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Determination of Adhesin Gene Sequences in, and Biofilm Formation by, O157 and Non-O157 Shiga Toxin-Producing *Escherichia coli* Strains Isolated from Different Sources[▽]

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Biofilm formation by Shiga toxin-producing *Escherichia coli* (STEC) has been associated with the expression of different adhesins (type 1 fimbria, curli, Ag43, Cah, and EhaA). In this study, biofilm formation and the presence of adhesin-related gene sequences were determined by PCR in 18 O157 strains and 33 non-O157 strains isolated from different sources (human, animal, food, and water). The expression of different adhesins was also assessed by reverse transcription-PCR (RT-PCR), Congo red agar plates, and mannose-sensitive hemagglutination (MSHA) assay. Biofilm formation occurred in 5/18 (28%) O157 STEC strains and 17/33 (51%) non-O157 STEC strains from different serotypes and sources, when the assays were performed at 28°C for 48 h. Among the non-O157 biofilm-producing isolates, 12/17 (71%) expressed type 1 fimbriae and 11/17 (65%) expressed curli and produced cellulose, while 8/17 (47%) were considered to be Ag43$^+$ by RT-PCR. Among O157 strains, a close correlation was observed between biofilm formation and expression of curli and cellulose. In non-O157 strains, it seems that, in addition to the presence of curli, the ability to form biofilm is associated with the presence of other factors such as type 1 fimbriae and autotransporter proteins, which may contribute to the persistence of these organisms in the environment.

Shiga toxin-producing *Escherichia coli* (STEC) is a foodborne pathogen that causes hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS). *E. coli* O157:H7 is the major STEC serotype involved in sporadic cases and outbreaks of HC and HUS worldwide (11). However, other serotypes, including serogroups O26, O103, O111, and O145, are also frequently isolated from severe illnesses (22). Ruminants, especially cattle, are considered the primary source of STEC (22), and contaminated undercooked beef has been most frequently implicated as a vehicle for STEC transmission (7). More recently, many O157:H7 outbreaks have also been associated with contaminated fresh vegetables, fruits, and sprouts (21, 25, 36).

Some previous studies showed that certain STEC O157:H7 strains have the abilities to attach, colonize, and form biofilm on food and other surfaces, and biofilm on various surfaces can serve as an important source and/or vehicle of contamination (6, 10, 17, 23, 29). Biofilm formation may also protect bacteria against adverse environmental conditions. The presence of O157 STEC in a diversity of food products also suggests that the expression of different types of adhesive structures may account for the ability of O157 to bind to several food surfaces. Indeed, some adhesins such as type 1 fimbriae (T1F), curli fimbriae, antigen 43 (Ag43), calcium-binding antigen 43 homologue (Cah), and autotransporter protein of enterohemorrhagic *E. coli* (EHEC) (EhaA) have been implicated in the formation of microcolonies and biofilms (4, 5, 24, 29, 31). In addition to curli, the production of cellulose, a major exopolysaccharide component of the biofilm matrix, has been shown to enhance bacterial adherence (5, 30). Despite this knowledge, there is little data in the literature concerning the ability of wild-type STEC strains belonging to different serotypes to form biofilms. Therefore, the aim of this study was to evaluate the capacity of biofilm formation in STEC strains isolated from different reservoirs and serotypes. The presence of adhesins associated with biofilm and the possibility of a link between biofilm formation and the expression of these adhesins were also examined.

MATERIALS AND METHODS

Bacterial strains. Fifty-one Shiga toxin-producing *Escherichia coli* (STEC) strains of different serotypes isolated from humans with infections ($n = 14$), animal reservoirs ($n = 35$), food ($n = 1$), and water ($n = 1$) samples belonging to the laboratory culture collection (1, 2) were studied (Tables 1 and 2). The strains were stored at −70°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) into which 15% glycerol was added after growth.

PCR assays. STEC strains were probed by PCR for the presence of *fimH* (type 1 fimbriae) (13), *csgA* (curli structural subunit) (20), *crl* (curli regulator gene) (20), *flu* (antigen 43) (12), *cah* (calcium-binding antigen 43 homologue) (26), *ehaA*$^\alpha$ (Eha passenger domain), and *ehaA*$^\beta$ (Eha translocation domain) (35) genes. PCR assays were also performed for the detection of *chuA*, *yjaA*, and TSPE4.C2 in order to determine the phylogenetic groups of the strains as described by Clermont et al. (3). Bacterial DNA to be amplified was released from whole organisms by boiling, and PCR was carried out in a total volume of 25 μl containing 3 μl of template DNA. The amplified DNA was visualized in 2% agarose gels stained with ethidium bromide, and a 100-bp ladder (Promega, Madison, WI) was used as the standard.

Detection of type 1 fimbriae, curli, and cellulose. Expression of type 1 fimbriae was examined by mannose-sensitive hemagglutination (MSHA) assay of guinea pig erythrocytes based on the method of Evans et al. (9) with some modifications. Bacterial suspensions (~3.0×10^8 cells/ml) in phosphate-buffered saline (PBS), prepared after bacterial growth in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI) at 37°C for 18 h, were mixed with guinea pig red blood cells at room temperature in the presence or absence of 1% D-mannose. *E. coli* DH5α and *E. coli* HB101 were used as positive and negative controls, respectively. The assay

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TABLE 1. Phenotypic and genotypic characteristics of non-O157 STEC strains

E. coli strain	Serotype	Origin[a]	PCR[b]						RT-PCR[c]			Fimbria expression		Cellulose[f]	Biofilm formation[g]				Phylogenetic group[h]
			fimH	*csgA* and *crl*	*flu*	*cah*	*ehaA*$^{\alpha}$	*ehaA*$^{\beta}$	Ag43	Cah	EhaA	Curli[d]	T1F[e]		48 h		7 days		
															28°C	37°C	28°C	37°C	
515/01	O22:H16	C	+	+	−	−	+	+	NT	NT	NT	−	+	+	+	+	+	+	B1
236/01	O77:H18	C	+	+	−	+	−	−	NT	−	NT	+	+	+	−	−	−	−	D
229/01	O79:H14	C	+	+	+	−	−	+	+	NT	NT	−	+	+	−	−	−	+	B1
240/02	O79:H14	C	+	+	+	−	+	+	+	NT	NT	−	+	+	−	−	−	−	D
243/01	O79:H14	C	+	+	+	−	−	+	+	NT	NT	−	+	+	+	+	−	−	B1
263/01	O87:H16	C	+	+	+	−	−	−	+	NT	NT	−	+	+	−	−	−	−	A
463/01	O91:H21	C	+	+	+	−	+	+	+	NT	+	+	+	+	+	+	+	+	B1
												−	+	−	−	−	−	−	
238/01	O98:H4	C	+	+	−	−	−	−	NT	NT	NT	−	+	−	+	+	+	+	B1
271/01	O98:H17	C	+	+	−	−	−	−	NT	NT	NT	+	+	+	+	+	+	+	B1
												−	+	−	−	−	−	−	
240/01	O105:H18	C	+	+	+	−	−	+	+	NT	NT	−	+	+	−	−	−	−	B1
473/01	O105:H18	C	−	+	+	−	+	+	+	NT	+	−	−	+	+	+	+	+	D
227/01	O113:H21	C	+	+	+	−	+	+	+	NT	NT	−	+	+	−	+	−	−	B1
258/01	O113:H21	C	+	+	+	−	+	+	+	NT	NT	−	+	−	−	−	−	−	B1
472/01	O113:H21	C	+	+	+	−	+	+	−	NT	NT	+	+	+	−	−	−	−	B1
												−	+	−	−	+	−	+	
444/01	O178:H19	C	+	+	+	−	+	+	−	NT	NT	−	+	+	−	−	−	−	B1
465/01	O178:H19	C	+	+	+	−	+	+	+	NT	NT	−	+	−	−	−	−	−	B1
239/01	O181:H4	C	+	+	−	−	−	−	NT	NT	NT	−	+	−	+	+	−	+	B1
455/01	O181:HNT	C	+	+	−	−	+	+	NT	NT	NT	−	+	−	−	−	−	−	B1
457/01	ONT:H16	C	+	+	−	−	+	+	NT	NT	NT	+	+	+	−	+	−	+	B1
281/01	ONT:H19	C	+	+	−	−	+	+	NT	NT	NT	−	+	−	−	−	−	−	B1
452/01	ONT:H19	C	+	+	−	−	+	+	NT	NT	NT	−	+	−	−	−	−	−	B1
495/01	ONT:H28	C	+	+	−	−	−	−	NT	NT	NT	+	+	+	+	−	+	−	D
252/01	ONT:H46	C	+	+	−	−	+	+	NT	NT	NT	−	+	−	+	−	−	−	B1
248/02	ONT:H49	C	+	+	+	−	+	+	+	NT	NT	+	+	+	+	+	+	−	B1
												−	+	−	−	−	−	−	
1137	O26:H11	HUS	+	+	+	+	+	+	+	−	NT	+	+	+	+	−	−	−	B1
												−	+	−	+	+	+	+	
100/00	O26:H11	D	+	+	+	+	+	+	+	−	NT	−	+	−	−	−	−	−	B1
234/85	O26:H11	D	+	+	+	+	+	+	+	−	−	+	−	+	+	+	+	+	B1
2567/81	O26:H11	D	+	+	+	+	+	+	+	−	NT	−	+	−	−	−	−	+	B1
36/01/87	O26:H11	D	+	+	+	+	+	+	+	−	NT	+	−	+	+	−	−	−	B1
												−	+	−	−	−	−	−	
429/84	O93:H19	D	+	+	+	−	+	+	−	NT	NT	+	+	+	+	−	+	−	B1
0651-1	O103:H2	D	+	+	+	−	+	+	+	NT	−	+	−	+	+	+	−	+	B1
												−	+	−	−	−	−	−	
495/00	O103:H2	D	+	+	+	−	+	+	−	NT	NT	+	−	+	+	−	−	−	B1
												−	−	−	+	+	+	+	
334/02	O93:H19	F	+	+	−	−	+	+	NT	−	NT	+	+	+	+	+	+	+	B1
												−	+	−	−	−	−	−	
Total no. of strains (%)	33		32 (97)	33 (100)	21 (64)	6 (18)	24 (73)	27 (82)	17 (51)	0	2	14 (42)	29 (88)	23 (69)	17 (51)	16 (48)	12 (36)	15 (45)	

[a] C, cattle; D, humans with diarrhea; F, food (ground meat); HUS, humans with hemolytic-uremic syndrome.
[b] No differences were observed between curli^{+} and curli^{-} variants among gene sequences detected by PCR.
[c] RT-PCR was performed only when the corresponding PCR result was positive. NT, not tested.
[d] Detection of curli on CFA agar plates containing Congo red dye is indicated as follows: +, red, curli-expressing colonies; −, white, non-curli-expressing colonies. Some strains presented both curli^{+} and curli^{-} colonies which were purified, and the two variants were tested separately.
[e] MSHA assay using guinea pig erythrocytes.
[f] Cellulose production after growth on Calcofluor plates is indicated as follows: +, fluorescent colonies; −, nonfluorescent colonies.
[g] In cases presenting curli^{+} and curli^{-} variants, the total number of biofilm-producing strains was considered when at least one of the variants was positive.
[h] Phylogenetic groups: A, *chuA* and TSPE4.C2 negative; B1, *chuA* negative and TSPE4.C2^{+}; D, *chuA*$^{+}$ and *yjaA* negative.

TABLE 2. Phenotypic and genotypic characteristics of O157 STEC strains

E. coli strain	Serotype	Origin[a]	PCR[b]						RT-PCR[c]			Fimbria expression		Cellulose[f]	Biofilm formation				Phylogenetic group[g]
															48 h		7 days		
			fimH	*csgA* and *crl*	*flu*	*cah*	*ehaA*$^{\alpha}$	*ehaA*$^{\beta}$	Ag43	Cah	EhaA	Curli[d]	T1F[e]		28°C	37°C	28°C	37°C	
581/1	O157:H7	C	+	+	−	+	+	+	NT	−	−	+	−	−	+	+	−	−	D
691/1	O157:H7	C	+	+	−	−	+	+	NT	−	−	+	−	+	+	+	+	−	D
1728/1	O157:H7	C	+	+	−	+	+	+	NT	−	−	+	−	+	+	+	+	−	D
1770/1	O157:H7	C	+	+	−	+	+	+	NT	−	NT	−	−	−	−	−	−	−	D
2004/1	O157:H7	C	+	+	−	+	−	+	NT	−	−	−	−	−	−	+	−	−	D
B1/1	O157:H7	C	+	+	−	+	+	+	NT	+	NT	−	−	−	−	−	−	−	D
B18/1	O157:H7	C	+	+	−	+	+	+	NT	−	NT	−	−	−	−	−	−	−	D
339/02	O157:H7	C	+	+	+	+	+	+	−	−	−	−	−	+	−	+	−	−	D
393/01	O157:H7	C	+	+	−	+	+	+	NT	−	NT	−	−	−	−	−	−	−	D
GC 148	O157:H7	C	+	+	−	+	+	+	NT	−	−	+	−	+	+	+	+	−	D
YB 20	O157:H7	C	+	+	−	+	+	+	NT	−	−	+	−	+	+	+	−	−	D
												−	−	+	−	−	−	−	
143/05	O157:H7	HUS	+	+	−	+	+	+	NT	−	NT	−	−	−	−	−	−	−	D
337/01	O157:H7	BD	+	+	−	+	+	+	NT	−	NT	−	−	+	−	−	−	−	D
385/01	O157:H7	BD	+	+	−	+	+	+	NT	−	NT	−	−	+	−	−	−	−	D
156/90	O157:H7	D	+	+	−	+	+	+	−	−	NT	−	−	−	−	−	−	−	D
255/03	O157:H7	BD	+	+	−	+	+	+	NT	−	NT	−	−	−	−	−	−	−	D
622/03	O157:H7	BD	+	+	−	+	+	+	NT	−	NT	−	−	+	−	−	−	−	A
102/97	O157:H7	W	+	+	−	+	+	+	NT	−	−	−	−	−	−	+	−	−	D
Total no. of strains (%)	18		18 (100)	18 (100)	1 (6)	17 (94)	17 (94)	18 (100)		1 (6)		5 (28)	0	8 (44)	5 (28)	8 (44)	3 (17)	0	

[a] C, cattle; D, humans with diarrhea; BD, humans with bloody diarrhea; HUS, humans with hemolytic-uremic syndrome; W, nontreated water.
[b] No differences were observed between curli^{+} and curli^{-} variants among gene sequences detected by PCR.
[c] RT-PCR was performed only when the corresponding PCR result was positive. NT, not tested.
[d] Detection of curli on CFA agar plates containing Congo red dye: +, red, curli-expressing colonies; −, white, non-curli-expressing colonies. Some strains presented both curli^{+} and curli^{-} colonies which were purified, and the two variants were tested separately.
[e] MSHA assay using guinea pig erythrocytes.
[f] Cellulose production after growth on Calcofluor plates was indicated as follows: +, fluorescent colonies; −, nonfluorescent colonies.
[g] Phylogenetic groups: A, *chuA* and TSPE4.C2 negative; D, *chuA*$^{+}$ and *yjaA* negative.

for curli expression was performed by the method of Kim and Kim (14). In brief, after growth in 3 ml of LB broth at 37°C for 18 h, bacterial strains were plated on colonization factor antigen (CFA) agar containing 40 mg/liter of Congo red (CR) (Sigma Chemical Co., St. Louis, MO) and incubated for 48 h at 28°C and for 24 h at 37°C. After these incubation periods, curli-expressing strains (curli$^+$) showed red colonies and non-curli-expressing (curli$^-$) strains displayed white colonies. Some STEC strains formed both curli$^+$ and curli$^-$ colonies on CR agar (from now on called phase-variant strains). The two variants were purified and used in the study as separate cultures. *E. coli* HB101 was used as a negative control. Evidence of cellulose production was obtained when fluorescent colonies were observed under a short-wavelength UV light lamp after growth on LB agar plates without salt but with 50 μM Calcofluor (CF; fluorescent brightener 28) (28). A *Salmonella enterica* serovar Typhimurium strain was used as a cellulose-positive control.

Detection of autotransporter proteins by RT-PCR. The expression of autotransporter proteins (Ag43, Cah, and EhaA) was indirectly detected by reverse transcription-PCR (RT-PCR). For this purpose, RNA extraction was performed with the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions after bacterial growth in LB broth for 18 h at 37°C. After extraction, approximately 1 μg of total RNA was digested with DNase I (Fermentas, Vilnius, Lithuania) for 30 min at 37°C, and the enzyme was then inactivated by adding 1 μl of 25 mM EDTA and heating the solution at 65°C for 10 min. To obtain cDNA, the ImpromII reverse transcription system kit (Promega, Madison, WI) was used according to the manufacturer's specifications. Primers for 16S ribosomal protein (18) were used to control the PCR, and the assay was then carried out using primers for Ag43, Cah, and EhaA.

Transmission electron microscopy (TEM). The presence of surface structures on selected STEC strains (two non-O157 strains, four non-O157 strains, and two O157 phase-variant strains) was determined as previously described (16). Strains grown in LB broth at 37°C for 18 h were subsequently grown on CR agar plates at 28°C for 48 h. The resulting colonies were scraped off the plates and transferred to microcentrifuge tubes containing PBS. After the cells were resuspended, the cell preparations were washed with PBS, negatively stained with 2% uranyl acetate, applied to Formvar carbon-coated 400-mesh nickel grids, dried, and examined in a LEO906E (Zeiss, Germany) electron microscope at 80 kV. For immunogold labeling (IGL), bacteria resuspended in PBS were directly applied to the grids, blocked with PBS containing 1% bovine serum albumin (BSA), and incubated with anti-curli antibody (1:100) prepared from *E. coli* E2348/69 (enteropathogenic *E. coli* prototype strain) for 1 h at room temperature. The preparations were then washed and incubated with 10-nm colloidal gold-labeled goat anti-rabbit serum (1:50) (Sigma Chemical Co.) for 1 h at room temperature. After the preparations were washed with PBS and distilled water, they were negatively stained with 2% uranyl acetate, dried, and examined in a LEO906E (Zeiss, Germany) electron microscope at 80 kV (16).

Biofilm formation assay. The assay for quantification of biofilm formation was performed in 96-well polystyrene microtiter plates (Corning Costar Corporation, Cambridge, MA) by the method of Uhlich et al. (34) with some modifications. Each test was performed in triplicate and in three separate experiments under the following conditions: at 28°C or 37°C for 48 h and at 28°C or 37°C for 7 days. The strains were grown in LB broth at 37°C for 18 h, and the cultures were diluted (1:10) in LB broth. After incubation of the microplates at the above temperatures and times, the plates were washed, fixed with methanol (Merck SA, Rio de Janeiro, Brazil), and stained with 0.1% crystal violet (Merck SA, Rio de Janeiro, Brazil). Absorbance at 540 nm was read after solubilization of the dye with 95% ethanol (Merck SA, Rio de Janeiro, Brazil) and in an enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystems Multiskan MS). Data for biofilm formation of all strains were compared with the data for the negative control (*E. coli* HB101) by Student's *t* test ($P < 0.05$). The visualization of biofilm formation was performed in individual plates (35 by 10 mm) (Corning Costar Corporation, Cambridge, MA) with selected strains (two non-O157 strains, two non-O157 phase variants, and two O157 phase variants). For this purpose, strains were grown in LB broth at 37°C for 18 h, cultures were diluted (1:10) in 1 ml of LB broth, and the preparation was kept at 28°C for different time periods (30 min and 2, 4, 6, 12, 36, and 48 h). At each time, bacterial cultures were discarded, and the plates were washed, fixed, and stained; adhesion was observed by light microscopy.

RESULTS

Genotypic and phenotypic identification of adhesins. Since STEC can carry gene sequences and express various adhesins that may be involved in biofilm formation (4, 14, 23, 26, 31, 32), we examined a collection of O157 and non-O157 STEC strains for their genotypic and phenotypic adhesin repertoire. The gene sequences related to curli (*csgA* and *crl*) were identified in all 33 non-O157 strains, and 14 (42%) of these isolates expressed curli as judged by characteristic red-colored colonies formed on CR agar plates after 48 h of incubation at 28°C (Table 1). All non-O157 curli$^+$ strains produced cellulose as confirmed by the Calcofluor (CF) binding assay, but production of cellulose was also identified in 9 (27%) non-O157 curli$^-$ isolates (Table 1). As previously mentioned, nine non-O157 strains formed both curli$^+$ and curli$^-$ colonies on CR agar plates (phase-variant strains), which were studied as separate cultures. As shown in Table 1, nonexpression of curli was accompanied by nonproduction of cellulose in all of these phase-variant isolates. The *fimH* sequence was observed in 32 (97%) of the non-O157 strains, and the expression of type 1 fimbriae was identified in 29 (88%) of the 33 isolates by MSHA assays (Table 1). The gene sequences related to the autotransporter proteins Ag43 (*flu*$^+$), Cah (*cah*$^+$), and EhaA (*ehaA*$^{\alpha+}$ *ehaA*$^{\beta+}$) occurred in 21 (64%), 6 (18%), and 24/27 (73%/82%) of the non-O157 strains, respectively. The RT-PCR results showed that 17 strains were Ag43$^+$, and assays for the *cah* gene showed that none of the 6 non-O157 strains *cah*$^+$ by PCR was considered positive (Cah$^+$) by RT-PCR (Table 1 and Fig. 1). Results for EhaA showed that only two of the four non-O157 strains, which showed a more pronounced biofilm production, were positive (EhaA$^+$) by RT-PCR (Table 1 and Fig. 1). As shown in Table 2, the presence of *csgA* and *crl* occurred in all O157 strains, but the expression of curli was observed in only five (28%) of these strains, which also produced cellulose, except for one isolate. Production of cellulose was also identified in four O157 curli$^-$ strains. The *fimH* gene was identified in all O157 isolates, but none of them expressed type 1 fimbriae. PCR assays showed that one (6%) of the 18 O157 strains was *flu*$^+$, and 17 (94%) were both *cah*$^+$ and *ehaA*$^+$ (Table 2). RT-PCR produced the following results: none of the O157 strains showed Ag43 under the conditions tested, only one isolate was *cah*$^+$, and none of the 8 biofilm-producing strains tested were positive for *ehaA* (Table 2 and Fig. 1). Pairs of

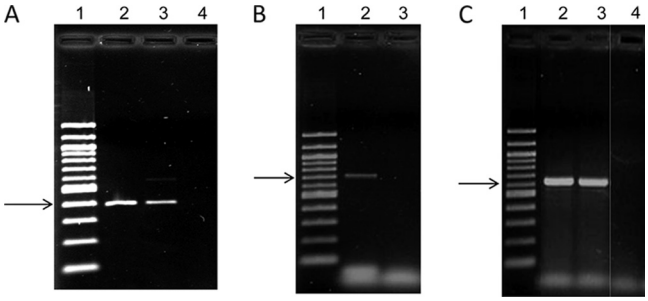

FIG. 1. Representative RT-PCR analysis for identification of Ag43 (A), Cah (B), and EhaA (C) autotransporter proteins from *E. coli* strains. Lanes 1 in panels A to C contained a 100-bp molecular size standard. (A) Lanes: 2, cDNA from strain 243/01 (O79:H14); 3, cDNA from strain 473/01 (O105:H18); 4, digested RNA from strain 243/01 (O79:H14). (B) Lanes: 2, cDNA from strain B1/1 (O157:H7); 3, digested RNA from strain B1/1. (C) Lanes: 2, cDNA from strain 463/01 (O91:H21); 3, cDNA from strain 473/01 (O105:H18); 4, digested RNA from strain 473/01 (O105:H18).

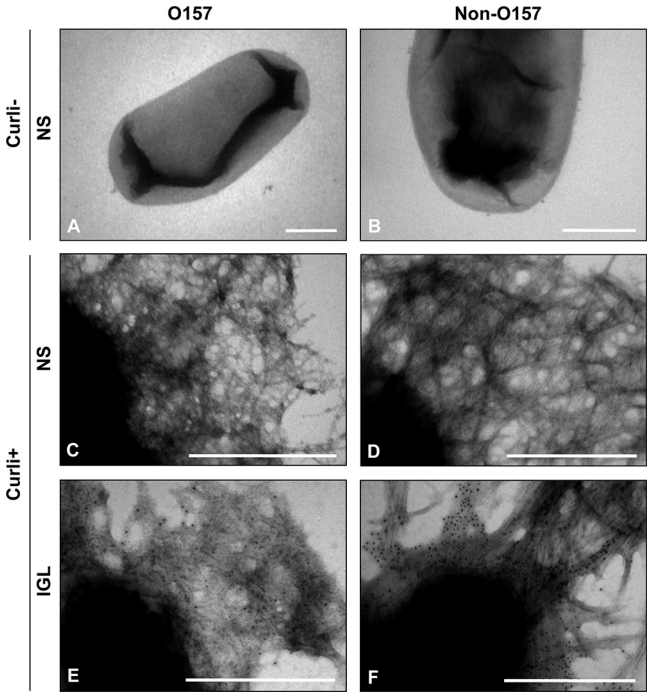

FIG. 2. Negative staining (NS) and immunogold labeling (IGL) of representative O157 and non-O157 STEC pair of strains previously detected as curli$^-$ and curli$^+$ after growth on CFA agar containing Congo red dye at 28°C for 48 h. (A to D) The absence of fimbrial structures in curli$^-$ variants (A and B) and the presence of hairlike fimbrial structures in curli$^+$ variants (C and D) can be observed. (E and F) IGL labeling using anticurli antibody and anti-rabbit antibody labeled with 10-nm colloidal gold confirm the presence of curli fimbriae. Bars, 0.5 μm.

phase-variant strains showed no difference in results when separately tested by PCR and RT-PCR.

TEM. The bacterial surface of the STEC strains studied was analyzed by TEM in two O157 strains and four non-O157 pairs of phase-variant strains detected on Congo red (CR) agar plates. The surfaces of curli$^-$ colonies did not display fimbrial structures (Fig. 2A and B). The presence of hairlike fimbrial structures similar to curli was observed only on the surfaces of previously detected curli$^+$ isolates (Fig. 2C and D). Immunogold labeling (IGL) with anticurli antibodies confirmed the identity of the fibers as curli (Fig. 2E and F).

Biofilm formation. To assess the ability of STEC strains to form biofilm, polystyrene microtiter plates were inoculated with diluted bacterial cultures and incubated under various conditions. As presented in Table 1, biofilm formation occurred in 17 (51%) and 16 (48%) of the non-O157 STEC strains after 48 h of incubation at 28°C and 37°C, respectively. Among the 17 non-O157 STEC strains able to form biofilm, the expression of type 1 fimbriae (MSHA) and coexpression of curli and cellulose (CR/CF) were observed in 12 (71%) and 11 (65%) strains, respectively, while 8 were considered Ag43$^+$. Among the four strains tested for *ehaA* by RT-PCR, which were able to form biofilm, two were positive for this autotransporter protein (Table 1). When the tests were performed at 28°C for 7 days, a reduction in the number of biofilm-producing strains was observed compared to assays performed at 28°C for 48 h (Table 1). Although no reduction in the number of biofilm-producing strains was identified at 37°C for 7 days (Table 1), a considerable reduction in absorbance values was found compared to the assays carried out for 48 h (data not shown). Not all curli$^+$ strains were able to form biofilm (Fig. 3A), but most of them were. Table 2 shows that biofilm formation occurred in 5 (28%) and 8 (44%) O157 STEC strains after incubation for 48 h at 28°C and 37°C, respectively. The ability to form biofilm at 28°C was identified only in curli-expressing O157 strains (Table 2 and Fig. 3B), and except for one isolate, all isolates also produced cellulose. A reduction in the number of strains capable of forming biofilm was observed when the tests were performed for 7 days at 28°C, and none of the strains formed biofilm after 7 days of incubation at 37°C (Table 2). Light microscopy showed that the tested non-O157 STEC strains adhered to the polystyrene plates after 2 h of incubation, and this adherence gradually increased until 48 h. Curli$^+$ colonies showed greater adherence than their respective curli$^-$ variants. Among the O157 phase-variant strains, only the curli$^+$ ones showed adherence (Fig. 3C).

Phylogenetic group. To seek a correlation between the ability to form biofilm and the genetic background of the STEC isolates, the phylogenetic group of the strains was determined by PCR. As shown in Table 1, most of the non-O157 STEC strains (85%) were classified as belonging to group B1, but one strain belonged to group A and four strains belonged to group D. In contrast, all O157 STEC strains were identified as belonging to phylogenetic group D, except for one strain which was characterized as belonging to group A (Table 2). Except for the two STEC strains classified as belonging to group A, which were unable to produce biofilm under the conditions tested, the ability to produce biofilm varied among the other isolates independent of their genetic background.

DISCUSSION

Studies on biofilm formation in STEC strains are mainly related to O157:H7 serotype, and there is little data on the ability of other STEC serotypes to form biofilm (4, 23, 29, 30, 34). The ability to form biofilm of several STEC strains belonging to different serotypes and isolated from humans with infections, food, water sample, and animal reservoir was described in the present study. Biofilm formation occurred in 28% (5 out of 18) and 51% (17 out of 33) of O157 and non-O157 STEC isolates, respectively, when the assays were performed after 48 h of incubation at 28°C. It should be mentioned that none of the human O157 strains studied produced biofilm, and this characteristic was identified only among O157 strains isolated from the animal reservoir and from a water sample. On the other hand, biofilm production was observed in several non-O157 STEC serotypes of human (6/8), animal (10/24), and food (1/1) origin. To the best of our knowledge, the ability to form biofilm in non-O157 STEC serotypes such as O26:H11 related to HC and HUS cases in several regions (22) has not been previously described.

A varied distribution of gene sequences related to fimbrial and afimbrial adhesins reported in the literature to be associated with biofilm formation was identified among the O157 and non-O157 STEC strains studied. Although the *fimH* gene was carried by most of the non-O157 strains (97%) and by all

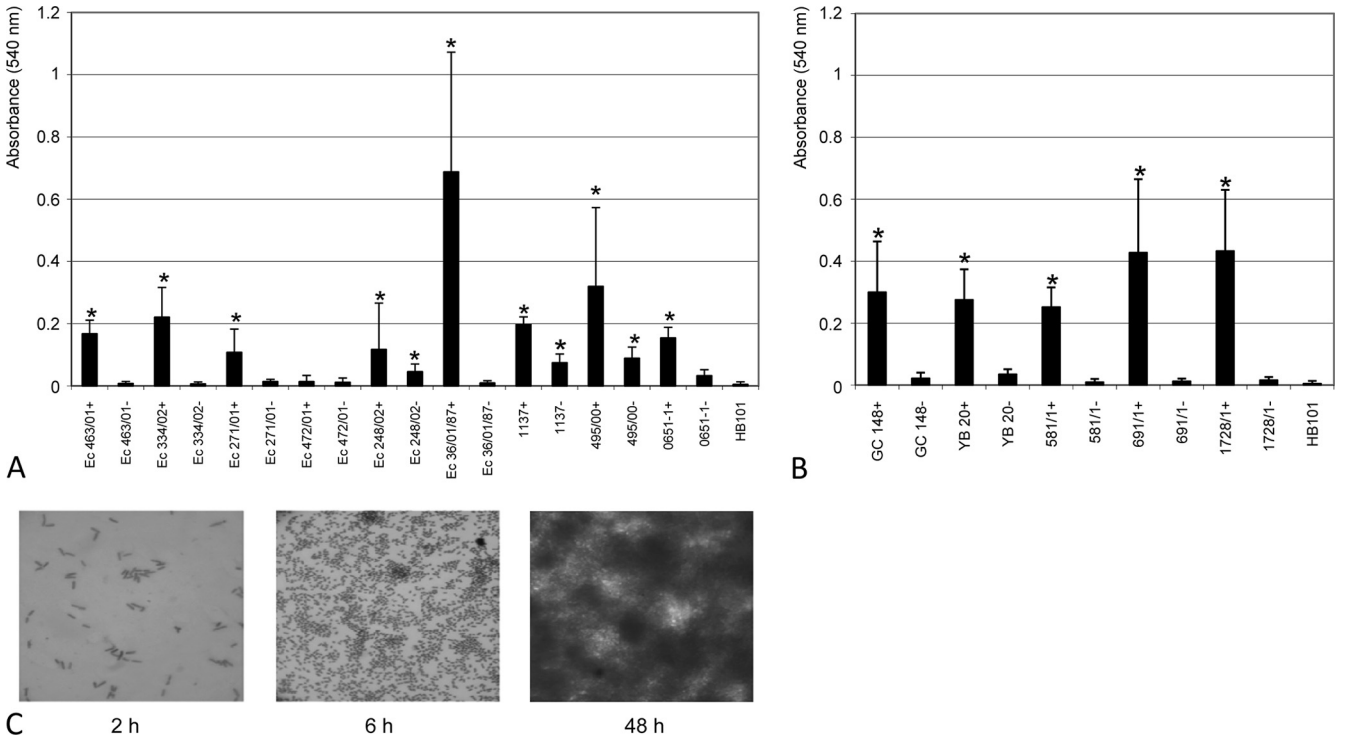

FIG. 3. Quantitative comparison of biofilm formation by pairs of curli$^+$ and curli$^-$ variants of non-O157 (A) and O157 (B) STEC strains in polystyrene plates after growth in LB broth at 28°C for 48 h. Biofilm assays were performed on pairs of curli$^+$ (+) and curli$^-$ (−) variants of *E. coli* strains as described in Materials and Methods, and each bar represents the mean absorbance value at 540 nm plus standard deviation (error bar) for three independent experiments. Values for the biofilm-forming strains that were significantly different ($P < 0.05$, Student's *t* test) from the values for negative-control *E. coli* HB101 are indicated by an asterisk. (C) Light microscopy of adherence of a curli$^+$ O157 STEC strain to polystyrene plates at 28°C (×1,000). Adherence starts after 2 h of incubation and gradually increases until 48 h.

O157 strains, the expression of type 1 fimbriae was identified in 88% of non-O157 strains, but in none of the O157 isolates. These data are in agreement with previously reported findings of the absence of type 1 fimbria expression among O157 STEC strains (4, 8). It has been shown that O157 strains lack the ability to express type 1 fimbriae due to a deletion of 16-bp fragment within the regulatory region that controls its expression (19), which suggests that in O157 STEC strains, the *fimH* gene is not involved in biofilm formation. The curli gene sequences, *csgA* and *crl*, were carried by all STEC strains studied here, independent of their serotype. However, the expression of these fimbriae occurred at a higher frequency among non-O157 strains compared to O157 isolates (42% versus 28%), and were identified in the latter only among those of animal origin. In contrast, curli expression was not identified among the O157 STEC strains studied by Cookson et al. (4) and occurred in 5 of 13 (38%) non-O157 STEC serotypes. Studies suggested that curli expression is uncommon in STEC O157:H7 but can occur in human strains in a temperature-independent phase-variant manner in association with *csg* promoter point mutations and with enhanced metabolic flexibility (33). In comparison, in the present study, the *flu*, *cah*, *ehaA*$^\alpha$, and *ehaA*$^\beta$ genes were detected in 64%, 18%, 73%, and 82% of the non-O157 STEC strains and in 6%, 94%, 94%, and 100% of the O157 strains, respectively. Ag43 has been reported in *E. coli* strains, including many pathogenic strains (15, 27), and Cah was identified in two O157:H7 strains (32). However, there are no studies on the frequency of these adhesins among a large group of STEC isolates. It is interesting that all *cah*$^+$ non-O157 STEC strains identified in the current study were also positive for the intimin (*eae*) gene (data not shown) and belonged to the O26:H11 serotype, except for one strain that belonged to the O77:H18 serotype (Table 1). *ehaA* genes were highly prevalent among the O157 and non-O157 STEC strains studied, in agreement with a previous report (35). In addition, more STEC strains showed a positive result for the *ehaA*$^\beta$ gene than for the corresponding *ehaA*$^\alpha$ gene, reinforcing a previous suggestion that a nucleotide sequence divergence occurred within the passenger-encoding domain of EhaA (35). In order to determine whether Ag43, Cah, and EhaA were expressed by the STEC strains under the conditions used, we analyzed their transcription by RT-PCR. All the O157 strains tested were negative for *flu*, *cah*, and *ehaA* by RT-PCR except for one *cah*$^+$ strain, showing that most of these genes were not transcribed during O157 growth in LB broth. Moreover, *cah* transcription was also not detected among the non-O157 STEC strains. These results are in accordance with those reported by Torres et al. (32), who found that the expression of Cah in the O157 EDL933 and 86-24 strains was not induced in LB broth. On the other hand, it is interesting that in this study *flu* and *ehaA* genes were transcribed by 81% (17 of 24 strains) and 2 of the 4 non-O157 strains tested, respectively.

Although some adhesins have been shown to enhance biofilm formation by *E. coli* O157 (35), it is clear from the reports

published so far that there are multiple genes in *E. coli* O157 and probably in other STEC serotypes that contribute to biofilm growth. In addition, the data described here suggest that none of the separate adhesin genes can explain the ability of different STEC strains to form biofilm. Moreover, one should be aware that the expression of these genes is, in general, related to particular culture conditions that may vary among different strains even within the same serotype. The culture conditions used in the present study (14) allowed the expression of curli by several O157 and non-O157 STEC strains, as well as the expression of type 1 fimbriae by most non-O157 isolates. It is noteworthy that a close correlation between biofilm formation and coexpression of curli and cellulose was identified among the O157 STEC strains, including all five phase-variant pairs of strains analyzed except for one pair of strains. Uhlich et al. (34), who studied only one O157 phase-variant pair of strains, also demonstrated that only the curli$^+$ variant produced biofilm, but no cellulose production was observed. Cookson et al. (4) also suggested that the elaboration of curli may enhance biofilm formation, despite the fact that this characteristic has not been found among the O157 STEC isolates included in their study. In relation to the non-O157 biofilm-producing strains studied here, a significant relationship between biofilm formation and coexpression of curli and cellulose was also identified. However, the expression of type 1 fimbriae alone or associated with cellulose may also be related to biofilm-forming ability in the curli$^-$ non-O157 isolates. Indeed, this was particularly true for the biofilm-producing *E. coli* strains 238/01 and 239/01, which expressed only type 1 fimbriae. On the other hand, it is interesting that the *E. coli* 473/01 strain produced a dense biofilm and cellulose and expressed two autotransporter proteins (Ag43 and EhaA), but it was negative for curli, Cah, and type 1 fimbriae. One can suggest that in this particular strain, biofilm formation may be related to cellulose, Ag43, and EhaA; however, further studies are necessary to confirm this hypothesis.

In conclusion, this study showed that the ability to adhere to abiotic surfaces forming biofilms, under defined culture conditions, is present in an array of wild-type O157 and non-O157 STEC strains, and not restricted to a particular set of serotypes. Although the experiments used here to study biofilm formation may not exactly reflect the natural environmental conditions experienced by STEC strains during biofilm formation in the food-processing industry, this ability may influence the persistence of these strains in the environment and the routes of transmission along the food chain. Knowledge of the bacterial structures involved in biofilm formation by STEC strains may contribute to our understanding of their adherence mechanisms, as well as the establishment of important control measures.

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