright-field microscopy or a Texas Red filter. All worms within the image frame of each colony picture were counted for that particular strain. The percentage of worms present on each colony was calculated (with the total number being the number of worms on the WT colony plus the number of worms on the *csgBA bcsA* colony for a particular plate).

***M. xanthus* predation assays.** *M. xanthus* predation assays were performed on YESCA agar medium. To support UTI89 rugose biofilm development and *M. xanthus* growth, YESCA medium was buffered to pH 7.4 with 10 mM MOPS (47). After being autoclaved with 1.5% agar, 1 ml of medium was added to each well of a 24-well plate. Plates were allowed to dry for 2 to 3 days at room temperature. Two-microliter dots of UTI89 and *M. xanthus* were spotted roughly 0.5-cm apart in a single well. UTI89 was grown ON at 37°C in LB, and *M. xanthus* was grown for 48 h at 30°C in CYE in shaking cultures prior to dotting, and each strain was normalized to an OD_{600} of 1. After dotting, plates were incubated for 2 days at 26°C to allow for biofilm formation by UTI89 and for *M. xanthus* growth. Plates were moved into a humidity chamber at 30°C to allow *M. xanthus* predation (designated day 0). Pictures were taken every 2 days. For CFU counts at day 10, the contents of each well were suspended in 1 ml kPi. WT UTI89, as well as the *csgBA* and *bcsA* mutants, was tissue homogenized in 1.5-ml Eppendorf tubes along with the buffer that had been added to each well. The *csgBA bcsA* double mutants went into suspension without homogenization. Suspensions were serially diluted on LB agar plates, which do not support *M. xanthus* growth (48), and *E. coli* CFU were determined. As with *C. elegans* predation assays, each well with *M. xanthus* was randomly paired with a well without *M. xanthus*, and the percentage of survival was calculated as CFU from the well with *M. xanthus* divided by the CFU from the well without *M. xanthus*.

Washout assay. The washout assay was performed as previously described (29). Briefly, agar slabs including the colony of interest were cut out and moved into a well of a 24-well plate. The colony was flooded with 1 ml of kPi and shaken gently. For older colonies (>48 h), buffer was pipetted gently onto the edge of the colony to facilitate colony lift-off. The original 1 ml kPi was removed from each well, added to a 1.5-ml Eppendorf tube, and spun down for 1 min at 12,000 rpm. The supernatant was then aspirated off. An additional 1 ml kPi was added to each well and was shaken gently for 5 min to remove any residual washout cells from the matrix fraction. The matrix fraction was moved with a pipette tip into a new 1.5-ml Eppendorf tube filled with 1 ml kPi. The remaining kPi in each well was used to resuspend the original washout pellet. Matrix fractions were tissue homogenized at high speed for 15 s. For CFU counts, each fraction was then serially diluted and an appropriate dilution was plated. For *M. xanthus* washout assays, 1 ml of kPi was added to the top of each UTI89 strain that had been preyed upon by *M. xanthus*. The plate was gently shaken, and kPi was pipetted around the edges of the WT colonies to facilitate colony liftoff. Biofilms that had been fed on by *C. elegans* or *M. xanthus* broke into aggregates when suspended in buffer (see Fig. S3 and S5 in the supplemental material). Therefore, for rugose biofilms that had been exposed to predators, the washout assay was slightly modified so that the stable aggregates were allowed to settle to the bottom of the well. Bacteria from three buffer washes were collected as the washout fraction, and the aggregates were collected as the matrix fraction.

Western blot analysis. Western blotting for the major curli subunit CsgA was performed as previously described (29), with minor changes. Briefly, 150 μl of an OD_{600} of 1.0 suspension of each respective strain/fraction was spun down at 12,000 rpm for 1 min. Pellets were resuspended in 150 μl of hexafluoroisopropanol (HFIP) and incubated at room temperature for 10 min, and then HFIP was removed using a Thermo Savant SPD SpeedVac. Samples were resuspended in 150 μl of 2× SDS running buffer, boiled for 10 min, and electrophoresed in 15% polyacrylamide gels. HFIP-negative samples were directly suspended in 150 μl of 2× SDS running buffer. For CsgA blots, samples were then transferred onto polyvinylidene difluoride membranes by standard techniques. Blots were blocked with 5% milk in Tris-buffered saline–Tween 20 (TBST) ON at 4°C, followed by incubation with 1:8,500 anti-CsgA peptide antibody for 1 h at room temperature. After TBST washes, blots were then incubated with 1:15,000 LI-COR IRDye 800CW goat anti-rabbit IgG secondary antibody. For blots that also include σ^{70} probing, samples were transferred onto nitrocellulose membranes in a wet transfer apparatus in 25 mM CAPS [3-cyclohexylamino)-1-propanesulfonic acid] transfer buffer (pH 11.2) with 10% methanol. After transfer, the blot was blocked in 5% milk in TBST for 1 h at room temperature. CsgA probing was performed the same, and blots were also incubated with 1:5,000 Santa Cruz RNA pol σ D antibody primary antibody (1:5,000) for 1 h at room temperature, followed by incubation with 1:15,000 LI-COR IRDye 700CW goat anti-mouse IgG secondary antibody. Blots were washed with TBST and visualized on a LI-COR Odyssey CLX imager.

Statistical analysis. For all predation assays, each data point is an average of biological triplicates, and error bars represent the standard deviation. For the colony occupancy assay, five individual plates were averaged, and bars represent the number of worms on each strain divided by the total number of worms counted on that particular plate. Error bars represent the standard deviation. Where indicated, a Student's *t* test was employed to compare between strains, and asterisks represent $P < 0.05$.

RESULTS

The role of biofilm formation in protecting *E. coli* against *C. elegans* predation. To determine whether *E. coli* biofilm formation protects against nematode predation, we grew four UTI89 strains, WT (curli$^+$ cellulose$^+$), *bcsA* (curli$^+$ cellulose$^-$), *csgBA* (curli$^-$ cellulose$^+$), and *csgBA bcsA* (curli$^-$ cellulose$^-$), on a single agar plate under biofilm-inducing conditions. *C. elegans* was then added to the plate (day 0). After 9 days of *C. elegans* feeding, the number of CFU/colony of all the strains decreased by roughly 10-fold (Fig. 1A). The WT rugose colony remained visible through the entire time course of the experiment, and the percentage of survival decreased from ~10% on day 9 to ~3% on day 12 to ~2.5% on day 15. Conversely, the *csgBA bcsA* mutant visually disappeared from the plate by day 12, and the percentage of survival decreased to ~0.008% by day 15 (Fig. 1A and B). No significant differences in percentages of survival between the WT and *csgBA* mutant were seen at day 12 or 15 (Fig. 1A and B), implying that cellulose alone provided robust *C. elegans* resistance. The *bcsA* mutant demonstrated a slight but significant survival advantage compared to the *csgBA bcsA* double mutant ($P = 0.044$) (Fig. 1A and B), indicating that curli alone provided a small degree of *C. elegans* resistance. CFU for all UTI89 strains rose slightly throughout the experimental time course when incubated without worms (see Fig. S1 in the supplemental material). When *C. elegans* was grown solely on WT UTI89 or on a matrix-deficient *csgD* mutant, the worms were able to propagate and decrease *E. coli* CFU in a similar manner to that in the competition assay (see Fig. S2 in the supplemental material).

Attraction of *C. elegans* to *E. coli* colonies. To determine whether matrix expression affects colony occupancy, fluorescent worms were moved onto the center of an agar plate that contained one WT colony and one *csgBA bcsA* colony (Fig. 2A). At 1, 6, or 24 h postinoculation, there was no significant difference between the number of worms on the WT colony and the number of worms on the *csgBA bcsA* colony (Fig. 2C). Therefore, it is likely that the *E. coli* biofilm matrix provides a mechanical barrier to predation by *C. elegans* instead of affecting *C. elegans* feeding preference.

Determination of the susceptibility of two biofilm populations to *C. elegans* feeding. After 12 days of biofilm development, a washout assay was performed on WT rugose colonies that had not been preyed on by *C. elegans* (see Fig. S3 in the supplemental material). The washout fraction contained 9.69 ± 8.67 $\log_{10}$ CFU, and the matrix fraction contained 9.38 ± 8.69 $\log_{10}$ CFU. Western blot analysis for the major curli subunit, CsgA, revealed that a bimodal population was maintained at least through 12 days (Fig. 3A) (29). We also performed the washout assay on WT UTI89 rugose colonies that had been preyed on by *C. elegans* for 6 or 12 days, and the numbers of CFU/fraction were determined (Fig. 3B). After 12 days of nematode feeding, 6.59 $\log_{10}$ CFU (~0.074% survival) were recovered from the washout fraction and 7.85 $\log_{10}$ CFU (~3.0% survival) were recovered from the matrix fraction (Fig. 3B), indicating that even within a single biofilm, *C. elegans* was able to feed more efficiently on non-matrix-encased bacteria. CsgA was SDS insoluble in both the absence and presence of nematode predation stress (Fig. 3A).

The role of biofilm formation in protecting *E. coli* against *M. xanthus* predation. When plated next to UTI89 colony biofilms, *M. xanthus* spread through the WT and *csgBA*, *bcsA*, or *csgBA bcsA* mutant cells with similar kinetics (Fig. 4A). The WT rugose biofilm maintained its original shape even after *M. xanthus* feeding (Fig. 4A). Approximately 1.8% of WT UTI89 CFU were recovered after *M. xanthus* predation (Fig. 4B). In contrast, no CFU could be recovered from the UTI89 *csgBA bcsA* mutant after *M. xanthus* predation, demonstrating that biofilm formation provided robust protection (Fig. 4B). Similarly, no colonies could be recovered from the *csgBA* mutant (Fig. 4B). The *bcsA* mutant retained some resistance to *M. xanthus*, indicating that curli is the more important matrix component with regard to *M. xanthus* protection (Fig. 4B). As with the nematode predation assay, matrix fraction bacteria demonstrated a survival advantage compared to washout bacteria after *M. xanthus* exposure (Fig. 4C).

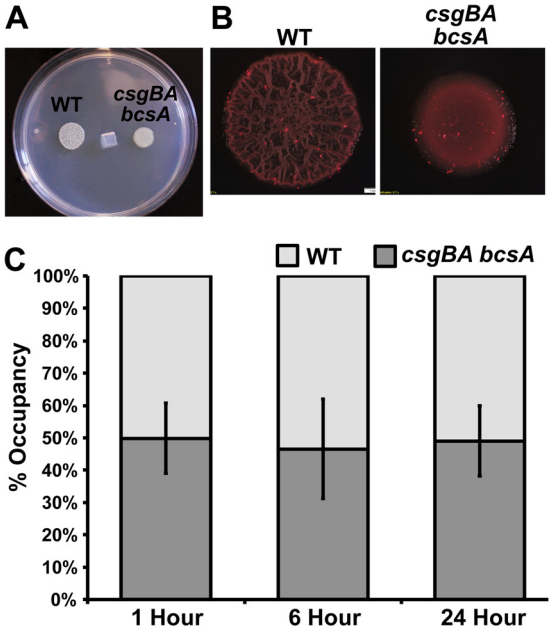

FIG 2 *C. elegans* is not preferentially attracted to the WT or UTI89 *csgBA bcsA* mutant. Cells of WT UTI89 or the *csgBA bcsA* mutant were spotted on opposite sides of a YESCA-cholesterol agar plate. After 2 days of biofilm development at 26°C, *C. elegans myo-2*::*rfp ceh22*::*gfp* worms were moved onto the center of the plate (A). Shown is a representative bright-field/Texas Red overlay of WT or *csgBA bcsA* colonies with fluorescent worms after 6 h of *C. elegans* exposure (B). At 1, 6, and 24 h post-*C. elegans* addition, fluorescent images were taken, and the numbers of worms on each strain were counted (C). Worm totals for each plate at each time point fell between 200 and 1,200. Five individual plates were averaged, and bars represent the number of worms on each strain divided by the total number of worms counted on that particular plate. Error bars represent standard deviations.

Curli remained SDS insoluble even after *M. xanthus* predation (see Fig. S4 in the supplemental material). Interestingly, multiple species of CsgA were visible after *M. xanthus* predation of WT UTI89 (see Fig. S4), demonstrating that *M. xanthus* may alter CsgA fibers. Even though curli remained SDS insoluble, WT rugose biofilms exposed to *M. xanthus* were fragile and broke into pieces upon buffer washing (see Fig. S5 in the supplemental material). After *M. xanthus* predation, the *csgBA* colony remained intact upon washing with buffer, suggesting that the cellulose component of the matrix remained intact (see Fig. S5).

Growth and matrix expression of enteric bacteria under environment-mimicking conditions. Common lab media, such as LB, typically do not support curli expression (49) (see Fig. S6 in the supplemental material). To determine if environmental con-

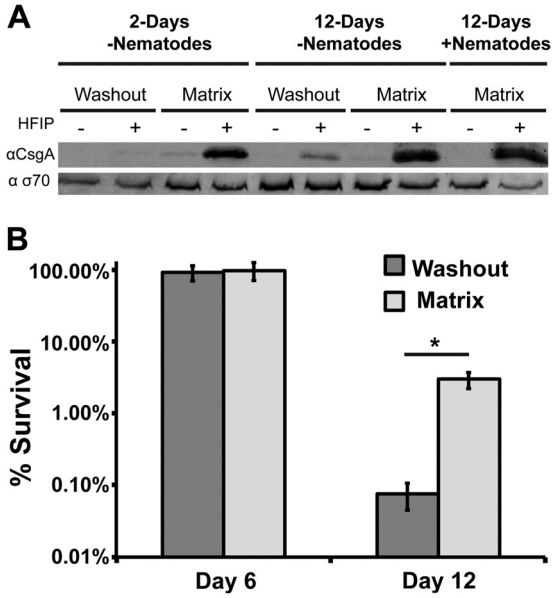

FIG 3 Washout cells are more susceptible to *C. elegans* predation. Western blots probing for the major curli subunit CsgA were performed on 2- or 12-day-old biofilms following the washout assay (A). Not enough cells could be harvested from the washout fraction at 12 days with nematode predation to allow for Western blot analysis of that fraction. σ^{70} represents a loading control. The washout assay was performed on WT UTI89 colony biofilms that had been fed on by *C. elegans* for 6 or 12 days before biofilms were harvested, and the percentage of survival was determined (B). Each bar represents an average of biological triplicates, and error bars represent standard deviations. Asterisks represent a *P* value of <0.05 using Student's *t* test.

ditions support biofilm formation, we monitored growth and curli production by the *Enterobacteriaceae* UTI89, *S. enterica* serovar Typhimurium, and *C. koseri* on dung agar plates. UTI89 and *S. enterica* serovar Typhimurium formed rugose colonies on pig dung agar plates, and no such colony structure was apparent in the UTI89 *csgBA bcsA* mutant (Fig. 5A). Western blot analysis of CsgA demonstrated that UTI89, *S. enterica* serovar Typhimurium, and *C. koseri* produced curli on pig dung agar plates (Fig. 5B). As expected no CsgA was detectable in the UTI89 *csgBA bcsA* mutant (Fig. 5B). Curli production and rugose biofilm formation were enhanced at 26°C compared to 37°C (Fig. 5A and B), which agrees with previous findings (50). To further test the ability of dung to support biofilm formation, we isolated two *E. coli* strains from the same pig dung we used to make agar plates (denoted WADS1 and WADS2). When these isolates were plated on pig dung agar plates, they also produced CsgA (Fig. 5B). Cow dung was also tested for its ability to support biofilm formation. Although growth was very limited on cow dung agar plates, rugose biofilm formation was apparent in UTI89 and *S. enterica* serovar Typhimurium at 26°C (see Fig. S7 in the supplemental material).

We also tested whether extracts from commonly contaminated foods provided the nutritional environment to support matrix production. UTI89, *C. koseri*, and both pig dung *E. coli* isolates produced curli on beef, chicken, and spinach agar plates (see Fig. S8A, B, and C in the supplemental material). CsgA was detected from *S. enterica* serovar Typhimurium on chicken agar plates (see Fig. S8B in the supplemental material). On all three food plates, incubation at 26°C supported more curli production than incubation at 37°C (50) (see Fig. S8A, B, and C). UTI89 and *C. koseri* produced rugose biofilms on beef plates (see Fig. S9 in the supplemental material).

DISCUSSION

Biofilm formation is a common bacterial coping mechanism for resisting environmental stresses. Rugose biofilm formation by *V. cholerae* protects the bacteria against flagellate predation, chlorinated water, and osmotic and oxidative stress (51–53). *P. aeruginosa* biofilm formation can likewise protect against amoeba feeding (37, 38). *Y. pestis*, *S. epidermidis*, and *M. xanthus* utilize biofilms to inhibit either feeding or digestion by *C. elegans* (40–42). CsgD-mediated biofilms protect *S. enterica* serovar Typhimurium against various nonhost stresses, such as desiccation and disinfectants (31, 32).

Pathogenic *Enterobacteriaceae* reside in the gut of cattle and pigs (7–9, 54) and after being shed in feces can contaminate food products, particularly if untreated feces is used as manure (7, 10, 55, 56). Indeed, *E. coli* can survive in dung for months, and survival is increased when temperatures are <23°C (57). CsgD is under complex transcriptional control, but in general, environments that present low salts, low temperatures (<30°C), and an air interface allow for high *csgD* transcription (29, 58–61). We hypothesized that habitats such as dung, where *E. coli* could encounter predators like nematodes and *M. xanthus* (43, 44), would provide the nutritional environment for biofilm formation. Our data reiterate that regulation of biofilm formation can vary substantially among *E. coli* strains (25, 62). Indeed, one of our pig dung *E. coli* isolates, WADS2, produced very little CsgA on YESCA plates, which normally allow for substantial curli production. The other isolate, WADS1, made CsgA on LB plates, which normally do not support curli production. In support of our hypothesis, however, pig dung was a unifying medium in that it induced robust CsgA expression by all tested WT strains, demonstrating that common nonhost niches of *E. coli* can provide the nutritional environment for biofilm development.

Some *E. coli* strains, particularly EPEC, can colonize the gut of *C. elegans*, leading to worm death (63). However, colonization is dependent on genes in the locus of enterocyte effacement (LEE), a pathogenicity island that only a subset of *E. coli* strains possess (3, 63). In this study, we demonstrated that biofilm formation protected UTI89 against *C. elegans* killing. It is possible that *E. coli* biofilm matrix components could act as a chemo-repellant to nematodes. Indeed, *P. aeruginosa* polysaccharide production changes the feeding behavior of *C. elegans* (64). However, a similar number of worms were attracted to either WT or *csgBA bcsA* mutant colony biofilms in our colony occupancy experiment, demonstrating that the biofilm matrix likely acts as a mechanical barrier to nematode feeding.

Intriguingly, a *csgBA* mutant resisted *C. elegans* killing to the same degree as WT UTI89, demonstrating that cellulose was more important than curli for resistance to *C. elegans*. *E. coli* biofilms producing only curli are more rigid than *E. coli* biofilms producing only cellulose (61). The elasticity imbued by cellulose may allow the biofilm community to better resist the mechanical forces applied by *C. elegans* movement and feeding. Indeed, a cohesive biomass was still visible after *C. elegans* preyed on the *csgBA* mutant (only producing cellulose).

While *C. elegans* mechanically swallows prey bacteria, predators such as *M. xanthus* utilize antibacterial and lytic agents to kill their prey extracellularly (45, 46). However, since efficient *M. xan-*

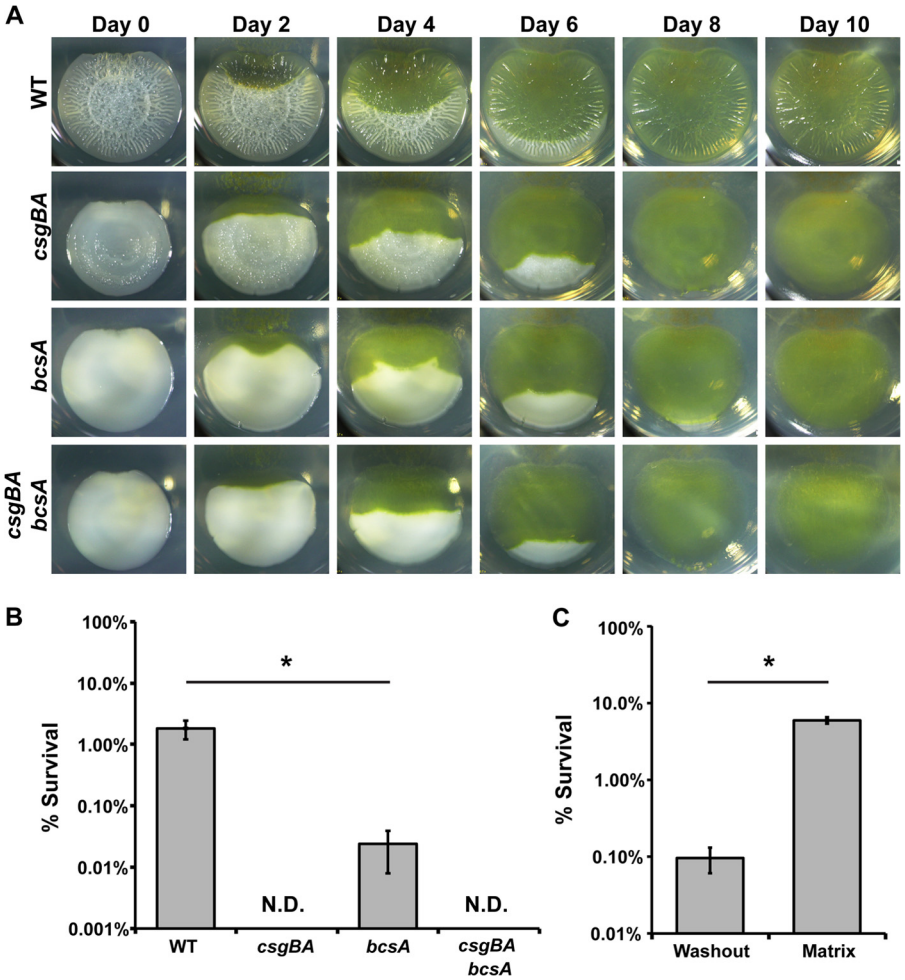

FIG 4 UTI89 biofilm formation protects against *M. xanthus* predation. *M. xanthus* was spotted next to UTI89 colony biofilms in 24-well plates. Pictures were taken at 2-day intervals (A). After 10 days, CFU were harvested from each UTI89 strain. The percentage of survival compared to *M. xanthus*-free UTI89 colonies was calculated (B). The washout assay was performed on WT UTI89 that had been preyed on by *M. xanthus* for 10 days. CFU from both the washout and matrix fractions were determined, and the percentage of survival compared to that in fractions from *M. xanthus*-negative UTI89 colonies subjected to the washout assay was calculated (C). Each data point is an average of biological triplicates, and error bars represent standard deviations. "N.D." denotes that no CFU were detected. A Student's *t* test was employed to determine significant differences between strains or fractions, and asterisks represent a *P* value of <0.05.

thus killing requires direct predator-prey contact and depends on prey cell density (65–67), we hypothesized that an extracellular matrix would protect *E. coli* in an *M. xanthus* killing assay. Indeed, WT UTI89 demonstrated ~1.8% survival after *M. xanthus* killing, while no colonies were detected from the *csgBA bcsA* mutant. In contrast to *C. elegans* killing, curli was more important than cellulose in preventing *M. xanthus* killing. The small size of *M. xanthus* (compared to nematodes) might allow the predatory bacteria to intercalate into the *E. coli* biofilm matrix. Therefore, the dense curli matrix that surrounds individual *E. coli* cells might be more efficient at preventing *M. xanthus-E. coli* contact than the more diffuse cellulose filaments (59, 61). A recent study demonstrated that protozoan growth on *E. coli* correlated positively with the ability of the *E. coli* strain to produce curli (68). In light of our results showing that different matrix components can offer various levels of resistance to different predators, an interesting possibility is that protozoan resistance could depend on cellulose or yet another *E. coli* matrix component.

Bimodal population development within rugose biofilms allows for analysis of fitness differences between distinct populations in a single biofilm community (29). We previously found that the two rugose biofilm populations demonstrate different stress resistance profiles, as the washout cells are more vulnerable to hydrogen peroxide killing than the matrix cells (29). As predicted, the interior, washout bacteria were more susceptible to predation by both *C. elegans* and *M. xanthus*. The increased susceptibility of the interior fraction of rugose colonies to oxidative stress as well as predation raises the question as to the functional role of this population (29). An intriguing possibility is that washout bacteria serve as an easily disseminated population of the biofilm. Indeed, even gentle washing of rugose colonies with liquid releases washout cells from the biofilm community (29). A pair of elegant scanning electron microscopy (SEM) studies have recently demonstrated that the interior bacteria of W3110 *E. coli* rugose biofilms produce flagella as an integral component of the biofilm matrix (60, 61). The structural division of labor, with matrix-encased bacteria lining the air-biofilm interface while covering a non-matrix-encased, flagellated population is also present in

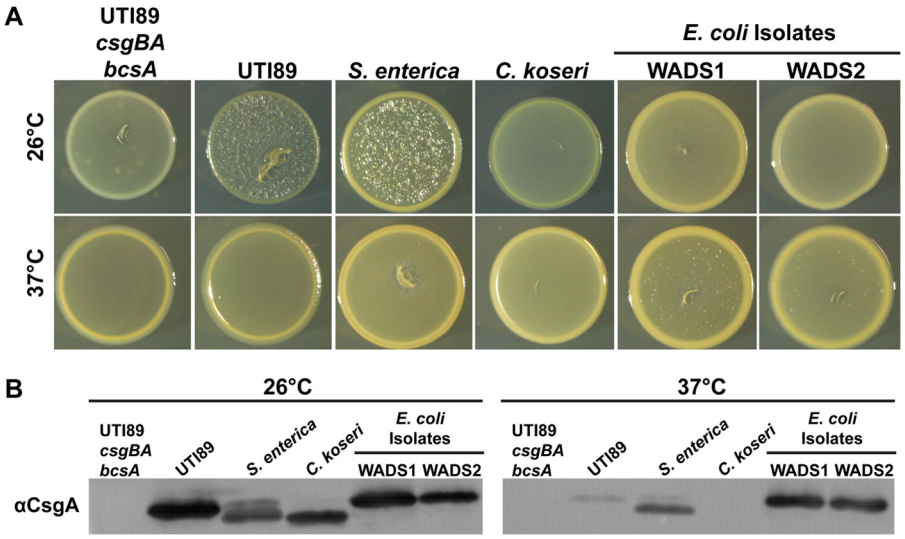

FIG 5 Growth and curli expression on pig dung agar plates. UTI89, *S. enterica* serovar Typhimurium, *C. koseri*, and two *E. coli* strains isolated from pig dung (WADS1 and WADS2) were grown on pig dung agar plates for 2 days at 26°C or 37°C. Rugose biofilm development was apparent by UTI89 and *S. enterica* serovar Typhimurium (A). Western blot analysis probing for the major curli subunit CsgA revealed that pig dung plates allowed for robust curli production by all strains (B). All Western blot samples were treated with HFIP to depolymerize CsgA.

UTI89 pellicle biofilms (59). Intriguingly, *P. aeruginosa* mushroom biofilms produce a similar architecture, with polysaccharide production being limited to the biofilm exterior (69). Flagellated *P. aeruginosa* cells that populate the biofilm interior are considered to be the chief agents of biofilm dispersal (69). It seems then that the formation of a protective outer coating surrounding an interior population of easily dispersed cells is a common architectural pattern in a variety of bacterial biofilms.

Altogether, our data support a model in which *E. coli* and other *Enterobacteriaceae* produce CsgD-dependent biofilms under environmental conditions commonly encountered outside the host. Biofilm development involves formation of a stress-resistant, matrix-producing population along with an easily dispersed but stress-susceptible interior population. Furthermore, the nutritional environment provided by nonhost reservoirs, such as dung, beef, chicken, and spinach, supported curliated biofilm formation by *E. coli* and other *Enterobacteriaceae*.

ACKNOWLEDGMENTS

We thank EMMA Acres Farm for their hospitality and the kind donation of animal dung. Additionally, we thank Lawrence Shimkets and Ann Miller for donations of strains and reagents and use of equipment. Finally, we thank members of the Chapman and Boles labs for helpful discussion and insight.

This work was supported by National Institutes of Health grants RO1 A1073847-6 to M.R.C., AT32 GM007544-32 to W.H.D., NIAID AI081748 to B.R.B, and American Heart Association Fellowship 13PRE13810001 to A.K.S.

REFERENCES

1. **Tauxe RV.** 1997. Emerging foodborne diseases: an evolving public health challenge. Emerg. Infect. Dis. **3:**425–434. http://dx.doi.org/10.3201/eid0304.970403.
2. **Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625. http://dx.doi.org/10.3201/eid0505.990502.
3. **Kaper JB, Nataro JP, Mobley HL.** 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. **2:**123–140. http://dx.doi.org/10.1038/nrmicro818.
4. **Foxman B, Brown P.** 2003. Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. Infect. Dis. Clin. North Am. **17:**227–241. http://dx.doi.org/10.1016/S0891-5520(03)00005-9.
5. **Russo TA, Johnson JR.** 2003. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. Microbes Infect. **5:**449–456. http://dx.doi.org/10.1016/S1286-4579(03)00049-2.
6. **Pitout JD.** 2012. Extraintestinal pathogenic *Escherichia coli*: an update on antimicrobial resistance, laboratory diagnosis and treatment. Expert Rev. Anti Infect. Ther. **10:**1165–1176. http://dx.doi.org/10.1586/eri.12.110.
7. **Ferens WA, Hovde CJ.** 2011. *Escherichia coli* O157:H7: animal reservoir and sources of human infection. Foodborne Pathog. Dis. **8:**465–487. http://dx.doi.org/10.1089/fpd.2010.0673.
8. **Fatica MK, Schneider KR.** 2011. *Salmonella* and produce: survival in the plant environment and implications in food safety. Virulence **2:**573–579. http://dx.doi.org/10.4161/viru.2.6.17880.
9. **Zweifel C, Schumacher S, Beutin L, Blanco J, Stephan R.** 2006. Virulence profiles of Shiga toxin 2e-producing *Escherichia coli* isolated from healthy pig at slaughter. Vet. Microbiol. **117:**328–332. http://dx.doi.org/10.1016/j.vetmic.2006.06.017.
10. **Locking ME, O'Brien SJ, Reilly WJ, Wright EM, Campbell DM, Coia JE, Browning LM, Ramsay CN.** 2001. Risk factors for sporadic cases of *Escherichia coli* O157 infection: the importance of contact with animal excreta. Epidemiol. Infect. **127:**215–220.
11. **Chen SL, Wu M, Henderson JP, Hooton TM, Hibbing ME, Hultgren SJ, Gordon JI.** 2013. Genomic diversity and fitness of *E. coli* strains recovered from the intestinal and urinary tracts of women with recurrent urinary tract infection. Sci. Transl. Med. **5:**184ra160. http://dx.doi.org/10.1126/scitranslmed.3005497.
12. **Russo TA, Stapleton A, Wenderoth S, Hooton TM, Stamm WE.** 1995. Chromosomal restriction fragment length polymorphism analysis of *Escherichia coli* strains causing recurrent urinary tract infections in young women. J. Infect. Dis. **172:**440–445. http://dx.doi.org/10.1093/infdis/172.2.440.
13. **Hooton TM.** 2001. Recurrent urinary tract infection in women. Int. J. Antimicrob. Agents **17:**259–268. http://dx.doi.org/10.1016/S0924-8579(00)00350-2.
14. **Yamamoto S, Tsukamoto T, Terai A, Kurazono H, Takeda Y, Yoshida O.** 1997. Genetic evidence supporting the fecal-perineal-urethral hypothesis in cystitis caused by *Escherichia coli*. J. Urol. **157:**1127–1129. http://dx.doi.org/10.1016/S0022-5347(01)65154-1.
15. **Manges AR, Johnson JR.** 2012. Food-borne origins of *Escherichia coli*

causing extraintestinal infections. Clin. Infect. Dis. **55:**712–719. http://dx.doi.org/10.1093/cid/cis502.
16. **Johnson JR, Kuskowski MA, Smith K, O'Bryan TT, Tatini S.** 2005. Antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* in retail foods. J. Infect. Dis. **191:**1040–1049. http://dx.doi.org/10.1086/428451.
17. **Phillips I, King A, Rowe B, Gross RJ, Eykyn S, Gransden WR, Frost JA.** 1988. Epidemic multiresistant *Escherichia-coli* infection in West Lambeth Health District. Lancet **i:**1038–1041.
18. **Ramchandani M, Manges AR, DebRoy C, Smith SP, Johnson JR, Riley LW.** 2005. Possible animal origin of human-associated, multidrug-resistant, uropathogenic *Escherichia coli*. Clin. Infect. Dis. **40:**251–257. http://dx.doi.org/10.1086/426819.
19. **Belanger L, Garenaux A, Harel J, Boulianne M, Nadeau E, Dozois CM.** 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunol. Med. Microbiol. **62:**1–10. http://dx.doi.org/10.1111/j.1574-695X.2011.00797.x.
20. **Hufnagel DA, Tukel C, Chapman MR.** 2013. Disease to dirt: the biology of microbial amyloids. PLoS Pathog. **9:**e1003740. http://dx.doi.org/10.1371/journal.ppat.1003740.
21. **Hammar M, Arnqvist A, Bian Z, Olsen A, Normark S.** 1995. Expression of two *csg* operons is required for production of fibronectin- and Congo red-binding curli polymers in *Escherichia coli* K-12. Mol. Microbiol. **18:**661–670. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_18040661.x.
22. **Romling U, Bian Z, Hammar M, Sierralta WD, Normark S.** 1998. Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. J. Bacteriol. **180:**722–731.
23. **Dueholm MS, Albertsen M, Otzen D, Nielsen PH.** 2012. Curli functional amyloid systems are phylogenetically widespread and display large diversity in operon and protein structure. PLoS One **7:**e51274. http://dx.doi.org/10.1371/journal.pone.0051274.
24. **Chapman MR, Robinson LS, Pinkner JS, Roth R, Heuser J, Hammar M, Normark S, Hultgren SJ.** 2002. Role of *Escherichia coli* curli operons in directing amyloid fiber formation. Science **295:**851–855. http://dx.doi.org/10.1126/science.1067484.
25. **Zogaj X, Nimtz M, Rohde M, Bokranz W, Romling U.** 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol. Microbiol. **39:**1452–1463. http://dx.doi.org/10.1046/j.1365-2958.2001.02337.x.
26. **Zhou Y, Smith DR, Hufnagel DA, Chapman MR.** 2013. Experimental manipulation of the microbial functional amyloid called curli. Methods Mol. Biol. **966:**53–75. http://dx.doi.org/10.1007/978-1-62703-245-2_4.
27. **Romling U, Rohde M, Olsen A, Normark S, Reinkoster J.** 2000. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. Mol. Microbiol. **36:**10–23. http://dx.doi.org/10.1046/j.1365-2958.2000.01822.x.
28. **Ryjenkov DA, Simm R, Romling U, Gomelsky M.** 2006. The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. J. Biol. Chem. **281:**30310–30314. http://dx.doi.org/10.1074/jbc.C600179200.
29. **DePas WH, Hufnagel DA, Lee JS, Blanco LP, Bernstein HC, Fisher ST, James GA, Stewart PS, Chapman MR.** 2013. Iron induces bimodal population development by *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **110:**2629–2634. http://dx.doi.org/10.1073/pnas.1218703110.
30. **Zogaj X, Bokranz W, Nimtz M, Romling U.** 2003. Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract. Infect. Immun. **71:**4151–4158. http://dx.doi.org/10.1128/IAI.71.7.4151-4158.2003.
31. **White AP, Gibson DL, Kim W, Kay WW, Surette MG.** 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. J. Bacteriol. **188:**3219–3227. http://dx.doi.org/10.1128/JB.188.9.3219-3227.2006.
32. **White AP, Gibson DL, Grassl GA, Kay WW, Finlay BB, Vallance BA, Surette MG.** 2008. Aggregation via the red, dry, and rough morphotype is not a virulence adaptation in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **76:**1048–1058. http://dx.doi.org/10.1128/IAI.01383-07.
33. **Wang R, Bono JL, Kalchayanand N, Shackelford S, Harhay DM.** 2012. Biofilm formation by Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157 strains and their tolerance to sanitizers commonly used in the food processing environment. J. Food Prot. **75:**1418–1428. http://dx.doi.org/10.4315/0362-028X.JFP-11-427.
34. **Macarisin D, Patel J, Bauchan G, Giron JA, Sharma VK.** 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodborne Pathog. Dis. **9:**160–167. http://dx.doi.org/10.1089/fpd.2011.1020.
35. **Pawar DM, Rossman ML, Chen J.** 2005. Role of curli fimbriae in mediating the cells of enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. J. Appl. Microbiol. **99:**418–425. http://dx.doi.org/10.1111/j.1365-2672.2005.02499.x.
36. **Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, Aberg V, Walker JN, Seed PC, Almqvist F, Chapman MR, Hultgren SJ.** 2009. Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nat. Chem. Biol. **5:**913–919. http://dx.doi.org/10.1038/nchembio.242.
37. **Matz C, Bergfeld T, Rice SA, Kjelleberg S.** 2004. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environ. Microbiol. **6:**218–226. http://dx.doi.org/10.1111/j.1462-2920.2004.00556.x.
38. **Weitere M, Bergfeld T, Rice SA, Matz C, Kjelleberg S.** 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environ. Microbiol. **7:**1593–1601. http://dx.doi.org/10.1111/j.1462-2920.2005.00851.x.
39. **Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S.** 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc. Natl. Acad. Sci. U. S. A. **102:**16819–16824. http://dx.doi.org/10.1073/pnas.0505350102.
40. **Darby C, Hsu JW, Ghori N, Falkow S.** 2002. *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. Nature **417:**243–244. http://dx.doi.org/10.1038/417243a.
41. **Begun J, Gaiani JM, Rohde H, Mack D, Calderwood SB, Ausubel FM, Sifri CD.** 2007. Staphylococcal biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defenses. PLoS Pathog. **3:**e57. http://dx.doi.org/10.1371/journal.ppat.0030057.
42. **Dahl JL, Ulrich CH, Kroft TL.** 2011. Role of phase variation in the resistance of *Myxococcus xanthus* fruiting bodies to *Caenorhabditis elegans* predation. J. Bacteriol. **193:**5081–5089. http://dx.doi.org/10.1128/JB.05383-11.
43. **De Mesel I, Derycke S, Moens T, Van der Gucht K, Vincx M, Swings J.** 2004. Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. Environ. Microbiol. **6:**733–744. http://dx.doi.org/10.1111/j.1462-2920.2004.00610.x.
44. **Reichenbach H.** 1999. The ecology of the myxobacteria. Environ. Microbiol. **1:**15–21. http://dx.doi.org/10.1046/j.1462-2920.1999.00016.x.
45. **Berleman JE, Kirby JR.** 2009. Deciphering the hunting strategy of a bacterial wolfpack. FEMS Microbiol. Rev. **33:**942–957. http://dx.doi.org/10.1111/j.1574-6976.2009.00185.x.
46. **Avery L, You YJ.** 21 May 2012. *C. elegans* feeding. *In* The *C. elegans* Research Community, WormBook (ed), Wormbook. http://dx.doi.org/10.1895/wormbook.1.150.1.
47. **Ramsey WS, Dworkin M.** 1968. Microcyst germination in *Myxococcus xanthus*. J. Bacteriol. **95:**2249–2257.
48. **Xiao Y, Wei X, Ebright R, Wall D.** 2011. Antibiotic production by myxobacteria plays a role in predation. J. Bacteriol. **193:**4626–4633. http://dx.doi.org/10.1128/JB.05052-11.
49. **Romling U, Sierralta WD, Eriksson K, Normark S.** 1998. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. Mol. Microbiol. **28:**249–264. http://dx.doi.org/10.1046/j.1365-2958.1998.00791.x.
50. **Olsen A, Jonsson A, Normark S.** 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature **338:**652–655. http://dx.doi.org/10.1038/338652a0.
51. **Wai SN, Mizunoe Y, Takade A, Kawabata SI, Yoshida SI.** 1998. *Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. Appl. Environ. Microbiol. **64:**3648–3655.
52. **Morris JG, Jr, Sztein MB, Rice EW, Nataro JP, Losonsky GA, Panigrahi P, Tacket CO, Johnson JA.** 1996. *Vibrio cholerae* O1 can assume a chlorine-resistant rugose survival form that is virulent for humans. J. Infect. Dis. **174:**1364–1368. http://dx.doi.org/10.1093/infdis/174.6.1364.
53. **Yildiz FH, Schoolnik GK.** 1999. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc. Natl. Acad. Sci. U. S. A. **96:**4028–4033. http://dx.doi.org/10.1073/pnas.96.7.4028.

54. **Mughini-Gras L, Enserink R, Friesema I, Heck M, van Duynhoven Y, van Pelt W.** 2014. Risk factors for human salmonellosis originating from pigs, cattle, broiler chickens and egg laying hens: a combined case-control and source attribution analysis. PLoS One **9:**e87933. http://dx.doi.org/10.1371/journal.pone.0087933.
55. **Solomon EB, Yaron S, Matthews KR.** 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. **68:**397–400. http://dx.doi.org/10.1128/AEM.68.1.397-400.2002.
56. **Mootian G, Wu WH, Matthews KR.** 2009. Transfer of *Escherichia coli* O157:H7 from soil, water, and manure contaminated with low numbers of the pathogen to lettuce plants. J. Food Prot. **72:**2308–2312.
57. **Kudva IT, Blanch K, Hovde CJ.** 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. Appl. Environ. Microbiol. **64:**3166–3174.
58. **Evans ML, Chapman MR.** 2014. Curli biogenesis: order out of disorder. Biochim. Biophys. Acta **1843:**1551–1558. http://dx.doi.org/10.1016/j.bbamcr.2013.09.010.
59. **Hung C, Zhou YZ, Pinkner JS, Dodson KW, Crowley JR, Heuser J, Chapman MR, Hadjifrangiskou M, Henderson JP, Hultgren SJ.** 2013. *Escherichia coli* biofilms have an organized and complex extracellular matrix structure. mBio **4**(5)**:**e00645-13. http://dx.doi.org/10.1128/mBio.00645-13.
60. **Serra DO, Richter AM, Klauck G, Mika F, Hengge R.** 2013. Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. mBio **4**(2)**:**e00103-13. http://dx.doi.org/10.1128/mBio.00103-13.
61. **Serra DO, Richter AM, Hengge R.** 2013. Cellulose as an architectural element in spatially structured *Escherichia coli* biofilms. J. Bacteriol. **195:**5540–5554. http://dx.doi.org/10.1128/JB.00946-13.
62. **Bokranz W, Wang X, Tschape H, Romling U.** 2005. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J. Med. Microbiol. **54:**1171–1182. http://dx.doi.org/10.1099/jmm.0.46064-0.
63. **Mellies JL, Barron AM, Haack KR, Korson AS, Oldridge DA.** 2006. The global regulator Ler is necessary for enteropathogenic *Escherichia coli* colonization of *Caenorhabditis elegans*. Infect. Immun. **74:**64–72. http://dx.doi.org/10.1128/IAI.74.1.64-72.2006.
64. **Reddy KC, Hunter RC, Bhatla N, Newman DK, Kim DH.** 2011. *Caenorhabditis elegans* NPR-1-mediated behaviors are suppressed in the presence of mucoid bacteria. Proc. Natl. Acad. Sci. U. S. A. **108:**12887–12892. http://dx.doi.org/10.1073/pnas.1108265108.
65. **McBride MJ, Zusman DR.** 1996. Behavioral analysis of single cells of *Myxococcus xanthus* in response to prey cells of *Escherichia coli*. FEMS Microbiol. Lett. **137:**227–231. http://dx.doi.org/10.1111/j.1574-6968.1996.tb08110.x.
66. **Hillesland KL, Lenski RE, Velicer GJ.** 2007. Ecological variables affecting predatory success in *Myxococcus xanthus*. Microb. Ecol. **53:**571–578. http://dx.doi.org/10.1007/s00248-006-9111-3.
67. **Pan H, He X, Lux R, Luan J, Shi W.** 2013. Killing of *Escherichia coli* by *Myxococcus xanthus* in aqueous environments requires exopolysaccharide-dependent physical contact. Microb. Ecol. **66:**630–638. http://dx.doi.org/10.1007/s00248-013-0252-x.
68. **Ravva SV, Sarreal CZ, Mandrell RE.** 2014. Strain differences in fitness of *Escherichia coli* O157:H7 to resist protozoan predation and survival in soil. PLoS One **9:**e102412. http://dx.doi.org/10.1371/journal.pone.0102412.
69. **Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ.** 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathog. **5:**e1000354. http://dx.doi.org/10.1371/journal.ppat.1000354.