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Dual-Serotype Biofilm Formation by Shiga Toxin-Producing *Escherichia coli* O157:H7 and O26:H11 Strains

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***Escherichia coli* O26:H11 strains were able to outgrow O157:H7 companion strains in planktonic and biofilm phases and also to effectively compete with precolonized O157:H7 cells to establish themselves in mixed biofilms. *E. coli* O157:H7 strains were unable to displace preformed O26:H11 biofilms. Therefore, *E. coli* O26:H11 remains a potential risk in food safety.**

Shiga toxin-producing *Escherichia coli* (STEC) strains of various serotypes are important food-borne pathogens responsible for numerous outbreaks, with symptoms ranging from bloody diarrhea to more severe diseases, such as hemolytic-uremic syndrome (HUS). Of the many STEC serotypes, O157:H7 is the most frequently identified serotype that causes food-borne outbreaks worldwide. Meanwhile, six non-O157 STEC serotypes, including O26, O45, O103, O111, O121, and O145, have attracted significant attention from the scientific and regulatory fields lately, since they have been increasingly associated with serious outbreaks. It was reported that these six STEC serotypes, now referred to as the "big six," were responsible for approximately 70% of all non-O157 STEC infections from 1983 to 2002 (4). In 2010, these non-O157 STEC serotypes collectively caused more human infections in the United States than STEC O157:H7. The Food Safety and Inspection Service (FSIS) published a Federal Register notice in September 2011 announcing their intent to regulate these non-O157 STECs as adulterants in certain raw beef products (11). Among the big six, O26 is the most common non-O157 STEC isolated from specimens submitted to the Centers for Disease Control and Prevention (CDC) for serotyping. Recovery of STEC O26 from cattle and beef products has been reported in different countries (1, 3, 8, 13). In particular, serotype O26:H11 has been regarded as one of the most dangerous non-O157 STEC due to its enhanced virulence and ability to cause diarrhea and HUS (2, 3). The recent FSIS recall of 8,500 pounds of Cargill ground beef contaminated by STEC O26:H11 was prompted by food-borne outbreaks in Maine and New York. These outbreaks highlight the critical need to prevent the transmission of and food contamination by these pathogens.

Biofilm formation is one of the major strategies that support bacterial survival under adverse circumstances. In nature, bacteria are able to form single-species biofilms or coexist in multispecies communities and form mixed biofilms on a wide variety of solid surfaces. In the food industry, attachment of food-borne pathogens to food products and contact surfaces can be enhanced by biofilm formation, and detached biofilms can become a continuous source of cross-contamination. It has been shown that multiple bacteria, including *E. coli*, *Salmonella*, *Staphylococcus*, *Bacillus*, and *Pseudomonas*, can coexist and form biofilms in meat-processing plants (7). Studies focusing on STEC O157:H7 have shown that surface attachment of an O157:H7 strain unable to form a single-serotype biofilm was enhanced by formation of mixed biofilms with an *E. coli* O−:H4 companion strain (10). Similarly, preformed *Acinetobacter calcoaceticus* biofilms enhanced O157:H7 colonization on solid surfaces under both static and dynamic growth conditions (6). However, an area that has been not thoroughly explored is non-O157 STEC biofilms and the effects of coexistence of STEC O157 and non-O157 serotypes on dual-serotype biofilm formation. Interactions between serotypes could profoundly affect biofilm development, composition, and structure. Research in this area should be of great interest from a food safety standpoint, because coexistence of multiple bacterial serotypes is frequently observed in food-processing plants, and multiserotype biofilms often demonstrate higher resistance to common sanitizers than single-serotype biofilms or the corresponding planktonic cells (10). Therefore, the objective of this study was to investigate how coexistence of STEC O157:H7 and O26:H11 serotypes affects bacterial growth, colonization, and the mixed-biofilm composition.

Two STEC O157:H7 strains and three O26:H11 strains that exhibited curli expression at different levels and demonstrated various biofilm forming ability on polystyrene surfaces in our previous study (12) were selected for the investigation (Table 1). We first compared the growth capability of these STEC strains while they replicated individually. Each strain was grown statically in Lennox broth without salt (LB-NS medium) at room temperature (22 to 25°C), the experimental condition used in the biofilm assays. Bacterial growth was examined after 24, 48, and 72 h by diluting and plating individual cultures onto Tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) plates or ChromAgar O157 agar (DRG International Inc., Mountainside, NJ) plates supplemented with 5 mg/liter novobiocin and 2.5 mg/liter tellurite (Sigma, St. Louis, MO) (ntChromAgar). On each type of the agar plate, no significant difference in bacterial growth as measured by colony numeration was observed among the five STEC strains (data not shown).

Since multiple bacterial species often coexist, compete, and colonize on solid surfaces in food processing environments, we investigated how coexistence of these two serotypes would affect bacterial planktonic growth and mixed-biofilm formation. Equal numbers of bacteria ($\sim 1 \times 10^6$ CFU) from one O157:H7 strain and one O26:H11 strain were mixed to make dual-serotype cul-

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TABLE 1 Characteristics of bacterial strains used and measurement of single-serotype biofilm formation

Serotype	Strain	Origin[a]	Presence of: stx_1	stx_2	*eae*	*hlyA*	Curli expression[b]	Biofilm (10^6 CFU/well)[c]	Source or reference[d]
O157:H7	FSIS11	G		Y	Y	Y	−	4.50 ± 0.24	MH
	FSIS62	G	Y	Y	Y	Y	±	9.83 ± 1.18	MH
O26:H11	7–14 50A	B		Y	Y	Y	++	13.17 ± 3.06	MARC
	DEC 10B	H	Y		Y	Y	±	9.50 ± 1.18	15
	DEC 10E	B	Y	Y	Y	Y	−	10.83 ± 2.12	15

[a] G, ground beef; B, bovine; H, human.
[b] Curli expression was confirmed by streaking the strains onto Congo red indicator agar plates, and the expression level was evaluated based on colony color and morphology on the plates.
[c] Each strain was allowed to form biofilms on 96-well polystyrene plates at room temperature for 72 h. Data are means ± standard deviations; $n = 3$.
[d] MH, Marcus Head, USDA, FSIS; MARC, U.S. Meat Animal Research Center strain collection.

tures. The cultures were grown statically on 96-well polystyrene plates at room temperature for 72 h. Bacterial cells in planktonic suspensions and in biofilms were harvested as previously described (12), serially diluted in LB-NS broth, and plated onto ntChromAgar plates for colony enumeration after overnight incubation at 37°C. The two serotypes were distinguished by colony morphology, as the O157:H7 and O26:H11 strains form pink and blue colonies on the plates, respectively. Substantially higher cell numbers of O26:H11 than O157:H7 strains were observed in all dual-serotype planktonic suspensions (Table 2), indicating that the O26:H11 strains were able to effectively compete with and outgrow O157:H7 strains in coinoculated suspensions, even though the two serotypes reached similar cell densities when cultured individually for 72 h. Similarly, in the strain pairs tested, the percentages of O26:H11 cells comprising the 72-h mixed biofilms were consistently higher than those of the O157:H7 companion strains. These data indicated that the O26:H11 strains could successfully outcompete O157:H7 strains during the biofilm-forming process as well, thus maintaining a higher population density and becoming the major component of the mixed biofilms. Although the percentages of the O26:H11 cells in the mixtures varied among the different strain pairs, there was a clear trend for serotype O26:H11 to constitute the majority in mixed biofilms and in medium suspension. This suggests that the ability of serotype O26:H11 to outcompete serotype O157:H7 during planktonic growth and biofilm formation may be serotype related rather than strain specific. To further confirm these observations, indirect enzyme-linked immunosorbent assays (ELISA) were performed as previously described using monoclonal anti-O157 or anti-O26 antibodies (9, 14) to measure each serotype in the mixtures. The

TABLE 2 Bacterial planktonic growth and biofilm formation on 96-well polystyrene plates[a]

Strain combination (O157:H7-O26:H11)	Growth (% of mixture) of: Planktonic cells (10^7 CFU/ml) O157:H7	Planktonic cells O26:H11	Biofilms (10^6 CFU/well) O157:H7	Biofilms O26:H11
Coinoculated				
FSIS11–7-14 50A	8.75 ± 1.34 (38.2)	14.15 ± 1.35 (61.8)	3.00 ± 0.94 (31.0)	6.67 ± 0.94 (69.0)
FSIS11-DEC 10B	0.15 ± 0.07 (0.5)	29.30 ± 5.23 (99.5)	0.83 ± 0.70 (8.2)	9.33 ± 0.94 (91.8)
FSIS11-DEC 10E	1.00 ± 0.14 (3.4)	28.65 ± 3.04 (96.6)	0.67 ± 0.47 (7.7)	8.00 ± 0.10 (92.3)
FSIS62–7-14 50A	<LOD[a]	13.00 ± 2.97 (100)	4.67 ± 0.94 (28.6)	11.67 ± 3.30 (71.4)
FSIS62-DEC 10B	<LOD[a]	28.50 ± 4.95 (100)	4.67 ± 1.41 (31.1)	10.33 ± 0.94 (68.9)
FSIS62-DEC 10E	<LOD[a]	31.35 ± 1.77 (100)	3.67 ± 0.0 (26.5)	10.17 ± 3.06 (73.5)
O26:H11 precolonized				
FSIS11–7-14 50A	0.30 ± 0.0 (2.3)	12.75 ± 0.78 (97.7)	0.38 ± 0.29 (2.3)	15.58 ± 0.59 (97.7)
FSIS11-DEC 10B	0.05 ± 0.07 (0.2)	25.35 ± 1.20 (99.8)	0.21 ± 0.18 (3.4)	5.92 ± 1.30 (96.6)
FSIS11-DEC 10E	<LOD	30.10 ± 3.11 (100)	0.17 ± 0.11 (2.1)	7.88 ± 0.53 (97.1)
FSIS62–7-14 50A	<LOD[a]	17.25 ± 1.06 (100)	1.42 ± 0.24 (7.3)	17.92 ± 0.94 (92.7)
FSIS62-DEC 10B	<LOD	23.45 ± 0.21 (100)	0.63 ± 0.06 (9.5)	5.96 ± 0.18 (90.5)
FSIS62-DEC 10E	<LOD	19.00 ± 0.57 (100)	0.38 ± 0.06 (2.9)	12.42 ± 5.42 (97.1)
O157:H7 precolonized				
FSIS11–7-14 50A	8.90 ± 1.70 (79.1)	2.35 ± 0.64 (20.9)	1.58 ± 0.12 (82.6)	0.33 ± 0.0 (17.4)
FSIS11-DEC 10B	7.25 ± 0.64 (25.3)	21.35 ± 0.07 (74.7)	1.67 ± 0.71 (50.0)	1.67 ± 0.94 (50.0)
FSIS11-DEC 10E	9.50 ± 1.41 (81.5)	2.15 ± 0.49 (18.5)	3.00 ± 0.90 (56.3)	2.33 ± 1.41 (43.7)
FSIS62–7-14 50A	1.75 ± 0.07 (20.2)	6.90 ± 0.71 (79.8)	16.50 ± 1.18 (95.2)	0.83 ± 0.0 (4.8)
FSIS62-DEC 10B	1.20 ± 0.42 (4.2)	27.40 ± 0.80 (95.8)	2.75 ± 0.82 (54.1)	2.33 ± 1.18 (45.9)
FSIS62-DEC 10E	2.80 ± 0.14 (19.6)	11.45 ± 0.92 (80.4)	1.92 ± 0.59 (37.1)	3.25 ± 0.35 (62.9)

[a] Data are means ± standard deviations ($n = 3$). <LOD, the value was below the limit of detection (1×10^5 CFU/ml).

ELISA data were consistent with the colony enumeration results, indicating that the O26:H11 strains were the predominant serotype within the mixtures (data not shown).

To further explore how bacteria colonize and form biofilms on a solid surface with a preformed biofilm of another serotype, which represents a likely event in commercial meat plants, pure cultures of O157:H7 or O26:H11 strains were allowed to form single-serotype biofilms on 96-well plates for 72 h as described above. After removal of supernatants and the loosely attached cells by washing, overnight cultures of the other serotype were diluted and added to the plates to allow mixed planktonic growth and biofilm formation for another 72 h in the presence of the pre-existing biofilms. At the end of the incubation period, samples were harvested and processed as described above.

We first tested the development of an O157:H7 biofilm on an established O26:H11 biofilm. In all cases, the O26:H11 cells were present in substantially higher numbers than O157:H7 cells at 72 h following O157:H7 inoculation, constituting over 90% of the total populations in the mixed biofilms as well as in planktonic growth (Table 2). These observations indicated that in the presence of the O26:H11 biofilms, the O157:H7 strains were unable to grow efficiently and compete with the O26:H11 strains under heterologous culture conditions to displace the pre-existing O26:H11 biofilms. With the coinoculated or precolonized O26:H11 companion strains, the fact that O157:H7 strain FSIS62 was not detected in the suspensions but was present in the mixed biofilms suggested that biofilm formation could offer protection and enhance O157:H7 cell survival during competition with companion strains.

In additional experiments, each O26:H11 strain was inoculated onto an O157:H7 biofilm that had been developed for 72 h. After incubation for an additional 72 h, all three O26:H11 strains successfully outgrew O157:H7 strain FSIS62 in planktonic suspensions (>80%). When cultured with biofilms formed by O157:H7 strain FSIS11, O26:H11 strain DEC 10B outgrew strain FSIS11 in the planktonic phase (~75%), and the other two O26:H11 strains each occupied approximately 20% of the cell populations in the suspensions. Therefore, the preformed O157:H7 biofilms did not inhibit cell growth of the O26:H11 strains. Meanwhile, biofilm measurements indicated that the O26:H11 strains in all strain pairs except one (FSIS62 plus 7-14 50A) were able to establish themselves in the mixed biofilms after 72 h of incubation in the presence of the preformed O157:H7 biofilm. The FSIS62–7-14 50A strain pair did not have a low percentage of the O26:H11 cells in planktonic suspension, indicating that the small amount of O26:H11 cells in the mixed biofilms was not due to cell outgrowth by the precolonized O157:H7 cells. However, the percentages of O26:H11 cells in these mixed biofilms were generally lower than those in the dual-serotype biofilms formed when the two serotypes were inoculated simultaneously. These observations suggested that coexistence and growth competition in planktonic phase plays a role in the effective outgrowth of the O26:H11 strains during mixed-biofilm development, while early-stage precolonization favors O157:H7 cell survival and biofilm maintenance.

Bacterial species could compete for essential nutritional resources or inhibit the growth of the competitors by producing inhibitory agents. To understand the mechanism for the domination of serotype O26:H11, O26:H11 culture supernatants were filter sterilized using syringe filters (0.20-μm pore size; Corning, New York, NY) and then applied at 20 μl/drop onto the surface of TSA plates seeded with 5-h broth culture of the O157:H7 strains, which formed a confluent lawn after overnight incubation. No growth inhibition zone was observed on the plates within the area covered by the filtered O26:H11 supernatants, suggesting that the capability of the O26:H11 strains to outgrow O157:H7 strains was not mediated by the production of inhibitory agents. To further identify the genetic determinants responsible for such dominating capability, future studies should focus on generating O26:H11 mutant strains deficient in certain replication- or virulence-associated genes and comparing the competition results from O157:H7 and O26:H11 mixtures, using either O26:H11 wild-type strains or the mutant strains.

In conclusion, we have shown that STEC O26:H11 strains were able to effectively outgrow O157:H7 strains in both planktonic and biofilm phases during coexistence via simultaneous inoculation. In the presence of preformed O157:H7 biofilms, the O26:H11 strains could still compete with the colonized O157:H7 cells and establish themselves in the mixed biofilms, while serotype O157:H7 appeared to be a poor colonizer if O26:H11 cells already occupied the solid surfaces. The fact that the O26:H11 strains have the strong ability to outcompete serotype O157:H7 highlights the potential risk of this non-O157 STEC serotype in food safety and calls for increased attention to non-O157 STEC biofilms in food processing facilities.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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