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Potentially Pathogenic *Escherichia coli* Can Form a Biofilm under Conditions Relevant to the Food Production Chain

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The biofilm-producing abilities of potentially human-pathogenic serotypes of *Escherichia coli* from the ovine reservoir were studied at different temperatures and on different surfaces. A possible influence of the hydrophobicity of the bacterial cells, as well as the presence of two virulence factors, the Shiga toxin-encoding (Stx) bacteriophage and the *eae* gene, was also studied. A total of 99 *E. coli* isolates of serotypes O26:H11, O103:H2, and O103:H25 isolated from sheep feces were included. The results show that isolates of all three *E. coli* serotypes investigated can produce biofilm on stainless steel, glass, and polystyrene at 12, 20, and 37°C. There was a good general correlation between the results obtained on the different surfaces. *E. coli* O103:H2 isolates produced much more biofilm than those of the other two serotypes at all three temperatures. In addition, isolates of serotype O26:H11 produced more biofilm than those of O103:H25 at 37°C. The hydrophobicity of the isolates varied between serotypes and was also influenced by temperature. The results strongly indicated that hydrophobicity influenced the attachment of the bacteria rather than their ability to form biofilm once attached. Isolates with the *eae* gene produced less biofilm at 37°C than isolates without this gene. The presence of a Stx bacteriophage did not influence biofilm production. In conclusion, our results show that potentially human-pathogenic *E. coli* from the ovine reservoir can form biofilm on various surfaces and at several temperatures relevant for food production and handling.

Diarrheagenic strains of *Escherichia coli* are the most common bacterial pathogens implicated in diarrhea worldwide, causing disease ranging from mild diarrhea to hemorrhagic colitis. The life-threatening hemolytic-uremic syndrome (HUS) is caused by Shiga toxin-producing *E. coli* (STEC) (1). Two main virulence factors of STEC are the production of Shiga toxins (Stx) and the attaching and effacing mechanism of the locus of enterocyte effacement (LEE), which includes the *eae* gene encoding the outer membrane cell adhesion-mediating protein intimin (2). Sequence and antigenic polymorphisms in the C terminus of intimin are used to define a number of distinct intimin types which appear to influence the site of human intestinal mucosal colonization (3–6). STEC can harbor one or more *stx* genes, which are encoded by inducible lambda phages integrated into their genomes, and the entire phage and specific regions within the phage can be gained or lost through horizontal gene transfer (7).

STEC O157 is the serogroup most commonly identified in human outbreaks. However, six non-O157 STEC serogroups, namely, O26, O45, O103, O111, O121, and O145, have also attracted significant attention because they have been increasingly associated with serious outbreaks. It has been reported that these six STEC serogroups, often referred to as "the big six," were responsible for approximately 70% of all non-O157 STEC infections from 1983 to 2002 in the United States (8). In particular, serotype O26:H11 has been regarded as one of the most severe non-O157 STEC due to its enhanced virulence and ability to cause diarrhea and HUS (9).

The reported annual incidence rate of STEC infections in humans in Norway has varied between 0 and 9.9 per 100,000 inhabitants for the last 20 years. STEC O157 has been the serogroup most frequently isolated from reported cases, followed by STEC O103, STEC O26 and STEC O145 (10). Nationwide STEC outbreaks caused by STEC serotypes O157:H7, O103:H25, and O103:H2 have been registered in Norway. The most severe outbreak was in 2006, the outbreak strain being *E. coli* O103:H25, with genes encoding both Stx2 and intimin (11). Sheep were implicated as the original reservoir for the outbreak strain, as fermented mutton sausages were shown to be the outbreak food source. It was also suggested that strains from the reservoir possibly could have contaminated the meat handling facilities and that a subsequent cross contamination of food products was the cause of the outbreak (12).

Food-borne microorganisms can attach to and form biofilm on various food contact surfaces (13). Bacterial biofilms are defined as microbial sessile communities that are attached to a substance, to an interface, or to each other (14). In the biofilm, the cells are embedded in a self-produced matrix and protected against environmental stress, e.g., disinfectants (14–17). A correlation between biofilm-forming abilities and persistence in food and feed-producing facilities has been reported for *Salmonella enterica* (18) and *Listeria monocytogenes* (19), and it has been suggested that biofilms in food-producing environments may serve as reservoirs for bacteria that may contaminate the food products. Furthermore, we have previously shown that dissemination of virulence genes like *stx* can occur in *E. coli* biofilm (20). Consequently, biofilms can also be environments for the evolution of new pathogenic *E. coli* isolates.

Most studies on the biofilm-forming potentials of *E. coli* have

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been performed on the laboratory strain K-12 and on isolates of the serogroup O157. Little is known about the biofilm-forming abilities of other *E. coli* serogroups. The aim of the present study was to investigate the biofilm-producing abilities of potentially human-pathogenic serotypes of *E. coli* from the ruminant reservoir at different temperatures and on different surfaces relevant for food production and handling. The serotypes included were O103:H2, O103:H25, and O26:H11. A possible influence on biofilm production of the hydrophobicity of the bacterial cells, as well as the presence of the two virulence genes *stx* and *eae*, was also studied.

MATERIALS AND METHODS

Bacterial isolates. The *E. coli* isolates were originally collected during a national survey of *E. coli* in sheep in Norway during the years 2006 and 2007. First, a total of 511 isolates (142 *E. coli* O26 and 369 *E. coli* O103) was selected from this survey and investigated for *eae*, *stx*, and H type, as previously described (21, 22). Among these isolates, 99 isolates of serotypes O26:H11 ($n = 25$), O103:H2 ($n = 42$), and O103:H25 ($n = 32$) were semirandomly selected for the present study, making sure that isolates from as many counties as possible were included within each serotype. The *E. coli* O26:H11 and O103:H2 isolates came from 13 counties, and O103:25 isolates from 10 counties. In the national survey, all *E. coli* O26:H11 and O103:H25 isolates were *eae* positive, whereas both *eae*-positive and *eae*-negative *E. coli* O103:H2 isolates were identified. This was also reflected in our study, which included 23 *eae*-negative and 19 *eae*-positive isolates. Due to the very low number of *stx*-positive isolates in the national survey, we chose to include only *stx*-negative isolates in order to have properly sized research groups in our study. The importance of *stx* in biofilm production was studied by constructing *stx*-positive strains by incorporation of a Stx bacteriophage.

All strains were stored at −80°C in brain heart infusion broth (BHI; Difco, BD, Franklin Lakes, NJ, USA) supplemented with 15% glycerin (Merck KGaA, Darmstadt, Germany) and recovered on blood agar (sheep blood) at 37.0 ± 1.0°C overnight. The bacterial cultures were then transferred into Luria-Bertani broth (LB; Merck) and incubated statically overnight at 37.0 ± 1.0°C (mean ± standard deviation [SD]). LB without NaCl (LB^{wo}/NaCl; Bacto-tryptone 10 g/liter, yeast extract 5 g/liter) was used as the test broth in the biofilm assays.

Genotyping. Pulsed-field gel electrophoresis (PFGE) was carried out using XbaI (Sigma-Aldrich, St. Louis, MO, USA) restriction endonuclease as described by Sekse et al. (21). Subtyping of *eae* for detection of *eae*β and *eae*ε was previously described by Zhang and coworkers (23), and subtyping of *eae*γ2/θ was performed as described by Blanco et al. (24), with a change in annealing temperature to 52°C.

Construction of *stx*-positive *E. coli* isolates. The Stx2-encoding bacteriophage Φ731 (Δstx_2::*cat*) (hereinafter called Φ731) was inserted into the chromosome of *E. coli* isolates and confirmed to be stable and functioning, as described previously (20). The original bacteriophage was carried by an *E. coli* O103:H25 strain from a Norwegian HUS patient (25).

Biofilm production on polystyrene (microtiter plate assay). Biofilm production on polystyrene was measured in the microtiter plate assay and performed as described previously (18) using 96-well Nunc microtiter plates with Nunclon (Nunc A/S, Roskilde, Denmark). In short, 30-μl amounts of an overnight culture were added to 100 μl LB^{wo}/NaCl in the wells of the microtiter plate (in three parallel assays). The microtiter plates were incubated statically for 2 days at 20.0 ± 1.0°C and 37.0 ± 1.0°C and for 7 days at 12.0 ± 1.0°C. After incubation, the wells were washed with tap water, stained with 1% crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature, and washed at least three times to remove excess dye. The remaining dye was dissolved in ethanol-acetone (70:30, vol/vol) for 10 min at room temperature, and the optical density at 595 nm (OD_{595}) (Multiscan MS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was measured. For each strain, the result was calculated by subtracting the median OD_{595} of the three parallel assays of the control (test broth only) from the median OD_{595} of the three parallel assays of the sample.

Biofilm production on glass slides and stainless steel coupons. Overnight cultures were inoculated (1 ml) into sterile centrifuge tubes (Greiner bio-one GmbH, Frickenhausen, Germany) containing 9 ml LB^{wo}/NaCl. An autoclaved microscope slide (76 by 26 mm; Menzel GmbH + CoKG, Braunschweig, Germany) or an autoclaved stainless steel (AISI304, 2B) coupon of approximately the same size was placed in each tube. The tubes were incubated at 20.0 ± 1.0°C or 37.0 ± 1.0°C for 3 days or at 12.0 ± 1.0°C for 5 days. During incubation, biofilm was formed on both sides of the microscope slides or steel coupons at the liquid-air interface. After incubation, the slides/coupons were transferred to a test tube with 1% crystal violet solution (Sigma-Aldrich) for staining of the biofilm for 30 min. Excess crystal violet solution was rinsed from the slides/coupons in tap water. The isolates were then examined visually and given a score from 0 to 3 according to the amount of stained biofilm observed.

Hydrophobicity. Hydrophobicity was determined using the microbial adhesion to hydrocarbons (MATH) assay as described by Mattos-Guaraldi et al. (26), with minor modifications. Briefly, 10-μl amounts of overnight cultures were mixed with 10 ml LB broth in 50-ml tubes and incubated for7 days at 12°C, 3 days at 20°C, or 24 h at 37°C. The bacteria were washed once with 5 ml phosphate-buffered saline (PBS) (pH 7.2) at the test temperature (for 10 min at 3,000 rpm) and resuspended in PBS (to an OD_{600} of 0.6 to 0.8, using a Hitachi U-1100 spectrophotometer). Then, 2-ml amounts of diluted bacterial suspension were transferred to glass tubes, and each was overlaid with 400 μl hexadecane (ReagentPlus, 99%; Sigma-Aldrich). The mixtures were vigorously vortexed for 1 min and incubated for 30 min to allow phase separation, and then the OD_{600} of the lower, aqueous phase was recorded. Controls consisted of untreated bacterial suspensions. The percentage of hydrophobicity for each strain was calculated according to the following formula: % hydrophobicity = [(OD_{600} untreated sample − OD_{600} aqueous phase)/OD_{600} untreated sample] × 100. Each strain was tested in three independent experiments at all temperatures.

Statistical analyses. The computer program JMP 9.0.0 (2010; SAS Institute, Inc., Cary, NC, USA) was used for all statistical calculations. For comparing responses when the data were normally distributed, analysis of variance (ANOVA) and the Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons of means were used. When data were not normally distributed, responses were compared by using the Wilcoxon each pair test for multiple nonparametric comparisons. Differences were considered statistically significant if the P value was <0.05.

RESULTS

Genotypes. All of the *eae*-positive *E. coli* O103:H2 isolates had the *eae*ε gene, all of the O103:H25 isolates carried the *eae*γ2/θ gene, and all of the O26:H11 isolates carried the *eae*β gene. Dendrograms based on the results from PFGE of all the isolates displayed a high variability within each serotype (Fig. 1, 2, and 3). Within the serotype O103:H2, *eae*-positive and -negative isolates showed a tendency toward clustering separately (Fig. 1). All *eae*-negative isolates except three had PFGE profiles with higher similarities to profiles of other *eae*-negative isolates than to those of *eae*-positive isolates. However, as many as 13 of the *eae*-positive isolates displayed higher similarities to *eae*-negative isolates than to the remaining 6 *eae*-positive isolates.

Biofilm production on polystyrene (microtiter plate assay). When biofilm production by all isolates was tested in the microtiter plate assay, the results displayed significant differences between the serotypes (Table 1). *E. coli* O103:H2 isolates produced significantly more biofilm at all three temperatures than isolates of the other two serotypes (ANOVA, $P < 0.0001$). *E. coli* O26:H11 produced more biofilm than *E. coli* O103:H25 at 37°C ($P = 0.003$).

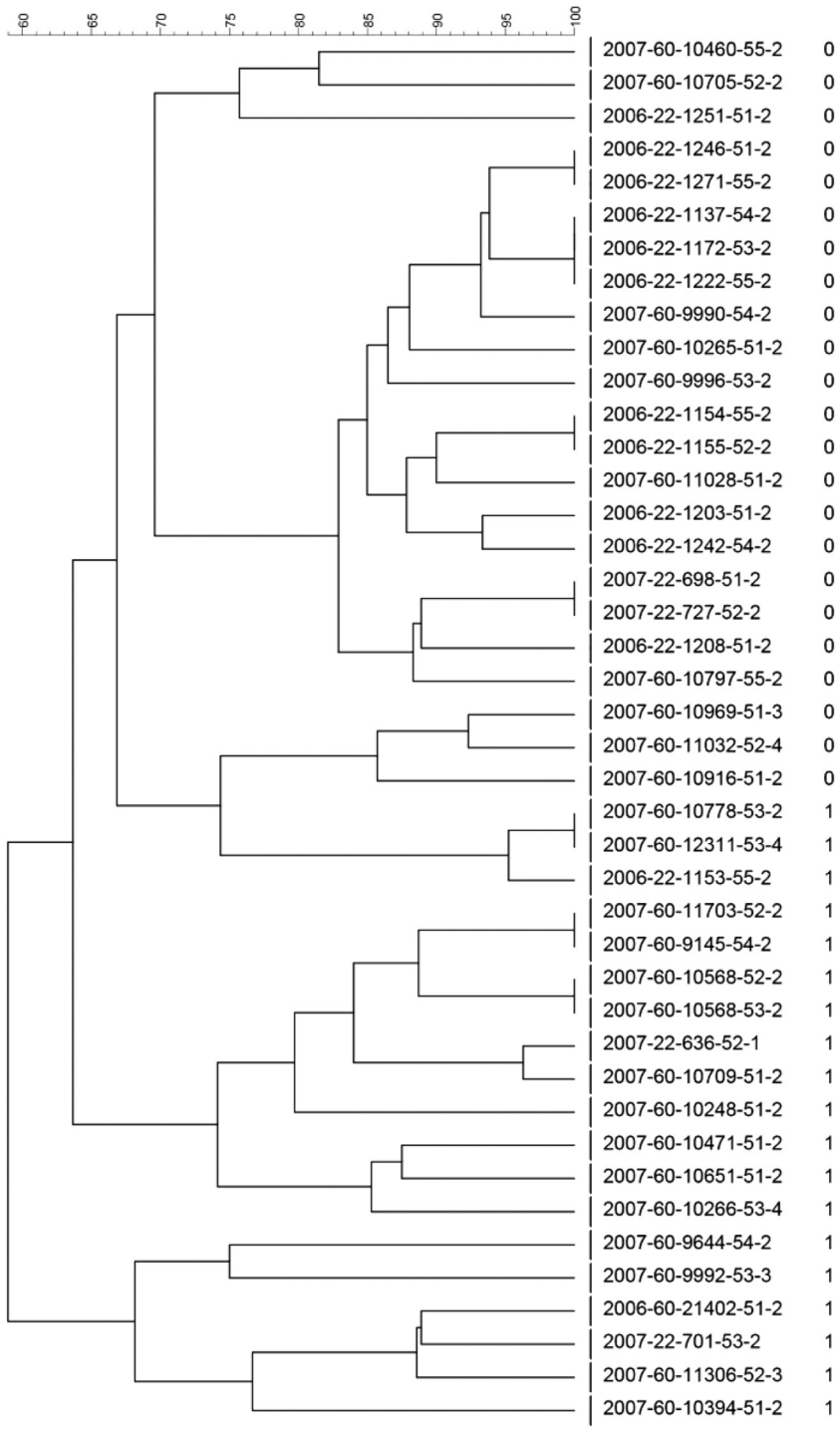

FIG 1 Dendrogram with percent similarity of PFGE profiles of *E. coli* O103:H2 isolates (isolate numbers are given for all isolates). In addition, the number 0 or 1 to the right of the isolate numbers signifies the absence or presence of the *eae* gene, respectively.

Biofilm production was also influenced to some extent by temperature. *E. coli* O103:H2 produced significantly less biofilm at 37°C than at 12°C and 20°C (ANOVA, $P < 0.0001$), whereas the biofilm production of *E. coli* O26:H11 isolates was higher at 20 and 37°C than at 12°C (Wilcoxon rank test, $P = 0.04$ and 0.0002, respectively).

Two of the *E. coli* O26:H11 isolates (2007-60-10125-53-2 and 2007-60-10710-54-4) produced more biofilm than the other isolates of this serotype both at 12 and 20°C. At 12°C, the OD_{595} values of the two isolates were 0.388 and 0.485, respectively, whereas the rest of the isolates had a mean of 0.020 (range, 0 to 0.136). The corresponding results at 20°C were 0.301 and 0.431 for the two isolates, while the rest of the isolates had a mean of 0.048 (range, 0.002 to 0.224). The two isolates were from two farms

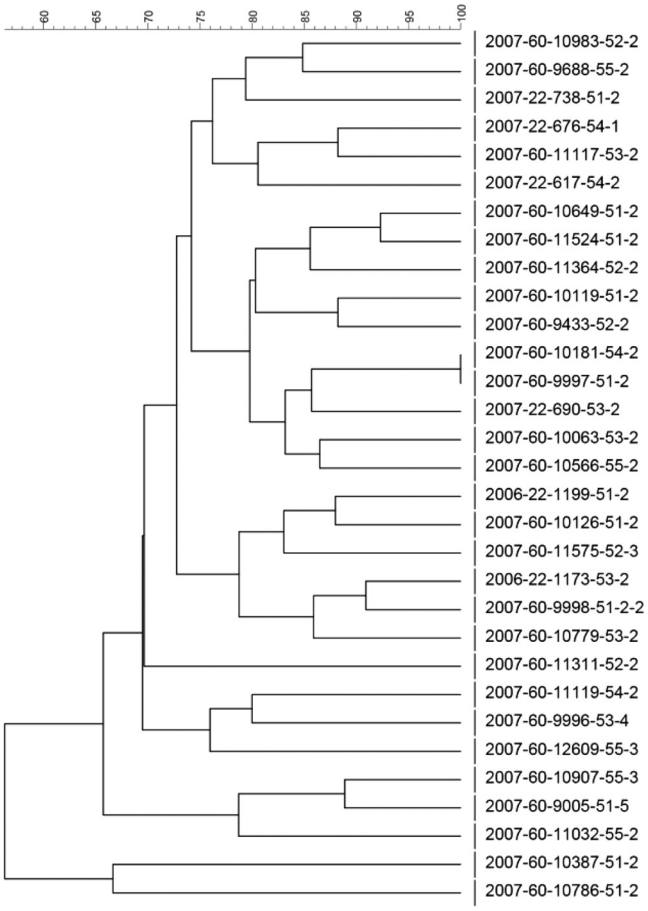

FIG 2 Dendrogram with percent similarity of PFGE profiles of *E. coli* O103:H25 isolates (isolate numbers are given for all isolates).

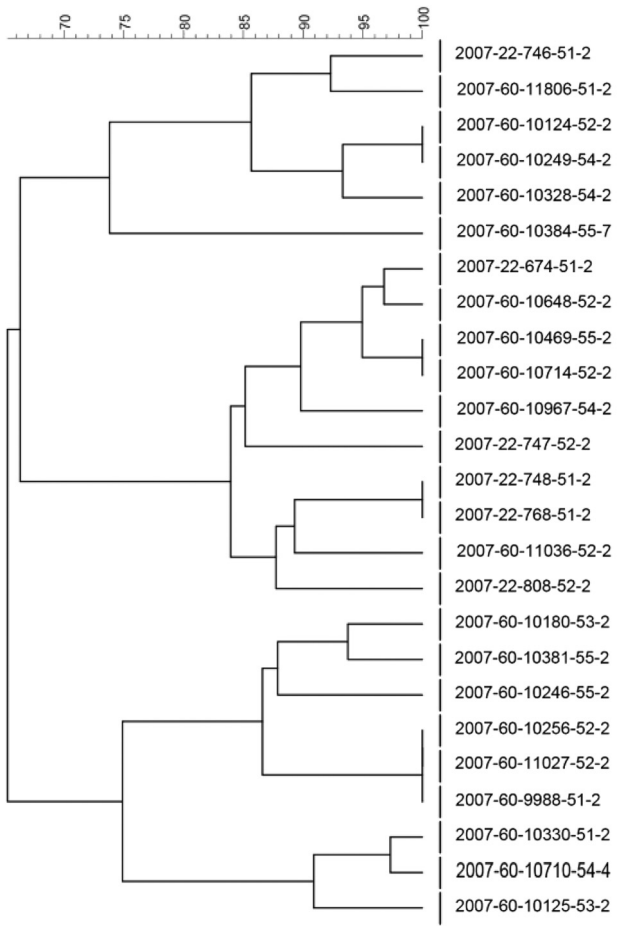

FIG 3 Dendrogram with percent similarity of PFGE profiles of *E. coli* O26:H11 isolates (isolate numbers are given for all isolates).

located in different counties, but they clustered together when genotyped by PFGE (Fig. 3). The third isolate in this cluster (2007-60-10330-51-2) also had relatively high OD_{595} values at these temperatures (0.101 and 0.224), but they were not as high as the two others.

Serotype O103:H2 was the only serotype that encompassed both *eae*-positive and *eae*-negative isolates. Within this serotype, the mean biofilm production at 37°C (as indicated by the OD_{595} in the microtiter plate assay) was significantly higher in *eae*-negative isolates than in *eae*-positive isolates (ANOVA, $P < 0.0001$), whereas no differences in mean OD_{595} were observed between *eae*-negative and *eae*-positive isolates at 12°C and 20°C (Table 1).

As there were no *stx*-positive isolates in our material, we infected three isolates with a Stx bacteriophage and compared their biofilm production with and without this bacteriophage. Two transductants were produced from each of the isolates 2006-22-1153-55-2 (serotype O103:H2) and 2006-22-1199-51-2 (serotype O103:H25) and one from the laboratory strain C600. When comparing the biofilm-forming abilities of isolates with and without the Stx bacteriophage in the microtiter plate assay, only minor variations in the total biofilm production (as indicated by the OD_{595} in the microtiter plate assay) were observed between mother strains and transductants (Table 2).

Biofilm production on glass and stainless steel. A subset of 12 isolates, including all three serotypes, different levels of biofilm production, and both *eae*-positive and -negative isolates, was tested on glass and stainless steel coupons with the semiquantitative method for biofilm production (Table 3). The isolates displayed various degrees of biofilm formation, ranging from no visible biofilm (scored as 0) to a thick biofilm at the liquid-air interface, which was scored on a scale from 1 to 3. In general, there was a good correlation between the amount of biofilm formed in microtiter plates and on glass and stainless steel, i.e., isolates with high OD_{595} values in the microtiter plates achieved high scores on glass and stainless steel and vice versa (ANOVA, $P < 0.0001$) (Fig. 4). There was also a relatively good correlation between the scores obtained on glass and stainless steel, although a tendency to higher scores on steel than on glass was observed (Table 3). This was particularly pronounced at 12°C, where all of the *E. coli* O103:H2 isolates displayed low scores (1) for biofilm production on glass and yet achieved higher scores (2 or 3) for biofilm production on steel.

Hydrophobicity. The subset of 12 isolates tested for hydrophobicity showed differences both between and within serotypes at all three temperatures tested (Table 3; Fig. 5). The O103:H2 isolates displayed significantly higher hydrophobicities at both 20 and 37°C than the other two serotypes (ANOVA, *P* value range of 0.001 to 0.04). Within the serotype O103:H2, the hydrophobicity was higher at 37°C than at 12 and 20°C ($P = 0.0001$ and 0.005).

TABLE 1 Biofilm production in the microtiter plate assay at 12, 20, and 37°C

Serotype	No. of isolates tested	Mean OD_{595}[a] ± SD (range) at temp (°C) of:		
		12	20	37
O103:H2	42	1.35 ± 0.60 (0.05–2.51) A	1.38 ± 0.45 (0.07–2.01) A	0.58 ± 0.64 (0.04–2.15) B
O103:H25	32	0.03 ± 0.01 (0.00–0.06) C	0.04 ± 0.03 (0.00–0.10) CD	0.04 ± 0.01 (0.02–0.08) C
O26:H11	25	0.06 ± 0.12 (0.00–0.49) C	0.09 ± 0.11 (0.00–0.43) DE	0.15 ± 0.18 (0.02–0.66) E
O103:H2 *eae* positive	19	1.34 ± 0.52 (0.05–2.18) A	1.38 ± 0.51 (0.07–1.99) A	0.12 ± 0.09 (0.04–0.34) F
O103:H2 *eae* negative	23	1.37 ± 0.68 (0.16–2.51) A	1.37 ± 0.41 (0.72–2.01) A	0.95 ± 0.65 (0.04–2.15) G

[a] Different letters signify that the means are statistically different at a P value of <0.05.

Similar differences in hydrophobicities could not be seen between or within *E. coli* O103:H25 and O26:H11. Furthermore, the *eae*-negative O103:H2 isolates had higher hydrophobicities at 37°C than the *eae*-positive isolates ($P = 0.0001$) (Table 3).

Isolates with no visible biofilm on the glass and stainless steel coupons (score of 0) displayed a lower mean hydrophobicity than isolates with biofilm scores of 1 to 3 (mean of 32% and SD of 11% versus mean of 50% and SD of 20%, respectively; ANOVA, $P = 0.001$). These results were the same on both glass and steel coupons. Similarly, isolates with very low biofilm production in the microtiter plate assay, i.e., OD_{595} of <0.1, displayed a significantly lower mean hydrophobicity than isolates with OD_{595} values of 0.1 and higher (mean of 33% and SD of 13% versus mean of 54% and SD of 19%, respectively; ANOVA, $P = 0.002$).

DISCUSSION

The results from the present study show that potentially pathogenic *E. coli* isolates of serotypes O103:H2, O103:H25, and O26:H11 can produce biofilm on stainless steel, glass, and polystyrene at 12, 20, and 37°C. There was a good general correlation between the results obtained on different surfaces. The main exception was found at 12°C, where isolates of *E. coli* O103:H2 produced large amounts of biofilm on polystyrene and stainless steel but little on glass. The microtiter plate assay is well suited for screening a large number of strains, and our results indicate that this assay can provide relevant information on biofilm-forming abilities on stainless steel and glass as well. This has previously been observed in *Salmonella enterica* subsp. *enterica*, where correlations between biofilm formation in microtiter plates, on stainless steel, and at the liquid-air interface were found (18).

Furthermore, we observed significant differences in the biofilm-forming abilities of the serotypes in all assays used. *E. coli* O103:H2 isolates produced much more biofilm than those of the other two serotypes at all three temperatures, and *E. coli* O26:H11 produced more biofilm than *E. coli* O103:H25 at 37°C. In particular, three isolates of *E. coli* O26:H11 belonging to the same PFGE subcluster produced more biofilm than the rest of the isolates of this serotype, although not as much as the *E. coli* O103:H2 isolates.

TABLE 2 Biofilm production in the microtiter plate assay of three *E. coli* strains before and after the Stx bacteriophage Φ731 was inserted

Isolate[a]	Serotype	Mean OD_{595} ± SD at temp (°C) of:		
		12	20	37
2006-22-1153-55-2	O103:H2	1.14 ± 0.08	1.67 ± 0.06	0.15 ± 0.03
2006-22-1153-55-2:Φ731 A	O103:H2	1.47 ± 0.11	1.70 ± 0.08	0.17 ± 0.13
2006-22-1153-55-2:Φ731 B	O103:H2	1.27 ± 0.08	1.52 ± 0.06	0.17 ± 0.05
2006-22-1199-51-2	O103:H25	0.08 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
2006-22-1199-51-2:Φ731 A	O103:H25	0.06 ± 0.04	0.02 ± 0.01	0.03 ± 0.03
2006-22-1199-51-2:Φ731 B	O103:H25	0.06 ± 0.01	0.03 ± 0.04	0.03 ± 0.27
C600		0.06 ± 0.01	1.78 ± 0.01	0.10 ± 0.06
C600:Φ731		0.16 ± 0.02	2.03 ± 0.02	0.24 ± 0.24

[a] Two different transductant strains (A and B) were produced from each of the strains in serogroup O103, whereas one was produced from the laboratory strain C600.

Different biofilm-forming abilities between serotypes of *Listeria monocytogenes* (27, 28) and between serovars of *Salmonella* (18, 29) have also been reported. Little has previously been reported on possible differences in biofilm production between serotypes or serogroups of *E. coli* (30), although differences between strains have been published by several (31–34). When comparing serotypes in our study, we aimed at reducing bias by having relatively large groups of isolates with as low an epidemiological relationship as possible. To achieve this, isolates from as many different counties as possible were included. This made it more likely that a large number of different clones were represented within each serotype, as moving sheep from one county to another is prohibited in Norway. This assumption was supported by the results from the PFGE analyses. Consequently, the results of the present study indicate that biofilm-forming ability in general is more closely correlated to serotype than to strain or clone.

All of the *E. coli* O103:H25 isolates displayed very low biofilm formation at all temperatures in all assays. However, we showed previously that the total number of CFU in biofilm produced on glass slides by one of these isolates (2006-22-1199-51-2) at 20°C was as high as 10^6 (20). This indicates that even isolates of the serotypes that were poor biofilm producers in our test systems still can form some biofilm under conditions relevant for the food production industry.

In the present study, significant differences in hydrophobicity were observed between the serotypes, with *E. coli* O103:H2 displaying the highest hydrophobicity. In addition, the hydrophobicity of these isolates increased with increasing temperature, whereas the isolates of the other two serotypes displayed low hydrophobicity at all temperatures. The hydrophobicity of a bacterial cell is largely influenced by the residues and structures on the surface of the cell, and it varies between species and strains and even within the same strain depending on the mode and stage of growth and the composition of the growth medium (35). It is not surprising that the surface structures and, thereby, the surface hydrophobicity may vary between isolates of different serotypes. Variations due to adaptations to different temperatures could also be expected, as this has been reported in other species, e.g., *Listeria monocytogenes* (27) and *Serratia marcescens* (36).

We did not observe any general correlation between hydrophobicity and the amount of biofilm formed in any of our test systems. However, we did find that isolates which did not form a visible biofilm on stainless steel and glass displayed lower hydrophobicity than isolates that did form a visible biofilm. Similarly, isolates with very poor biofilm production in the microtiter plate assay had lower hydrophobicity than those with better biofilm

TABLE 3 Biofilm formation in a microtiter plate assay and on glass and stainless steel coupons and hydrophobicity of a subset of 12 ovine *E. coli* strains at different temperatures

Isolate	Serotype	*eae* type	Median OD_{595} in microtiter plate assay ($n = 3$) at temp (°C) of:			Mean score[a] at indicated temp (°C) on:						Mean hydrophobicity (%) ± SD ($n = 3$) at temp (°C) of:		
						Glass coupons			Steel coupons					
			12	20	37	12	20	37	12	20	37	12	20	37
2006-22-1242-54-2	O103:H2	Negative[b]	2.194	1.886	0.479	1	3	2	3	3	1	51 ± 19	52 ± 16	93 ± 2
2006-22-1246-51-2	O103:H2	Negative	1.048	1.744	1.364	1	3	1	3	3	2	47 ± 3	66 ± 5	90 ± 1
902006-22-1271-55-2	O103:H2	Negative	1.089	1.722	0.644	1	3	1	3	3	2	62 ± 1	57 ± 7	93 ± 0
2006-22-1153-55-2	O103:H2	*eae*ε	1.579	1.478	0.217	1	3	1	2	2	1	24 ± 3	34 ± 3	63 ± 1
2007-60-10651-51-2	O103:H2	*eae*ε	1.284	1.804	0.344	1	3	1	3	3	2	36 ± 2	55 ± 7	67 ± 3
2007-60-10709-51-51	O103:H2	*eae*ε	1.292	1.558	0.083	1	3	1	2	2	1	42 ± 8	63 ± 6	65 ± 8
2006-22-1173-53-2	O103:H25	*eae*γ2/θ	0.018	0.000	0.030	0	1	0	0	0	1	23 ± 7	29 ± 0	24 ± 10
2006-22-1199-51-2	O103:H25	*eae*γ2/θ	0.020	0.097	0.022	0	1	1	0	1	1	27 ± 12	25 ± 14	20 ± 3
2007-60-10125-53-2	O26:H11	*eae*β	0.388	0.301	0.061	3	3	2	3	3	2	37 ± 6	38 ± 7	33 ± 5
2007-60-10710-54-4	O26:H11	*eae*β	0.485	0.431	0.658	3	3	2	3	3	3	39 ± 5	39 ± 6	38 ± 0
2007-60-10180-53-2	O26:H11	*eae*β	0.002	0.012	0.025	0	0	0	0	0	1	22 ± 4	25 ± 9	32 ± 6
2007-60-10246-55-2	O26:H11	*eae*β	0.000	0.019	0.017	0	0	0	0	0	1	46 ± 3	43 ± 1	54 ± 3

[a] There was no variation between parallel assays in the coupon test, i.e., the standard deviation was 0.
[b] Negative, isolate did not have the *eae* gene.

production. These results strongly indicate that the hydrophobicity of the isolates influenced the attachment of the bacteria rather than their ability to form biofilm once attached. A positive correlation between the hydrophobic properties of *E. coli* isolates and their attachment abilities has been reported in some studies (37–40), whereas others have failed to find such a relationship (41–43). It has been suggested that the differences between these studies could be explained, e.g., by the number and variety of strains included in the study and/or a lack of sensitivity within methods (35).

One aim of the study was to investigate whether the two main virulence factors of STEC, i.e., Stx bacteriophages and the *eae* gene encoding intimin, could influence the biofilm-producing abilities of *E. coli*. A number of cell surface structures are involved in the attachment of *E. coli* to host cells and/or abiotic surfaces (44, 45). To the best of our knowledge, a possible role of intimin in biofilm production is unknown. In our material, *E. coli* O103:H2 was the only serotype where we had both *eae*-positive and *eae*-negative isolates. Interestingly, the *eae*-negative isolates were significantly better biofilm producers than the *eae*-positive isolates at 37°C within this serotype. The *eae*-negative isolates also displayed higher hydrophobicity than the *eae*-positive isolates at this temperature. Isolates of the other two serotypes, which were all *eae* positive, displayed both low hydrophobicity and biofilm formation at 37°C. Consequently, one could speculate that intimin contributed to the lower hydrophobicity and biofilm production at this temperature. On the other hand, *eae*-positive O103:H2 isolates were very good biofilm producers at 12 and 20°C, whereas the *eae*-positive isolates of the other serotypes produced the same amount of biofilm at these temperatures as at 37°C. One explanation could be that intimin was only expressed at 37°C and that other factors were more important for biofilm production at the other temperatures. It has been reported previously that the LEE operons are repressed at 27°C and expressed at 37°C (46). The

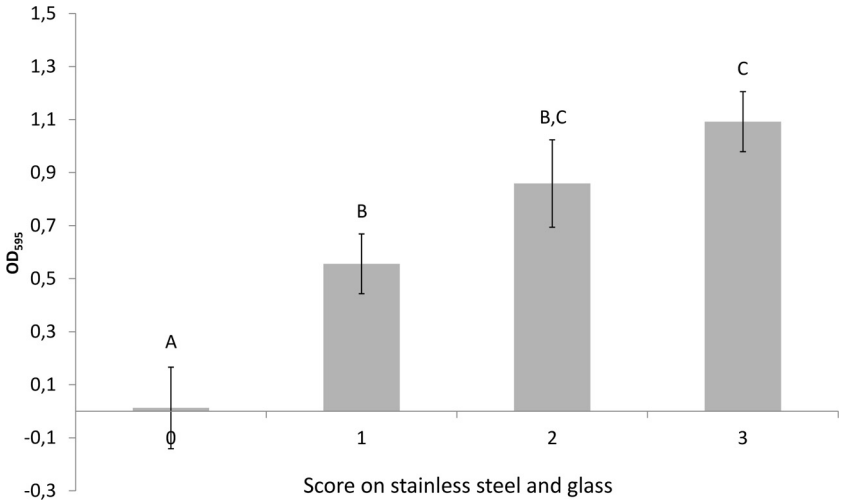

FIG 4 Mean OD_{595} values in microtiter plate assay of subset of 12 isolates with different biofilm formation scores on glass slides and steel coupons. Results from all temperatures are included. Bars represent standard deviations (SD). Different letters signify that the means are statistically different at a P value of <0.05.

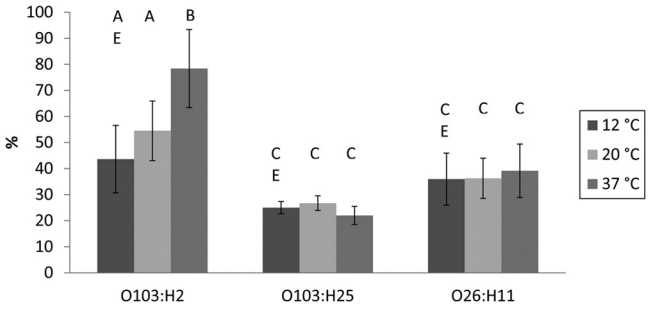

FIG 5 Mean hydrophobicities of different serotypes in subset of 12 isolates at 12, 20, and 37°C. Bars represent standard deviation (SD). Different letters signify that the means are statistically different at a P value of <0.05.

differences might also be attributed to the differences in intimin type found between the serotypes in our study. However, these are speculations only, as our investigations of *eae* were performed at the gene level, and we do not know whether intimin was expressed at the cell surface in any of our assays. An alternative explanation for the different biofilm-forming abilities of *eae*-positive and -negative *E. coli* O103:H2 isolates may be that *eae* was a confounding variable in our material. If so, the observed difference in biofilm formation would have been due to other factors that also differed between the two groups. In the PFGE analyses, the majority of the *eae*-negative *E. coli* O103:H2 isolates clustered together, but this was not as obvious for the *eae*-positive isolates. Thus, the results from the PFGE analyses are only partly supportive of this theory. Consequently, the observed difference in biofilm formation between *eae*-positive and -negative isolates remains interesting but unexplained. On the other hand, our results from testing strains before and after infection with a Stx bacteriophage strongly suggest that the presence of the bacteriophage does not influence biofilm-forming abilities.

In conclusion, our results show that potentially human-pathogenic *E. coli* from the ovine reservoir can form biofilm on various surfaces and at several temperatures relevant for food production and handling, although variation could be seen between and within serotypes. In food-producing facilities, the biofilm may serve as a reservoir for cross contamination of the products and as an environment for the dissemination of virulence genes and emergence of new pathogenic *E. coli* isolates.

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