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In Vitro Biofilm Formation of Commensal and Pathogenic *Escherichia coli* Strains: Impact of Environmental and Genetic Factors

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Our understanding of *Escherichia coli* biofilm formation in vitro is based on studies of laboratory K-12 strains grown in standard media. However, pathogenic *E. coli* isolates differ substantially in their genetic repertoire from *E. coli* K-12 and are subject to heterogeneous environmental conditions. In this study, in vitro biofilm formation of 331 nondomesticated *E. coli* strains isolated from healthy ($n = 105$) and diarrhea-afflicted children ($n = 68$), bacteremia patients ($n = 90$), and male patients with urinary tract infections ($n = 68$) was monitored using a variety of growth conditions and compared to in vitro biofilm formation of prototypic pathogenic and laboratory strains. Our results revealed remarkable variation among the capacities of diverse *E. coli* isolates to form biofilms in vitro. Notably, we could not identify an association of increased biofilm formation in vitro with a specific strain collection that represented pathogenic *E. coli* strains. Instead, analysis of biofilm data revealed a significant dependence on growth medium composition ($P < 0.05$). Poor correlation between biofilm formation in the various media suggests that diverse *E. coli* isolates respond very differently to changing environmental conditions. The data demonstrate that prevalence and expression of three factors known to strongly promote biofilm formation in *E. coli* K-12 (F-like conjugative pili, aggregative adherence fimbriae, and curli) cannot adequately account for the increased biofilm formation of nondomesticated *E. coli* isolates in vitro. This study highlights the complexity of genetic and environmental effectors of the biofilm phenotype within the species *E. coli*.

There is growing appreciation that formation of bacterial surface communities is a process that contributes to pathogenicity of microorganisms (36). It has become a common working hypothesis that the persistence of bacterial biofilms in the human body is a major cause of recurrent or chronic infections (15). Parsek and Singh (37) proposed four basic criteria to define biofilm-associated infections: (i) Bacterial cell adherence to or association with a surface, (ii) in vivo observation of bacterial cell clusters, (iii) a localized infection pattern, and (iv) increased resistance to antibiotic treatment in the host compared to resistance of genetically equivalent planktonic bacteria. A role for bacterial biofilms in pathogenesis is well established for a number of infections and opportunistic pathogens; for many other infections a link between biofilms and disease has been proposed, but the evidence remains less clear (37).

E. coli is a genetically diverse species that causes diarrheal diseases and a variety of extraintestinal infections which fulfill many or all of the proposed criteria for biofilm-associated infections (19). As a nonpathogenic member of the large intestine in vertebrates, *E. coli* appears to reside within the mucus layer without colonizing the underlying epithelium (38). In contrast, diarrheagenic *E. coli* strains are defined and characterized by their ability to penetrate the mucus layer and efficiently colonize the mucosa (45). The adherence patterns of bacteria to epithelial HEp-2 cells in vitro are in good agreement with those found on mucosal surfaces in animal models and distinguish different pathotypes, such as diffusely adhering *E. coli*, enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAggEC) strains. Uropathogenic *E. coli* strains are frequently isolated from biofilms formed in the lumen of catheters, where they resist antibiotic treatment and shear forces (28). In addition, formation of bacterial associations within bladder epithelium is characteristic of experimental urinary tract infections (UTI) (20). Based on these in vivo and in vitro observations, it is reasonable to predict that the pathotypes causing these infections possess genetic repertoires that enable formation of stable cell-cell interactions and biofilms under appropriate environmental conditions. If true, the establishment of an in vitro biofilm system that reflects the in vivo biofilm formation of *E. coli* pathotypes would enable development of drugs directed against this virulence strategy.

E. coli has been an important gram-negative model organism for in vitro analysis of biofilm formation on abiotic surfaces (35, 48). Many cell surface components [such as flagella, type I fimbriae, outer membrane proteins, colanic acid, and poly(β-1,6-GlcNAc)] were found to contribute to biofilm formation of K-12 strains during static growth conditions. The existence of other pathways that promote biofilm formation is indicated by rare studies utilizing hydrodynamic environments, characterized by increased shear forces, where constitutive expression of

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curli fibers (41) or plasmid-encoded conjugative pili (14, 42) can overcome the absence of other biofilm-promoting factors in K-12 strains under these conditions. As a consequence of the exclusive focus on domesticated, nonpathogenic *E. coli* K-12 strains in most studies, we lack significant insights regarding the biofilm phenotypes of pathogenic *E. coli* isolates in these model systems as well as the underlying molecular mechanisms. Indeed, among the variety of characterized or putative surface components that are encoded by pathogenic *E. coli*, only expression of aggregating adherence fimbriae (AAF) of EAggEC has been shown to mediate the strong cell-cell adherence required for extensive biofilm formation in vitro (44).

In this communication, we report the characterization of the ability of *E. coli* isolates to form biofilms in vitro in two model systems under a variety of growth conditions. To facilitate significant conclusions, we used a large number of *E. coli* strains isolated from humans representing many different *E. coli* pathotypes and a large genetic reservoir. In addition, we evaluated an association of good biofilm formation of natural *E. coli* isolates in vitro with the presence of potent biofilm-promoting factors previously described in domesticated laboratory strains. We found that the ability of biofilm formation in vitro varies extensively among *E. coli* isolates and is dependent on the applied growth condition. However, good biofilm formation was not found to be associated with disease-associated isolates. The presence of the evaluated factors, which are known to promote biofilm in domesticated K-12 strains, was not sufficient to explain the observed variation among natural isolates, indicating the involvement of additional yet unknown determinants in the process of *E. coli* biofilm formation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Laboratory *E. coli* K-12 reference strain MG1655 (5) was obtained from F. Blattner (University of Wisconsin, Madison). MG1655*ompR234* (50) and MG1655*csgA* (40) were kindly provided by P. Lejeune (Institut National des Sciences Appliquées, Lyon, France). Plasmid R1*drd*19 has been described previously (42). Prototypic pathogenic strains EAggEC 042 (27), EPEC E2348/69 (23), enterotoxigenic *E. coli* H10407 (24), neonatal meningitis-associated *E. coli* RS218 (1), and uropathogenic *E. coli* strains 536 (3) and CFT073 (52) were obtained from J. Hacker (Universität Würzburg). A total of 331 *E. coli* isolates of human origin were provided by F. Scheutz, World Health Organization International Collaborative Reference Center for *Escherichia* and *Klebsiella* (Statens Serum Institut, Copenhagen, Denmark). Ninety strains had been isolated from blood of hospitalized patients during episodes of bacteremia at Hvidovre Hospital, Denmark (30, 31). Sixty-eight strains had been cultivated from urine samples of male patients suffering from febrile UTI at Sahlgrenska University Hospital, Sweden (46, 47). A total of 173 *E. coli* strains originated from stools of Danish children under 5 years of age (32). Of these, 68 strains were isolated as potential pathogens associated with diarrhea, and 105 strains were from the normal intestinal flora of healthy controls. T. Whittam (STEC Center, Michigan State University) provided the *E. coli* reference (ECOR) collection (29).

Growth conditions. Unless otherwise stated, bacterial strains were propagated in Luria-Bertani (LB) medium containing 5 g NaCl per liter (4). For tests in the static biofilm model, AB minimal medium (11) containing 2.5 mg ml^{-1} thiamine was supplemented with 0.5% glucose (ABTG) or 0.5% Casamino Acids (ABTCAA). Porcine mucus was isolated from the small intestine of a piglet as previously described (6). Phosphate-buffered saline (PBS) was used to rinse intestinal sections and as suspension buffer. For biofilm assays, mucus was diluted in PBS to a protein concentration of 1 mg ml^{-1} and supplemented with 25 mM HEPES (pH 7.5). Human urine was pooled from late-morning midstream samples of two adults. Artificial urine was prepared according to two protocols (18, 25). Expression of curli was visualized by growth of test strains on LB, ABTG, and ABTCAA media containing 15 g $liter^{-1}$ agar, 20 μg ml^{-1} Congo red (CR; Sigma), and 10 μg ml^{-1} Coomassie brilliant blue (Sigma) for 48 h at 37°C. *E. coli* isolates carrying R1*drd*19 were selected on ABTG agar plates containing 50 μg ml^{-1} kanamycin sulfate following incubation of the isolates with *E. coli* CSH26(R1*drd*19) on LB agar for 24 h at 37°C.

Static biofilm assay. For tests in the static biofilm model, overnight cultures of test strains were prepared in 96-well plates (V-bottom; BioSterilin) containing 100 μl LB medium per well (37°C, 200 rpm). After 20 h, a 96-pin replicator (Boekel Scientific) was applied to inoculate 96-well test plates containing 150 μl of relevant preheated medium. Test plates were transferred to large plastic bags to avoid evaporation of medium and incubated at 37°C for 48 h without shaking. Biofilm formation was assayed by staining of polystyrene-attached cells with crystal violet (CV) using a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Inc.) equipped with a wash tool. After removal of medium and two washes with 150 μl of 0.9% NaCl solution, surface-attached cells were covered with 160 μl of 0.1% CV for 15 min. Following two subsequent washes with 170 μl of 0.9% NaCl solution, surface-bound CV was extracted by addition of 180 μl of ethanol (96%). Absorbance measurements obtained at 590 nm (A_{590}) with a VICTOR^2 multilabel counter (Perkin-Elmer, Inc.) were corrected for blanks.

Multiplex PCR. The presence of conjugative transfer genes *traA* and *finO* in the test strains was evaluated by duplex PCR. Primer pairs ar062 (5′-CGGAA AACCATCATCAATGTCAC-3′) and ar063 (5′-TCCTCCTGAGAAATATGC TCCGT-3′) as well as ar064 (5′-TTAAGTGTTCAGGGTGCTTCTGC-3′) and ar065 (5′-ACTTGACGTTTTTGGTCATCATGTA-3′) were designed based on conserved regions within published *finO* and *traA* sequences of F-like conjugative plasmids and produce amplicons of 406 bp and 289 bp, respectively. Bacterial lysates were prepared by suspension of a single bacterial colony in 50 μl MilliQ water and incubation at 95°C for 10 min. For each test reaction, 1 μl of lysate was added to 14 μl of PCR mix. The reaction mixtures containing 1× PCR buffer (Gibco), 0.1 mM deoxynucleoside triphosphates, 0.33 μM of each oligonucleotide, and 0.4 U of *Taq* polymerase (Gibco) were incubated at 94°C for 2 min, followed by 40 cycles of 25 s at 94°C, 30 s at 50°C, 50 s (plus 1 s per cycle) at 72°C, and a final extension for 2 min at 72°C. Bacterial lysates were also used for detection of plasmid-borne genes of EAggEC, as previously described (9).

Flow chamber biofilms. Cultivation of biofilms in laminar flow was done at 37°C as previously described (42). Flow channels irrigated with AB minimal medium (11) supplemented with 0.01 mM Fe-EDTA, 1.0 mg ml^{-1} thiamine, and 0.02% Casamino Acids were inoculated with 250 μl of normalized dilutions (optical density at 600 nm of 0.05) of 16- to 20-h-old bacterial cultures. After biofilm maturation (72 h to 96 h), cells were stained by injection of 250 μl medium containing 1.67 μM of the nucleic acid stain SYTO9 (Molecular Probes) into the channel. Biofilm structures were monitored using a Zeiss LSM510 scanning confocal microscope.

Statistical analysis. Data obtained from the static biofilm assay were log transformed to stabilize the variance and to make the approximation to the normal distribution applicable. Each strain was analyzed in four different media, and hence these four observations are not independent. The correlation induced by this sampling scheme was included in the analysis of the effects of strain origin and test media on the log-transformed A_{590} values. The statistical analysis was performed in SAS version 8.2. Sigmaplot (version 9.0; SyStat Software, Inc.) was used to perform Student's *t* tests and to create graphics.

RESULTS

Biofilm formation under static conditions. A microdish biofilm model system was established to evaluate biofilm-forming abilities of a large number of nondomesticated *E. coli* organisms on a polystyrene surface in batch culture. Full automation of biofilm quantification after staining with crystal violet guaranteed that biofilms formed in different microdishes were subject to the identical shear forces generated during consecutive liquid handling steps, thereby assuring highly reproducible assay conditions. A total of 331 diverse *E. coli* strains of human origin as well as 6 well-characterized prototype pathogens were included in the study. Three K-12 strains served as reference strains: wild-type *E. coli* MG1655, a MG1655*ompR234* mutant causing increased curli expression, and MG1655 elaborating conjugative pili encoded by the IncFII plasmid R1*drd*19.

These strains were tested for biofilm formation under various growth conditions. LB medium and minimal medium sup-

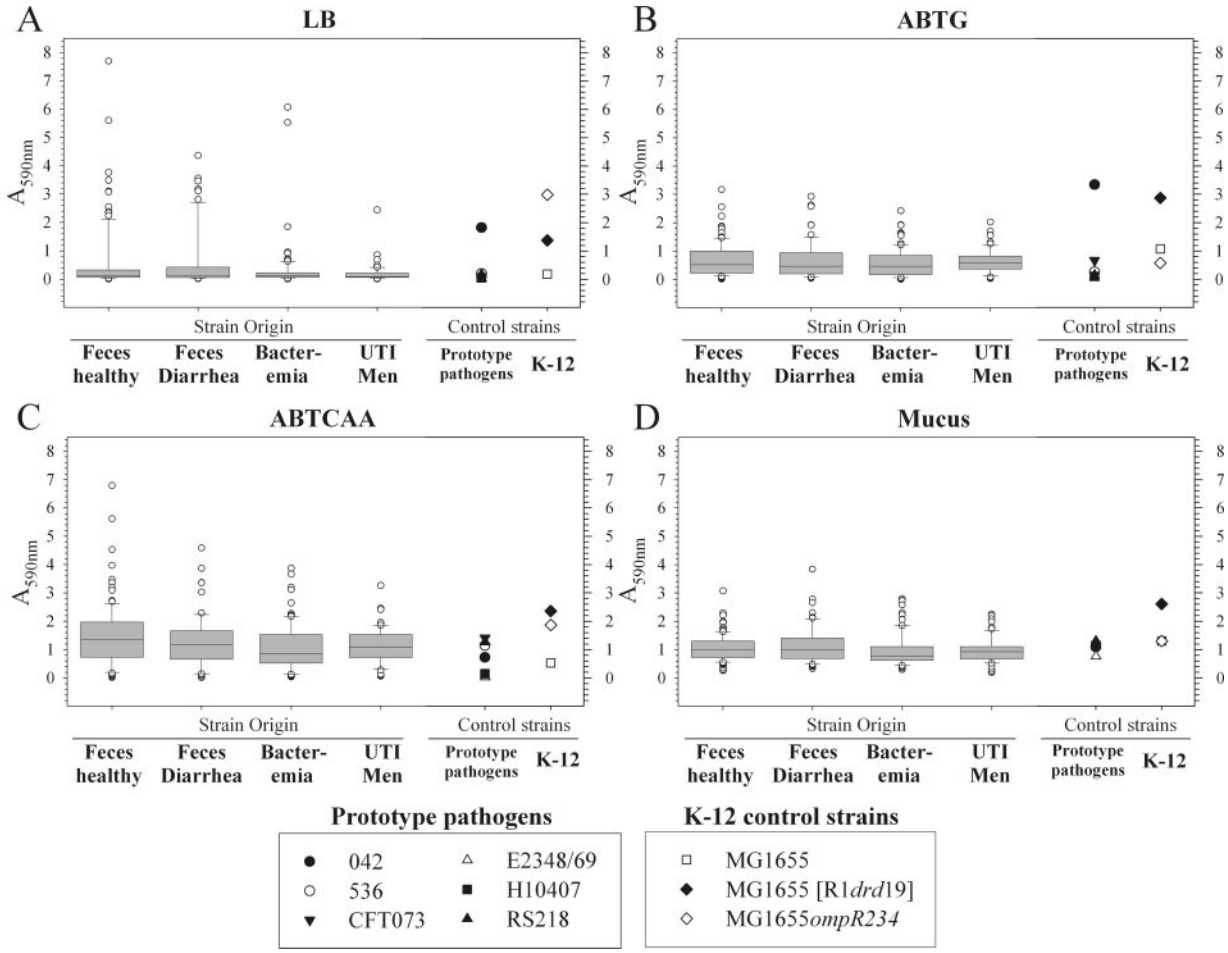


FIG. 1. Distribution of biofilm formation in static media. Diagrams illustrate A_{590} values obtained after dissolution of biofilm-bound CV for four *E. coli* strain collections of different origin and relevant control strains after growth in LB medium (A), ABTG (B), ABTCAA (C), and diluted mucus (D). Distribution of biofilm formation of each of the four strain collections is shown in a box and whisker format. Boxes range from the 25th to 75th percentile and are intersected by the median line. Whiskers extending below and above the box range from the 10th to the 90th percentile, respectively. Outliers are indicated as individual data points. Means of A_{590} values obtained for K-12 and prototypic pathogen control strains are shown.

plemented with glucose (ABTG) or with Casamino Acids (ABTCAA) served as standard laboratory media. To mirror conditions in the gastrointestinal and urinary tract, bacteria were cultivated in diluted porcine mucus and urine. Using a randomly chosen subset of test strains, we found that all strains reached similar cell densities after growth for 48 h in each of these media except for urine (data not shown). Since biofilm formation in urine was hardly detectable after 48 h and did not correlate with biofilm formation observed using artificial urine, the urine-derived data were excluded from analysis (data not shown). In a subsequent sample survey, we confirmed that absorption (A_{590}) of solutions obtained after dissolution of biofilm-bound CV was well correlated with the number of living cells obtained after mechanical harvest of biofilms in each of the four remaining media (data not shown). These results suggested that different growth rates of test strains and different abilities among test strain biofilms to bind CV would not bias the interpretation of the biofilm assay results.

(i) Variation in biofilm formation among natural *E. coli* isolates. For biofilm assays under static conditions, overnight cultures of all test strains were diluted in the relevant test medium and incubated at 37°C for 48 h. For an initial data analysis, the A_{590} values obtained after dissolution of biofilm-bound CV were grouped based on strain origin and growth medium (Fig. 1). In LB medium, the majority of isolates exhibited a poor ability to form biofilms (Fig. 1A). Distributions of A_{590} values obtained with the different strain collections after growth in minimal media and diluted mucus were less skewed compared to those grown in LB, since a larger number of strains was able to form significant biofilms (Fig. 1B to D). Similar to results in LB medium, biofilms formed by several isolates were stronger than that observed for any of the K-12 control strains. Interestingly, the pronounced effect of plasmid R1*drd*19 on MG1655 biofilm formation was discernible in all media tested. An *ompR234* mutation increased biofilm formation of MG1655 in LB and ABTCAA only, indicating reduced curli expression in ABTG and diluted mucus. In agreement with previous results, EAggEC 042 formed strong biofilms in LB and ABTG (44). Wild-type K-12 MG1655 and the other prototypic pathogens exhibited levels of biofilm formation

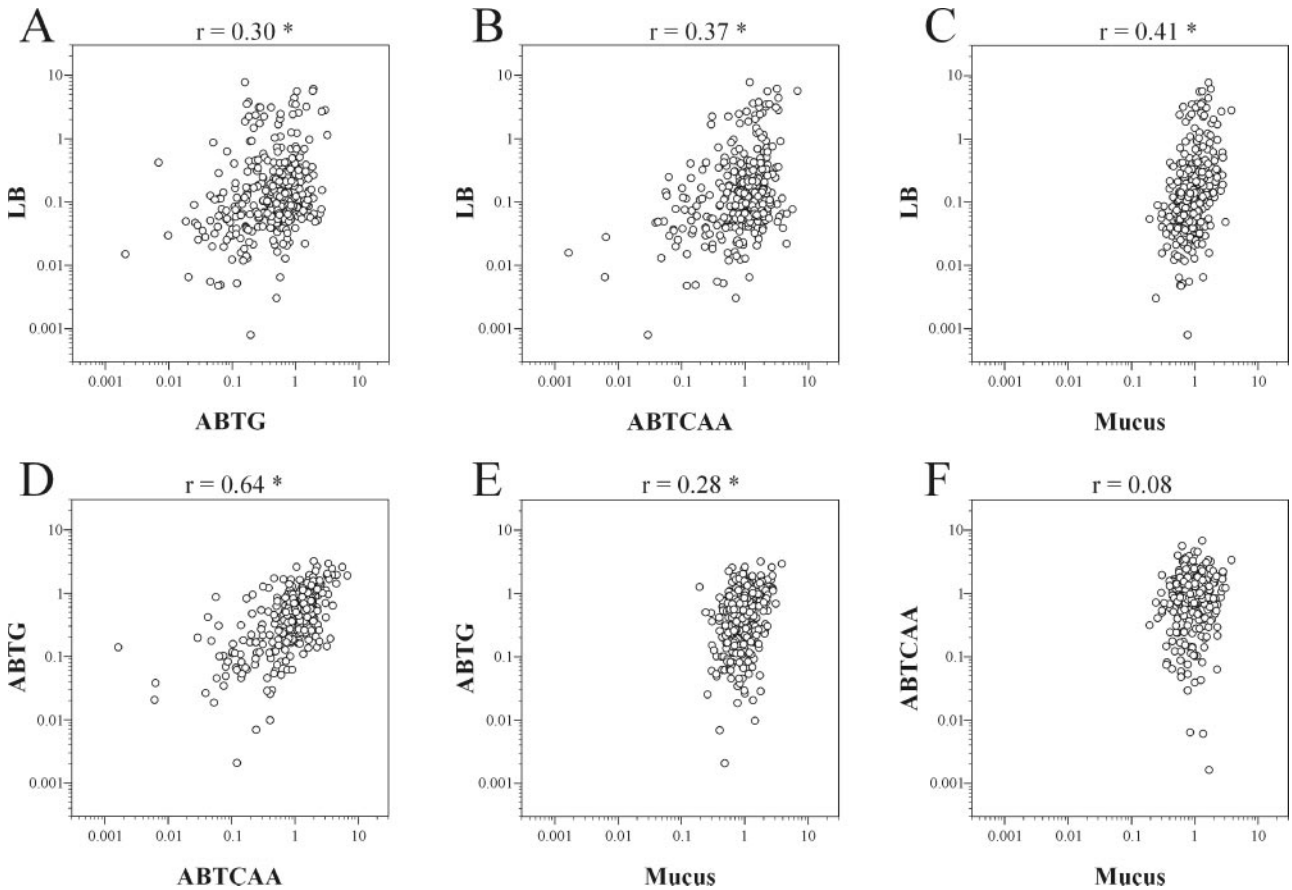
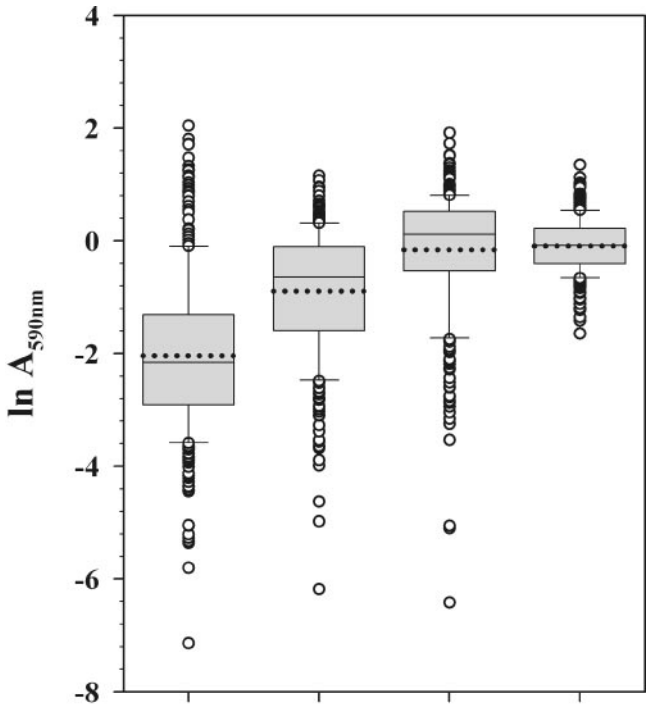


FIG. 2. Correlation of biofilm formation in different media. A_{590} values obtained after dissolution of biofilm-bound CV for all 331 *E. coli* isolates of the four strain collections in each medium are plotted against A_{590} values obtained in each of the other three media (LB versus ABTG [A], LB versus ABTCAA [B], LB versus mucus [C], ABTG versus ABTCAA [D], ABTG versus mucus [E], and ABTCAA versus mucus [F]). Both axes are log scaled. The calculated Pearson correlation coefficient r is indicated in each plot. Asterisks demonstrate significance of positive correlation ($P < 0.001$).

comparable to the median A_{590} values observed for the four strain collections indicated in Fig. 1. We concluded from this initial analysis that natural *E. coli* isolates exhibit a wide spectrum of biofilm-forming capabilities in each of the tested media.

(ii) Correlation of biofilm formation in different growth media. To determine whether strains that form strong biofilms in one medium also form strong biofilms in other media, we plotted the log-transformed A_{590} values of all *E. coli* isolates obtained in two different media against each other, followed by calculation of Pearson correlation coefficients ($r = 0$, no correlation; $r = 1$, perfect positive correlation) (Fig. 2). Biofilm formation in ABTG correlated well with biofilm formation in ABTCAA (Fig. 2D; $r = 0.64$). However, correlation between LB and each of the other media as well as between ABTG and diluted mucus was poor (Fig. 2A to C and E; $r = 0.28$ to 0.41). No significant correlation was found between biofilm formation in ABTCAA and diluted mucus (Fig. 2F; $r = 0.08$; $P = 0.124$). Similar results were obtained when the data of the strain collections of different origin were analyzed separately. Hence, due to the low level of correlation in most comparisons, biofilm formation of a given test strain in one medium does not enable prediction of biofilm formation of the same strain in a different medium.

(iii) Biofilm formation is not dependent on strain origin but on growth medium. Taking these correlation patterns into account, log-transformed A_{590} data of the 331 *E. coli* isolates was subjected to a two-way analysis of variance to reveal whether strain origin, growth medium, or an interaction of both factors affect biofilm formation. Neither an effect of strain origin nor an effect of a possible interaction of origin and medium on biofilm formation was significant ($P > 0.05$). Thus, biofilm formation of *E. coli* isolates under static conditions was solely dependent on growth medium ($P < 0.001$). Solution of the adjusted analysis of variance confirmed the picture seen in summary plots of biofilm data obtained in each of the four media (Fig. 3). Biofilm formation in ABTCAA and diluted mucus was not significantly different ($M_{\ln A} = -0.161$ for ABTCAA versus -0.089 for diluted mucus; $P = 0.25$) but was stronger than that in ABTG ($M_{\ln A} = -0.894$; $P < 0.001$) and LB medium ($M_{\ln A} = -2.038$; $P < 0.001$). Additionally, growth in ABTG led to increased biofilm formation of nondomesticated *E. coli* strains compared to growth in LB medium ($P < 0.001$). In summary, the four media stimulated biofilm forma-



Medium	LB	ABTG	ABTCAA	Mucus
$M_{\ln A_{590nm}}$	-2.038*	-0.894*	-0.161	-0.089
$SE_{\ln A_{590nm}}$	0.078	0.061	0.060	0.026
$M_{g, A_{590nm}}$	0.130	0.409	0.851	0.915

FIG. 3. Dependence of biofilm formation on growth medium composition. Distribution of log-transformed A_{590} values obtained after dissolution of biofilm-bound CV for all 331 *E. coli* isolates in four growth media is illustrated as a box-and-whisker diagram. Features of the box and whisker format are described in the legend Fig. 1. The mean value ($M_{\ln A}$) of each distribution is indicated by a dotted line in the box and is listed below the diagram together with the corresponding standard error. For easier comparison, $M_{\ln A}$ values were retransformed to the linear scale and resulted in the listed geometric means ($M_{g, A}$) of the original data. Asterisks indicate significant difference between the given $M_{\ln A}$ value and the $M_{\ln A}$ values obtained in all other growth media ($P < 0.05$). SE, standard error.

tion of the *E. coli* isolates in the order of LB < ABTG < ABTCAA ≈ mucus. Since strain origin had no significant impact on biofilm formation in vitro, we conclude that pathogenic *E. coli* strains do not exhibit better biofilm formation in vitro than nonpathogenic *E. coli* strains.

Prevalence and expression of known biofilm-promoting genes among *E. coli* isolates. The broad spectra of biofilm-forming capabilities exhibited by the *E. coli* isolates in the test media (Fig. 3) implied extensive diversity regarding carriage or expression of genes that enable increased biofilm formation. Since many test strains exhibited stronger biofilm formation than the laboratory K-12 reference strain MG1655 (Fig. 1), we predicted that carriage or expression of genes not present in MG1655 were contributing to the biofilm phenotype of those test strains. To evaluate this hypothesis, we tested the contribution of three factors which are known to improve biofilm formation in laboratory K-12 strains and biofilm formation of nondomesticated *E. coli* isolates: conjugative pili, aggregative adherence fimbriae, and curli.

(i) Carriage of F-like conjugative transfer systems is not associated with good biofilm formation. F-like conjugative plasmids comprise several incompatibility groups and are considered to be the predominant self-conjugative plasmid family in natural *E. coli* populations (8). Assembly of conjugative pili by F-like type IV secretion systems (T4SS) has been shown previously to exhibit a pronounced induction effect on biofilm formation of laboratory K-12 strains on glass surfaces (14, 42). Data collected in this study indicated that expression of conjugative pili from plasmid R1*drd*19 also contributed to increased biofilm formation of MG1655 on a polystyrene surface in all tested media (Fig. 1). However, plasmid R1*drd*19 is a regulatory mutant of the natural plasmid R1. In contrast to R1*drd*19, wild-type R1 harbors a functional fertility inhibition (fin) system that represses pilus synthesis efficiently in the majority of plasmid-carrying cells (21). Hence, plasmid R1 does not induce biofilm formation of *E. coli* K-12 in LB or minimal medium (14 and data not shown). At present, little is known about the distribution of functional fin systems among natural F-like plasmids and the environmental stimuli that enable escape from fin. Nonetheless, we reasoned that if natural F-like plasmids that are at least partially derepressed for pilus expression contribute significantly to biofilm formation of our test strains, then this effect would be discernible by a correlation of overall carriage of F-like T4SS with increased biofilm formation.

A multiplex PCR was established to evaluate the prevalence of F-like T4SS among the test strains. PCR primers were designed to specifically amplify homologs of two conserved genes, *traA* and *finO*, that normally flank the transfer operon necessary for conjugative pili expression. We evaluated the accuracy of the PCR screen by testing the ECOR collection ($n = 72$) followed by comparison of our results with a previous study that had scored the prevalence of F-like conjugative transfer genes among the ECOR strains by dot blot analysis (8). Among the 27 ECOR strains that gave a positive dot blot result for at least one of the three probed conjugative transfer genes *finO*, *traD*, or *traY* (8), 26 (96%) also allowed amplification of *traA*, *finO*, or both. Only ECOR7, which did not give a positive hybridization signal in the previous study, showed a positive result for *traA* and *finO* in our screen. Following this validation of the assay, we extended our PCR screen to all 331 *E. coli* isolates (Table 1).

TABLE 1. Prevalence of F-like conjugative transfer genes among *E. coli* isolate collections

Strain origin	No. of strains tested	No. of strains[a] with the following genes detected:			
		traA finO	*traA*	*finO*	None
Feces—healthy	105	39 (37)	4	5	57
Feces—diarrhea	68	28 (41)	3	14	23
Bacteremia	90	45 (50)	4	0	41
UTI in men	68	21 (31)	4	2	41
Total	331	133 (40)	15	21	162

[a] Percentages of strains relative to the number of tested strains are shown in parentheses.

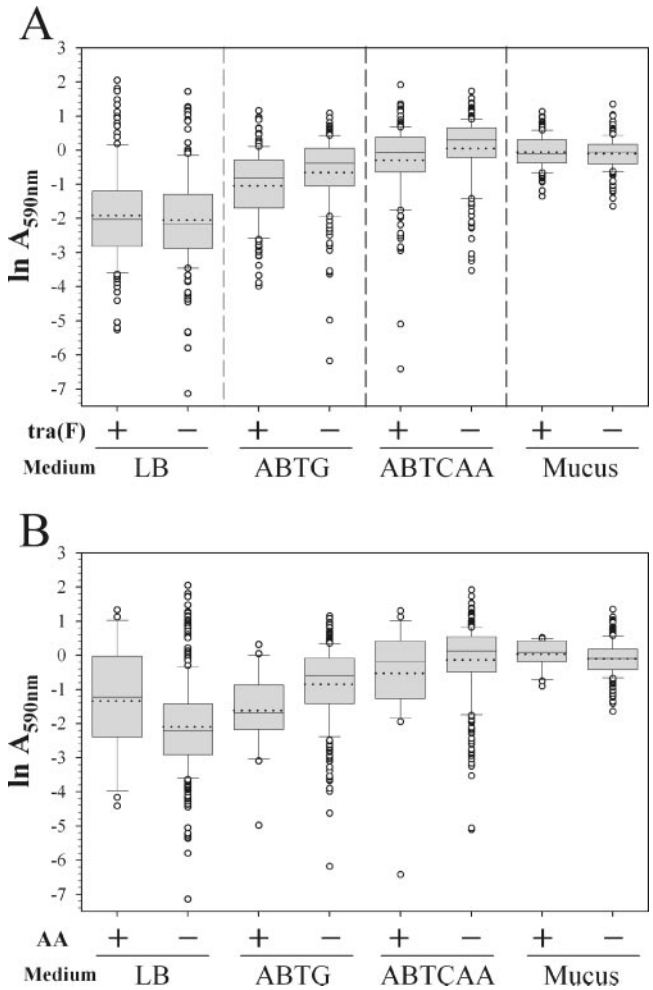


FIG. 4. Comparison of biofilm formation of strains that carry or lack genes specific for F-like conjugative plasmids or EAggEC. *E. coli* isolates were grouped based on positive PCR amplification of F-like *tra* genes *finO* and *traA* (A) or the EAggEC-specific gene probe AA (B). Distributions of log-transformed A_{590} values obtained after dissolution of biofilm-bound CV for these strain groups are illustrated as box-and-whisker diagrams. Features of the box-and-whisker format are described in the legend to Fig. 1. The mean value ($M_{\ln A}$) of each distribution is indicated by a dotted line in the box.

Amplification of *traA* and *finO* genes was observed for a total of 133 (40%) strains. Strains categorized as tra(F)$^+$ in accordance with these results were equally distributed among the four strain collections (χ^2 test, $P > 0.05$). To assess the contribution of these genes to biofilm formation of *E. coli* isolates, we compared the normalized A_{590} values obtained for the 133 tra(F)$^+$ strains with the biofilm data of the 162 tra(F)$^-$ strains, which did not support amplification of *traA* or *finO* (Fig. 4A). Carriage of genes encoding components of an F-like T4SS was not associated with increased biofilm formation in any growth medium ($P > 0.05$). Instead, tra(F)$^+$ strains formed less biofilm than the tra(F)$^-$ strains in ABTG ($M_{\ln} A_{590} = -1.041$ for tra(F)$^+$ versus -0.656 for tra(F)$^-$; $P = 0.002$) and ABTCAA ($M_{\ln} A_{590} = -0.290$ for tra(F)$^+$ versus 0.055 for tra(F)$^-$; $P = 0.005$).

Thus, this analysis did not support a role for F-like plasmids in biofilm formation of natural *E. coli* isolates. To rule out the possibility that the stimulatory effect of F-like conjugative pili on biofilm formation is restricted to a laboratory K-12 strain background, we transferred R1*drd*19 to 26 randomly chosen tra(F)$^-$ strains and tested biofilm formation in LB medium. Indeed, maintenance of the derepressed plasmid induced biofilm formation at least threefold in 18 (69%) of the strains ($P < 0.05$). Given that several natural virulence plasmids contain only remnants of an F-like T4SS (49), we could not exclude the possibility that several strains classified as tra(F)$^+$ in our screen actually lack a functional F-like T4SS. However, in the accompanying report we demonstrate that conjugative transfer can be readily detected for many tra(F)$^+$ strains (43). Thus, we conclude that the most logical explanation for the current observations is that a positive effect of F-like conjugative pili on biofilm formation is masked in many of the natural isolates due to limited expression of their structural genes, at least under some of the growth conditions. In the accompanying report (43), we further show that a substantial portion of the natural tra(F)$^+$ *E. coli* isolates promote superior biofilm formation in vitro when conditions permit bacterial conjugation. As previously proposed for *E. coli* K-12 (14), this enhancement may arise via increased conjugative pilus synthesis in transconjugants.

(ii) EAggEC strains exhibit increased biofilm formation in LB medium. Since outstanding biofilm formation of pathotypic EAggEC 042 in LB and ABTG media in the static model system (Fig. 1) confirmed previous results (44, 51), we expected that *E. coli* isolates belonging to this pathotype would be associated with the subgroup of isolates that exhibited exceptional biofilm formation under these conditions. In an earlier study, Cerna et al. (9) reported excellent correlation of the enteroaggregative adherence phenotype with PCR amplification of the commonly used AA gene probe and two other plasmid-borne genes, *aap* and *aggR*. Therefore, we chose to subject the 331 *E. coli* isolates to this established multiplex PCR screen. Among the 23 strains (7%) that gave positive PCR amplification of the genes *aap* and *aggR* or the AA probe, only those 22 strains positive for the AA probe were categorized as EAggEC (AA$^+$) (Table 2). As expected, most AA$^+$ strains (86%) were isolated from feces. Interestingly, 21 (95%) of these AA$^+$ strains also belonged to the tra(F)$^+$ strains. We compared the normalized A_{590} values obtained for the 22 AA$^+$ strains with data of the 309 AA$^-$ strains, which did not support AA probe amplification (Fig. 4B). In agreement with our ex-

TABLE 2. Prevalence of EAggEC-associated genes among *E. coli* isolates

Strain origin	No. of strains tested	No. of strains[a] with the following genes detected:			
		AA *aggR aap*	AA	*aap*	None
Feces—healthy	105	9 (8.6%)	1	1	94
Feces—diarrhea	68	7 (10.3%)	2	0	59
Bacteremia	90	3 (3.3%)	0	0	87
UTI in men	68	0 (0.0%)	0	0	68
Total	331	19 (5.7%)	3	1	308

[a] Percentages of strains relative to the number of tested strains are shown in parentheses.

pectation, AA^+ strains formed significantly more biofilm than the AA^- strains in LB medium ($M_{lnA} = -1.335$ for AA^+ strains versus -2.088 for AA^- strains; $P = 0.016$). In contrast, AA^+ strains formed less biofilm than AA^- strains in ABTG minimal medium ($M_{lnA} = -1.622$ for AA^+ strains versus -0.842 for AA^- strains; $P = 0.001$). No significant difference was found in ABTCAA and diluted mucus ($P > 0.05$). Thus, the occurrence of EAggEC can only partially explain exceptional biofilm formation among the test strains. The inconsistent results obtained in different media might reflect dependence of AAF expression on environmental conditions, as observed in a previous study (44), and imply that the majority of growth conditions used here were not permissive for AAF expression. It is also conceivable that strains positive in our PCR screen are missing components necessary to produce functional AAF.

(iii) Involvement of unknown biofilm-promoting factors is indicated. Unlike conjugative pili and AAF, curli fibers are produced from chromosomally located genes present in most *E. coli* isolates. Expression of curli is tightly regulated by various environmental signals (16, 33). In particular, low temperatures (<30°C) and low medium osmolarity favor curli expression and assembly. Genetic evidence suggests that strong curli expression, e.g., that caused by a point mutation in *ompR*, enables increased biofilm formation, but the level of curli expression required to produce this phenotype is unknown (50). To investigate the contribution of curli expression to biofilm formation of *E. coli* isolates observed in different growth media, we visualized curli expression of the test strains by a widely used Congo red (CR) binding assay after growth on LB, ABTG, and ABTCAA agar at 37°C for 48 h. In agreement with previous reports, curli overexpression from MG1655*ompR234* resulted in formation of intensely red colonies, whereas the negative control strains MG1655 and curli-deficient MG1655*csgA* formed rather pale colonies on all agar plates. In comparison, we observed a much broader color spectrum for colonies formed by the different test strains on the agar plates. Although 87 (26%), 71 (21%), and 62 (19%) of the 331 test strains gave more intensively stained colonies than wild-type MG1655 and MG1655*csgA* colonies on LB, ABTG, and ABTCAA agar, respectively, most of these isolates exhibited intermediate phenotypes in various red or brown hues (data not shown). Extensive variation of curli expression among strains is therefore likely, although the possibility that CR also binds to other surface appendages expressed by some test strains remains open. Given that this qualitative test did not identify a clear subset of strong curli producers, we were unable to draw an association between increased curli expression and increased biofilm formation. Instead, we combined the biofilm data of the strain group producing wild-type (MG1655) or lower levels of curli with the results of both multiplex PCR screens. We reasoned that *E. coli* isolates that exhibited exceptional biofilm formation and that were $tra(F)^-$ and AA^- and not indicative of curli expression would utilize additional, potentially novel genetic pathways for the elevated biofilm production. To determine the number of strains that fulfill these criteria, we focused our analysis on the three strain groups that produced the strongest biofilms (the upper 25% percentile included 88 strains) in either LB, ABTG, or ABTCAA. We found that the majority of the 88 top biofilm-forming strains in each medium were negative for curli overexpression in the same medium. Strikingly, of the 53 (64%), 57 (69%), and 63 (76%) strains that did not exhibit curli expression in LB, ABTG, and ABTCAA, respectively, as many as 24 (29%), 44 (53%), and 48 (58%) strains of these subsets were also $tra(F)^-$ and AA^-. Thus, we conclude that even if elaboration of curli, F-like conjugative pili, or AAF stimulates biofilm formation of some nondomesticated *E. coli* strains, the prevalence of these factors is not sufficient to explain exceptional biofilm formation for many strains. This suggests that additional, possibly new genetic pathways are involved.

Biofilm formation in a continuous flow model system. The flow chamber biofilm model system has been extensively used in biofilm research, because it allows nondestructive monitoring of biofilm development on glass surfaces by means of scanning confocal laser microscopy (SCLM) (10). Previous studies revealed that standard laboratory K-12 strains exhibit poor biofilm formation under these hydrodynamic conditions (14, 42). Since many nondomesticated *E. coli* isolates yielded more pronounced biofilms in the static biofilm system compared to the K-12 reference strain, we speculated that some of these isolates would also form strong biofilms when subjected to continuous flow conditions. Furthermore, we were interested to determine whether biofilms formed under these conditions would be characterized by similar biofilm architecture. Because biofilm architecture is thought to reflect the nature and strength of cell-cell and cell-surface interactions, diverse biofilm structure would indicate variation in the underlying molecular mechanisms.

Fifty-seven of the 256 *E. coli* isolates that had exhibited stronger biofilms than *E. coli* MG1655 in ABTCAA under static conditions were tested for biofilm formation in flow chambers irrigated with AB minimal medium supplemented with Casamino Acids. Biofilms were cultivated for 3 to 4 days prior to SCLM analysis. *E. coli* MG1655, MG1655*ompR234*, MG1655(R1*drd*19), and EAggEC 042 served as comparison strains. As expected, MG1655 hardly attached to the surface and only a few microcolonies were scattered over the surface (Fig. 5A). In agreement with previous observations (42, 44), MG1655*ompR234*, MG1655(R1*drd*19), and EAggEC 042 exhibited strong biofilm formation (Fig. 5B to D). Strikingly, we observed a potpourri of biofilm-forming capabilities and architectures among the natural *E. coli* isolates (Fig. 5). Phenotypes included very low levels of adherence (data not shown), single-cell layers, small microcolonies and loosely attached cell aggregates, large microcolonies of various shapes, and thick cell layers (Fig. 5E to L). Thus, in contrast to MG1655, several nondomesticated *E. coli* isolates express strong cell-cell and cell-glass interactions that resist the shear forces characteristic for this system.

DISCUSSION

The current perception of in vitro biofilm formation by the species *E. coli* has emerged from studies utilizing laboratory strains grown in standard laboratory media. In this report, we extend our understanding of the process into two previously neglected areas. (i) The contribution of genetic diversity among *E. coli* strains to biofilm formation in vitro was tested by investigating 331 nondomesticated human *E. coli* isolates of

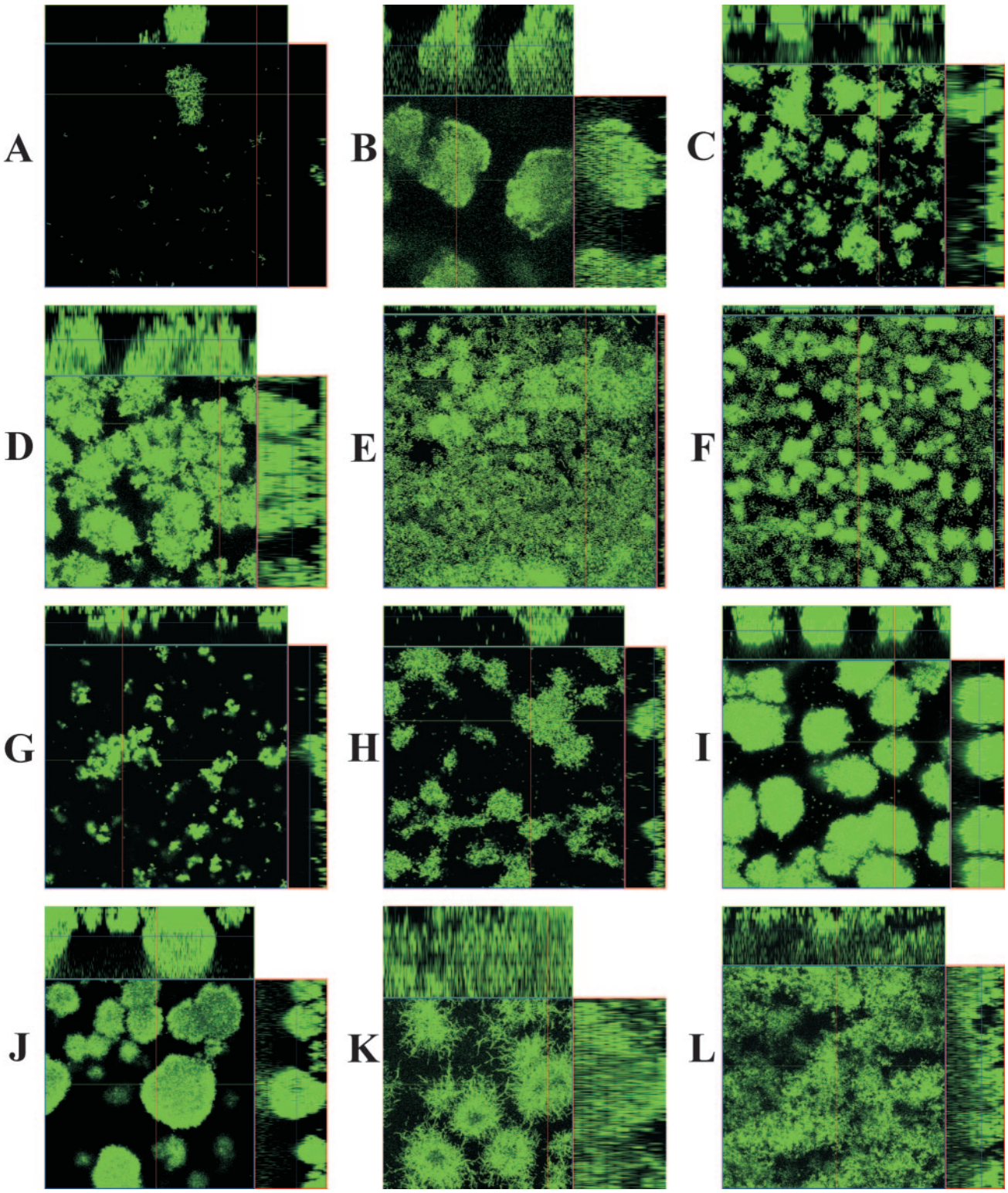


FIG. 5. Biofilm formation in a flow chamber model system. Micrographs represent horizontal sections of biofilms monitored by SCLM 72 h to 96 h after inoculation with the following strains: *E. coli* MG1655 (A), MG1655(R1*drd*19) (B), MG1655*ompR234* (C), EAggEC 042 (D), and *E. coli* isolated from humans suffering from diarrhea (I), bacteremia (E, G, H, and L), and UTI (F, J, and K). The horizontal sections correspond to a surface area spanning 230 μm by 230 μm and were collected at the substratum or within the biofilm as indicated by the blue lines in the vertical sections to the right and above. The positions of the vertical sections are indicated by the red lines in the horizontal sections.

various origin. Analysis of relevant reference and laboratory strains assured comparability with past and future studies. (ii) Application of four growth media and two biofilm model systems enabled us to evaluate the contribution of environmental conditions to biofilm formation of nondomesticated *E. coli* isolates. To our knowledge, the present study constitutes the most extensive comparative analysis of in vitro biofilm formation published thus far.

Our results revealed remarkable variation among the capacities of *E. coli* isolates to form biofilms in vitro. As expected, based on previous studies of in vitro biofilm formation of individual *E. coli* strains (12, 39, 44), growth medium composition had a significant impact on biofilm-forming capability. On average, growth in diluted mucus and ABTCAA led to increased biofilm formation compared to that in ABTG and LB medium. In agreement with a previous study on biofilm formation of *E. coli* O157:H7 on stainless steel (12), this tendency may suggest that adherence and/or biofilm formation of *E. coli* is increased under low-nutrient conditions and in media with lower osmolarity. However, we cannot exclude that adhesion of medium components such as amino acids or lipids to the abiotic surface may stimulate cell adherence. In addition, mucus-derived proteins may elicit bacterial cell agglutination as shown in prior studies using commensal and laboratory *E. coli* strains (7, 34).

Strikingly, we found poor correlation between biofilm formation in different media, suggesting that *E. coli* isolates respond very differently to the changing growth and environmental conditions. This finding emphasizes the relevance and difficulty involved in selecting proper conditions for in vitro biofilm studies which attempt to mirror natural environments in vivo. Studying biofilm formation of different strains in a single laboratory medium imposes a significant risk of obtaining biased results. The lack of correlation also supports the notion that reference strains cannot adequately reflect the biofilm-forming capabilities of other strains of the same species (13).

Notably, we could not identify an association of increased biofilm formation in vitro with a strain collection that represented pathogenic *E. coli* strains. Previous studies have reported enhanced biofilm formation by endocarditis isolates of *Enterococcus faecalis* as opposed to nonendocarditis isolates (26), whereas cystic fibrosis isolates of *Pseudomonas aeruginosa* did not display enhanced biofilm formation compared to other isolates (17, 22). Certainly, genotypic and/or phenotypic heterogeneity within the different strain collections may mask elevated biofilm formation by pathogenic subgroups that share specific virulence attributes in our study. However, additional analysis based on serotype and presence of known virulence factors in the 173 fecal *E. coli* isolates (32) did not reveal the presence of such a subgroup (A. Reisner, unpublished results). Thus, it appears that low and increased in vitro biofilm-forming capabilities are well distributed among the commensal and pathogenic *E. coli* isolates included in this study.

To gain insights into the mechanisms that contribute to exceptional biofilm formation of nondomesticated *E. coli* isolates, we focused our preliminary analysis on the distribution of those three factors known to profoundly induce biofilm formation in laboratory K-12 and EAggEC strains. On the population level, expression of curli and the presence of genes encoding F-like conjugative pili and AAF could not adequately explain increased biofilm formation in vitro. In light of our results in both biofilm model systems, we therefore predict that in natural *E. coli* isolates a plethora of biofilm-forming capabilities is mediated by many genetic pathways. These may well include factors already implicated from studies on *E. coli* K-12, such as flagella, type I fimbriae, Ag43, and exopolymeric substances [colanic acid and poly(β-1,6-GlcNAc)], but certainly also adhesions not present in the MG1655 genome. The possible interplay between these factors and their varying expression levels under different environmental conditions suggests that analysis of genetic factors contributing to biofilm formation of nondomesticated *E. coli* strains in vitro will need to be based on a case-by-case approach. In pursuit of this strategy, we have subjected eight *E. coli* isolates exhibiting different biofilm phenotypes in vitro to transposon mutagenesis and are currently analyzing mutants exhibiting an altered biofilm phenotype (A. Reisner, unpublished). The poor overlap between studies investigating the global gene expression patterns of *E. coli* K-12 biofilms formed in vitro indeed indicates that it will be difficult to find a common denominator (2).

Based on our current results, in vitro biofilm phenotypes cannot be correlated with the expected virulence phenotypes of the *E. coli* isolates in vivo. The tremendous impact of environmental conditions highlights the need to develop better biofilm model systems to approximate in vivo situations. Careful adjustment of the medium composition is an important first step. Incorporation of more adequate surfaces in the experimental design appears to be an additional measure, e.g., by studying biofilm formation directly on eukaryotic cells. However, given that multiple species are present in most environments, we also need to establish models that enable monitoring of possible antagonistic or synergistic interactions between community members (43).

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