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Escherichia coli O157:H7 Strains Isolated from High-Event Period Beef Contamination Have Strong Biofilm-Forming Ability and Low Sanitizer Susceptibility, Which Are Associated with High pO157 Plasmid Copy Number

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ABSTRACT

In the meat industry, a *high-event period* (HEP) is defined as a time period when beef processing establishments experience an increased occurrence of product contamination by *Escherichia coli* O157:H7. Our previous studies suggested that bacterial biofilm formation and sanitizer resistance might contribute to HEPs. We conducted the present study to further characterize *E. coli* O157:H7 strains isolated during HEPs for their potential to cause contamination and to investigate the genetic basis for their strong biofilm-forming ability and high sanitizer resistance. Our results show that, compared with the *E. coli* O157:H7 diversity control panel strains, the HEP strains had a significantly higher biofilm-forming ability on contact surfaces and a lower susceptibility to common sanitizers. No difference in the presence of disinfectant-resistant genes or the prevalence of antibiotic resistance was observed between the HEP and control strains. However, the HEP strains retained significantly higher copy numbers of the pO157 plasmid. A positive correlation was observed among a strain's high plasmid copy number, strong biofilm-forming ability, low sanitizer susceptibility, and high survival and recovery capability after sanitization, suggesting that these specific phenotypes could be either directly correlated to gene expression on the pO157 plasmid or indirectly regulated via chromosomal gene expression influenced by the presence of the plasmid. Our data highlight the potential risk of biofilm formation and sanitizer resistance in HEP contamination by *E. coli* O157:H7, and our results call for increased attention to proper and effective sanitization practices in meat processing facilities.

Key words: Biofilm; *Escherichia coli* O157:H7; High-event period; pO157 plasmid; Sanitizer

In the U.S. beef industry, a high-event period (HEP) refers to a time period when commercial meat plants experience an elevated rate of *Escherichia coli* O157:H7 contamination. HEPs pose a serious public health concern and often result in significant financial losses because of product recalls or devaluation. The precise cause or contamination source responsible for HEPs is not well understood. Our previous study *(1)* indicated that, within each HEP event, most of the *E. coli* O157:H7 strains belonged to a singular dominant strain type. This observation was in disagreement with the traditional beef contamination model, which states that contamination occurs when the incoming pathogen load on animal hides, which consists of widely diverse strain types of *E. coli* O157:H7, exceeds the intervention capacity of the plants. Thus, we hypothesize that certain types of in-house contamination might be responsible for HEPs that occur after carcasses exit the kill floor.

Biofilm formation can significantly benefit bacterial survival under adverse circumstances because bacterial cells in their biofilm stage are much more resistant to sanitization or other types of physical and chemical treatments than cells in planktonic suspensions. Bacteria can form biofilms in many different areas at food processing plants *(8)*; thus, biofilms formed by foodborne pathogens, such as *E. coli* O157:H7, present serious food safety concerns because the detached biofilm cells may lead to cross-contamination of food products. Therefore, one potential explanation for HEP contamination could be that certain *E. coli* O157:H7 strains are better able to survive the exposure to sanitizers, probably through biofilm formation to colonize in-plant contact surfaces. When the surviving bacteria in mature biofilms, which are unevenly distributed on equipment or contact surfaces, become detached as the meat products pass through the area, random occasions of high-level contamination (i.e., HEPs) could occur. These occasions would exhibit no apparent temporal-spatial patterns or association with any specific process control failure. This hypothesis was supported in our previous study *(17),* which showed

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that, under laboratory conditions using 96-well polystyrene plates, the *E. coli* O157:H7 strains isolated from multiple HEPs had a significantly higher biofilm-forming ability and sanitizer resistance than the control panel strains.

In the present study, we further phenotypically and genetically characterize and compare the *E. coli* O157:H7 HEP and control panel strains for their biofilm-forming ability using materials and temperatures commonly encountered in the meat industry under normal operating conditions. The sensitivity of the strains to common sanitizers was also assessed to determine the potential contribution of biofilm formation and sanitizer resistance to HEP contamination. Furthermore, the potential genetic basis for strong biofilm-forming ability and high sanitizer resistance was investigated as well.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and curli and cellulose expression. We used 45 HEP strains and 47 diversity control panel strains in the study. The HEP strains were recovered from beef trim enrichment samples during HEPs, as described in a previous article *(1),* and represented the pulsed-field gel electrophoresis types associated with 14 different HEPs. The diversity control panel strains were isolated from cattle hide swabs during harvest at commercial beef processing plants, which represented the breadth of *E. coli* O157:H7 pulsed-field gel electrophoresis types obtained from over 1,000 cattle hide samples collected previously *(2).*

Bacterial broth cultures at stationary phase were prepared in Lennox broth (Acumedia Manufacturers, Baltimore, MD) without salt (LB-NS), as described in a previous article *(16);* they were then further diluted in fresh sterile LB-NS medium for each experiment. The expressions of curli and cellulose fimbriae by the strains, the two bacterial extracellular polymeric substances (EPS) associated with biofilm-forming abilities and sanitizer resistance, were tested as previously described *(18)* using Congo red indicator plates and Lennox broth agar plates containing calcoflour dye, respectively.

Sanitizers. Two types of commercial sanitizers commonly used in beef processing establishments, Vanquish and Pro-Oxine, were tested in this study. The quaternary ammonium chloride (QAC)–based sanitizer Vanquish (Dawn Chemical Corp., Milwaukee, WI) is a product authorized by the U.S. Department of Agriculture (USDA) as category D2 for use in meat, poultry, and other food processing plants. We prepared Vanquish working solutions following the manufacturer's instructions, which contain an alkylbenzyldimethyl–ammonium chloride mixture as the active ingredients.

Pro-Oxine (DanMar, Arlington, TX) is a broad-spectrum fungicidal and bactericidal sanitizer that contains purified sodium chlorite. Its mechanism of action starts with product activation and then the production of chlorine dioxide (ClO_2), resulting in enhanced antimicrobial activity against bacterial biofilms. We prepared Pro-Oxine working solutions following the manufacturer's instructions, and the concentrations of total available or free chlorine dioxide were confirmed using a chlorine dioxide test kit provided by the manufacturer.

Biofilm formation on materials commonly used in the meat industry. Biofilm formation on materials commonly used in the meat industry, including stainless steel (SS) and polyvinyl chloride (PVC) plastic, was quantified by cell numbers using a colony enumeration method on agar plates. Sterile SS chips (18 by 18 by 2 mm) and PVC chips (14 by 12 by 3 mm) were prepared as platforms on which biofilms were developed by incubating the chips for 3 days at 22 to 25°C (room temperature conditions) or for 5 days at 7°C (fabrication conditions) in diluted bacterial cultures prepared overnight in LB-NS and containing approximately 5×10^6 CFU/ml cells. After incubation, each chip was rinsed with 10 ml of sterile phosphate-buffered saline (pH 7.2), 5 ml on each side, and then transferred to a centrifuge tube containing sterile LB-NS with glass beads (425 to 600 μm; Sigma-Aldrich, St. Louis, MO). The SS chips were transferred to 50-ml centrifuge tubes, each containing 1 g of glass beads in 10 ml of LB-NS; the PVC chips were transferred to 15-ml centrifuge tubes, each containing 0.5 g of glass beads in 5 ml of LB-NS. All tubes were vortexed at maximum speed for 2 min to remove the attached biofilm cells. The vortexed suspensions were 10-fold serially diluted in sterile LB-NS, and the appropriate dilutions were plated onto CHROM-Agar O157 plates (DRG International Inc., Mountainside, NJ) for colony enumeration after overnight incubation at 37°C. Biofilm formation was calculated as Log CFU per chip using the CFUs on the agar plates and their corresponding dilution factors.

Sanitizer and antimicrobial susceptibility tests. The MICs of the two sanitizers for each strain were determined using a broth microdilution method and sterile 96-well flat-bottom polystyrene plates (Costar, Corning, NY). *E. coli* O157:H7 cultures were diluted overnight in cation-adjusted Mueller-Hinton broth (Sigma) to reach a cell concentration of approximately 2×10^5 cells per ml and were then added to the plates at 100 μl per well. Control wells contained sterile Mueller-Hinton broth only. The sanitizer concentrations were examined in twofold increments. To reach the desired final concentrations of the sanitizer in each well, we prepared a twofold concentration of the sanitizer and added it to the plate at 100 μl per well. Bacterial growth was recorded after a 24-h incubation at 25°C using a microtiter plate reader set at 600 nm (Molecular Devices, Sunnyvale, CA). The lowest concentration of the sanitizer that completely inhibited bacterial growth was recorded as the MIC for each strain.

Antimicrobial susceptibility testing was performed using the Sensititre broth microdilution system and CMV2AGNF plates (TREK Diagnostic Systems, Cleveland, OH) to determine the MICs of each of the 15 antimicrobial agents, as described in previous work *(13).* The antimicrobials and the breakpoints for resistance were amoxicillin–clavulanic acid, ≥32 and 16 μg/ml; ampicillin, ≥32 μg/ml; azithromycin, ≥32 μg/ml; cefoxitin, ≥32 μg/ml; ceftiofur, ≥8 μg/ml; ceftriaxone, ≥4 μg/ml; chloramphenicol, ≥32 μg/ml; ciprofloxacin, ≥4 μg/ml; gentamicin, ≥16 μg/ml; kanamycin, ≥64 μg/ml; nalidixic acid, ≥32 μg/ml; streptomycin, ≥64 μg/ml; sulfisoxazole, ≥512 μg/ml; tetracycline, ≥16 μg/ml; trimethoprim, ≥4 mg/ml; and sulfamethoxazole, ≥76 mg/ml.

Detection of disinfectant-resistant genes. PCR amplification was conducted to detect the presence of 10 known QAC-resistance genes and their potential association with resistance to QAC and other sanitizer reagents in the examined strains. The primers used to amplify the 10 resistant genes (*qacE, qacEΔ1, qacF, qacG, emrE, sugE(c), sugE(p), mdfA,* and *ydgE/ydgF*) and the experimental protocols were described in a previous study *(20).*

Copy number of the pO157 plasmid. To determine the average copy number of the pO157 plasmid, six *E. coli* O157:H7 isolates, freshly recovered from cattle feces, were used as template DNA. These were applied in duplicate duplex qPCR reactions targeting the plasmid encoded gene *ecf1* and the single copy

number chromosomal gene *eae*. Duplex qPCR reactions were performed as described in a previous article *(7)*. The plasmid copy number was determined using a relative quantification method, as described in a previous article *(15)*. To further confirm the qPCR results, we analyzed direct sequencing data to determine the pO157 plasmid copy number from three additional *E. coli* O157:H7 strains, and we determined the plasmid copy numbers of the examined strains by comparing the chromosome sequence coverage with the plasmid sequence coverage, as described in a previous article *(12)*.

To address the potential variation of the pO157 plasmid copy number due to multiple passages of bacterial growth, we selected five HEP strains and five control strains that demonstrated biofilm formation at various levels, cultured them individually in LB-NS medium overnight, and used the cultures to inoculate fresh sterile LB-NS medium the next day. All cultures were transferred daily for up to five passages, and we determined the pO157 plasmid copy numbers after the fifth passage, as previously described, and compared them to the results we obtained from the original strains before the sequential growth passages.

Statistical analysis. We stratified the data on biofilm formation on the contact surfaces (in Log CFU per chip) and sanitizer MICs (in parts per million) into frequency classes. The strain distributions of the frequency classes in the HEP and control strain collections were compared using the Pearson chi-square statistic. We compared the data on the pO157 plasmid copy numbers, biofilm formation on 96-well plates (optical density at 570 nm [$OD_{570 nm}$]), and bacterial recovery growth after sanitization ($OD_{433 nm}$) for the HEP and control strains and for the high and low plasmid copy number groups using unpaired *t* tests. $P < 0.05$ was considered statistically significant.

RESULTS

Biofilm formation on materials commonly used in the meat industry. All the strains developed biofilms on the SS and PVC surfaces at various levels under both temperature conditions. The ability to form biofilms was highly strain dependent, and strains with positive EPS (curli and cellulose) expressions on bacterial cell surfaces exhibited overall a stronger biofilm-forming ability than did strains with negative EPS expression (data not shown). At room temperature, the mean of total biofilm cells recovered from the SS and PVC surfaces was approximately 6.0 Log CFU per chip; under fabrication conditions (7°C), the average amount of bacteria in biofilms reached approximately 4.5 to 4.9 Log CFU per chip. Thus, the amount of biofilm cells recovered after 3 days of incubation at room temperature (22 to 25°C) was significantly higher than that recovered after 5 days of incubation at 7°C; this indicates that temperature was critical for *E. coli* O157:H7 colonization on contact surfaces.

When we classified the HEP and control strains into biofilm potency groups based on the logarithmic counts of biofilm cells (in Log CFU per chip) recovered from the contact surfaces, we found that at room temperature the HEP strains overall developed significantly stronger biofilms on the PVC surfaces than did the control strains ($P = 0.044$). In addition, on the SS surfaces, even though not statistically significant, we observed a consistent pattern of higher numbers of the HEP strains than the control strains

belonging to strong biofilm-forming groups (Fig. 1). We obtained a similar pattern of results under fabrication conditions. The HEP strains exhibited significantly higher potency of biofilm formation on the PVC surfaces at 7°C ($P = 0.018$). On the SS surfaces at 7°C, although no significant statistical difference was observed, again a higher number of HEP strains belonged to the strong biofilm-forming group (>5 Log CFU per chip) than did the control strains.

Sanitizer MIC. The sanitizer MIC distribution profiles of the HEP and control strains are shown in Table 1. All tested strains exhibited QAC MICs between 0.75 and 6 ppm. However, statistical analysis indicated that the control strains were significantly more susceptible to QAC than were the HEP strains ($P < 0.0001$). A total of 39 HEP strains (87% of the total HEP strains) showed a QAC MIC at 6 ppm, whereas only 15 control strains (32% of the total control strains) had a QAC MIC at this high level. Moreover, fewer HEP strains exhibited low QAC MICs than did the control strains.

A wide range of ClO_2 MICs, from 1.5 to 50 ppm, were observed. Again, the control strains overall exhibited a significantly higher susceptibility to the sanitizer than did the HEP strains ($P = 0.041$). Forty-one HEP strains (91% of total HEP strains) showed a ClO_2 MIC at 50 ppm, whereas only 29 control strains (62% of total control strains) had the same ClO_2 MIC at this high level. Moreover, more control strains exhibited ClO_2 MICs also at much lower levels, indicating that they have overall a higher susceptibility to the sanitizer.

Antimicrobial resistance. We tested all the strains for antibiotic resistance against 15 commonly used antimicrobial agents. Overall, a very low prevalence of antibiotic resistance was observed. Only one HEP strain was resistant to chloramphenicol. Within the control group, two strains were found to be antibiotic resistant, one to sulfisoxazole only and the other to sulfisoxazole and tetracycline.

Presence of the disinfectant-resistant genes. The five QAC-resistant genes that are chromosome encoded (*emrE, mdfA, sugE(c),* and *ydgE*/*ydgF*) were found in all the strains. In contrast, the other five QAC-resistant genes that have been identified on mobile genetic elements (MGEs; *qacE, qacEΔ1, qacF, qacG,* and *sugE(p)*) were not detected in any of the examined strains. Thus, there was no difference in the presence of the QAC-resistant genes between the HEP and the control strains.

Copy number of the pO157 plasmid. For each individual strain, we measured the plasmid copy number using the relative quantification method and direct sequencing analysis, generating a result that was the average of the actual plasmid copy numbers of the cell population used for DNA extraction for each strain. Overall, the pO157 plasmid copy number of the examined *E. coli* O157:H7 strains ranged from 1.5 to 2.7 copies per genome, with an average of approximately 2 copies. Note that only 6 (13% of all the HEP strains) of the 45 HEP strains had fewer than two
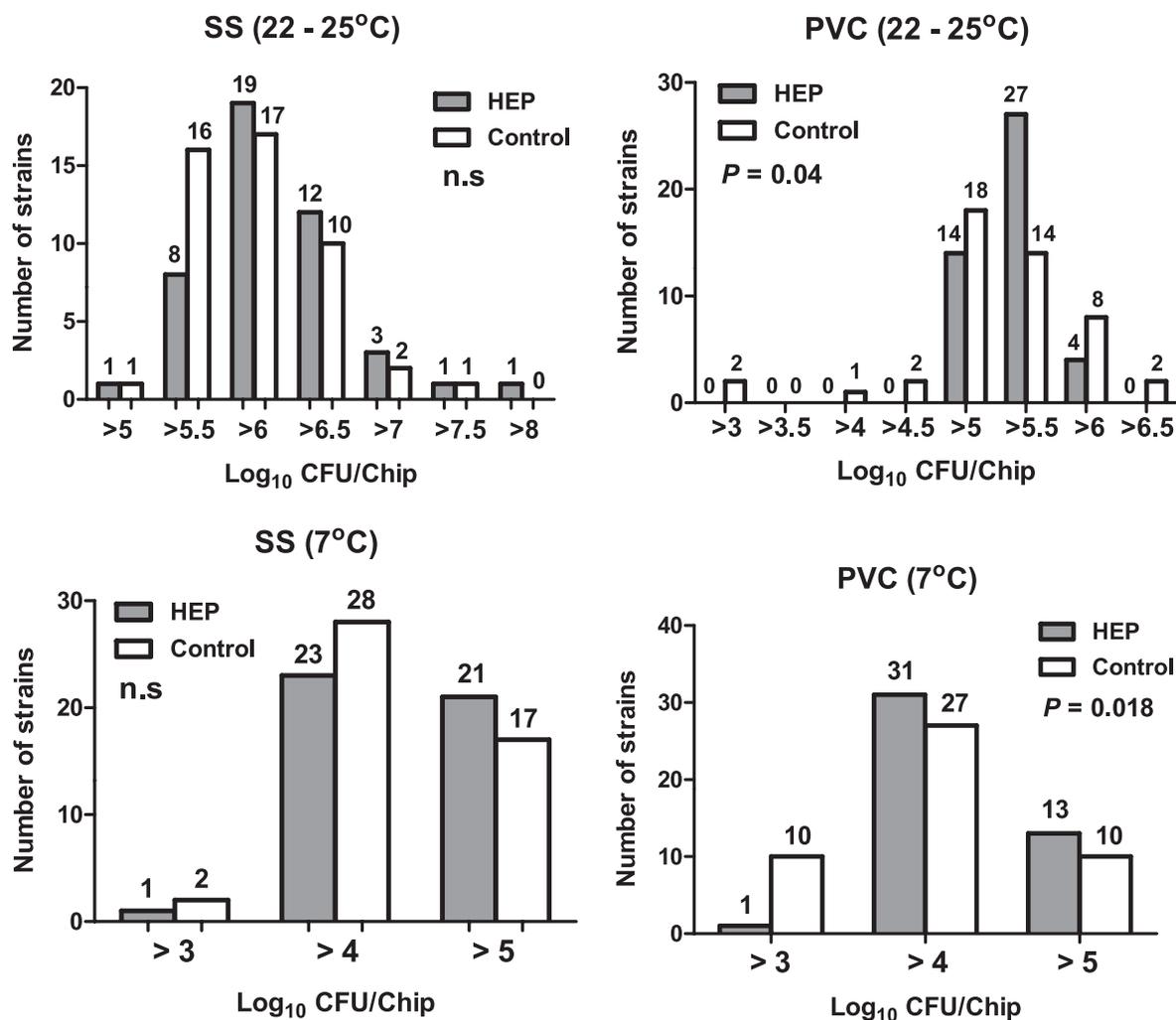
FIGURE 1. *Frequency distribution of* E. coli *O157:H7 HEP and control strains classified by their ability to form biofilms on SS and on PVC surfaces at room temperature (22 to 25°C; top left [SS] and top right [PVC]) and at 7°C (bottom left [SS] and bottom right [PVC]). Each strain was allowed to form biofilms on SS or PVC chip surfaces in LB-NS medium at 22 to 25°C for 3 days or at 7°C for 5 days. Data are the number of strains in each biofilm-forming potency group. Statistical analysis of frequency distributions was performed using the Pearson chi-square statistic; P values less than 0.05 were considered statistically significant. n.s., no statistical significance.*

TABLE 1. *MIC distribution for HEP and control* E. coli *O157:H7 strains for the two types of common sanitizers[a]*

Strain	No. (%) of strains with the MIC at a QAC concn (ppm) of:						P
	0.75	1.5	3	6			
HEP		1 (2)	5 (11)	39 (87)			
Control	2 (4)	3 (6)	27 (58)	15 (32)			<0.0001

Strain	No. (%) of strains with the MIC at a ClO$_2$ concn (ppm) of:						P
	1.5	3	6	12.5	25	50	
HEP				1 (2)	3 (7)	41 (91)	
Control	1 (2)	1 (2)	2 (4)	3 (6)	11 (24)	29 (62)	0.0408

[a] MIC tests were conducted using the microdilution method in cation-adjusted Mueller-Hinton broth on sterile 96-well polystyrene plates. Statistical analysis of the frequency distributions was performed using the Pearson chi-square statistic. P values less than 0.05 were considered statistically significant.

TABLE 2. *Distribution of* E. coli *O157:H7 HEP and control strains by their pO157 plasmid copy number*

No. of pO157 copies	No (%) of strains	
	HEP	Control
<2	6 (13)	27 (57)
>2	39 (87)	20 (43)

pO157 plasmid copy numbers; in contrast, 27 (57% of all the control strains) of the 47 control strains had fewer than two copies of the pO157 plasmid per genome (Table 2). When we averaged the plasmid copy numbers within each strain group and compared the HEP and control strains, the results again indicated that the HEP strains overall retained significantly higher plasmid copy numbers (Fig. 2) than the control strains ($P < 0.0001$). Experiments further indicated that the copy number of the pO157 plasmid was stable after multiple passages of bacterial growth (data not shown).

Association between the pO157 plasmid and bacterial biofilm-forming ability. Our previous study *(17)* using crystal violet (CV) staining and absorbance measurement ($OD_{570 nm}$) indicated that the HEP strains had a significantly higher level of mature biofilm formation on 96-well plates (after incubating 4 to 6 days) than the control strains but that there was no difference between them in early-stage biofilm formation (after incubating 1 to 3 days). To investigate the potential role of the pO157 plasmid in this phenomenon and because we identified an average of two copies of the plasmid per genome across the examined strains, we pooled the HEP and control strains and then classified them into two groups based on their plasmid copy numbers: the "above average" copy number group (more than two copies) and the "below average" copy number group (fewer than two copies). Biofilm formation ($OD_{570 nm}$) on each day from day 1 to 6, measured by CV staining in our previous study *(17)*, was averaged for each plasmid copy number group. Our statistical analysis indicated that no difference was observed between the two groups with respect to biofilm development on days 1, 2, and 3; however, the above average group exhibited significantly stronger biofilm formation on days 4, 5, and 6 (Fig. 3).

Association between the pO157 plasmid and bacterial survival and recovery capability. We developed an absorbance assay in our previous study *(17)* to measure bacterial survival and recovery growth after sanitizer treatment. The assay was based on the color shift of the Dey-Engley neutralizing broth, which was positively correlated to the amount of bacterial cells surviving sanitization and thus remaining culturable, and the assay was also further quantitatively correlated to the post-incubation cell density because the color shift of the medium from purple to yellow is a result of carbohydrate fermentation from bacterial growth that can be quantified by measuring the absorbance ($OD_{433 nm}$). To investigate the potential role of the pO157 plasmid in the bacterial survival
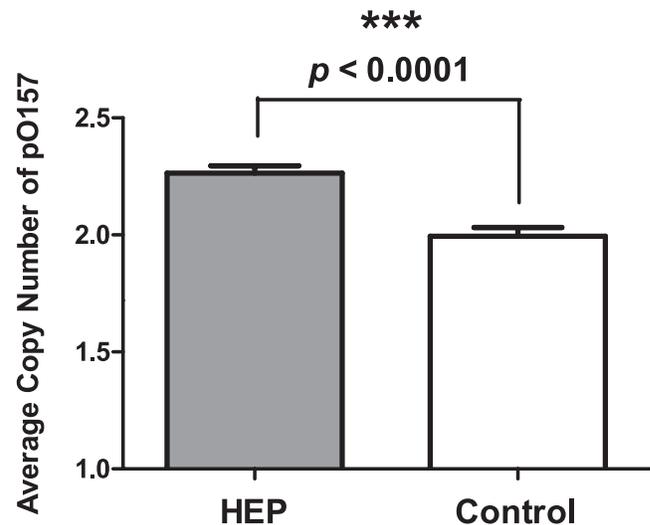

FIGURE 2. *Average copy numbers of the pO157 plasmid in the HEP and control strains. Statistical analysis was performed using unpaired* t *tests. Error bars represent the mean* ± *standard deviation.* P *values less than 0.05 were considered statistically significant.* ***, P < *0.001.*

and recovery capability, we pooled our previous data on bacterial recovery growth *(17)* in Dey-Engley neutralizing broth after 3-day biofilms were treated with sanitizers at recommended concentrations and averaged them for each plasmid copy number group. Our statistical analysis indicated that the above average group had significantly stronger survival and recovery growth after QAC (300 ppm) and ClO_2 (50 ppm) treatments than did the below average group (Fig. 4), even though no significant difference in biofilm formation on day 3 was observed between these two plasmid copy number groups (Fig. 3; $P = 0.46$).

Association between the pO157 plasmid and bacterial-sanitizer susceptibility. To investigate potential involvement of the pO157 plasmid in sanitizer susceptibility, we analyzed the MICs of the two sanitizers and compared them for the high and low plasmid copy number groups. Our statistical analysis indicated that the above average group had a significantly higher QAC MIC range than the below average group (Table 3; $P = 0.031$). Even though the difference in ClO_2 MICs of the two plasmid copy number groups was not statistically significant, more strains in the above average group exhibited high ClO_2 MICs than in the below average group (Table 3).

DISCUSSION

Biofilms formed by foodborne pathogens in the food industrial environments may have a significant impact on public health because of their potential to cause food contamination. Because of their enhanced resistance to sanitization, it is difficult to completely inactivate biofilms that have colonized food contact surfaces; thus, any improperly or incompletely sanitized contact surface or food processing equipment could reintroduce pathogens into food products. It was reported *(9)* that one of the most common transmission routes for bacteria that cause

FIGURE 3. *Biofilm formation on 96-well polystyrene plates by* E. coli *O157:H7 strains classified as "above average" (>2) or "below average" (<2) based on their pO157 plasmid copy numbers. Each strain was allowed to form biofilms on 96-well polystyrene plates in LB-NS medium at 22 to 25°C for 1 to 6 days. They were then stained with CV, and their absorbance was measured at 570 nm; n = 4. Error bars represent the mean ± standard deviation. Statistical analysis was performed using unpaired t tests. P values less than 0.05 were considered statistically significant. *, P < 0.05; **, P < 0.01.*
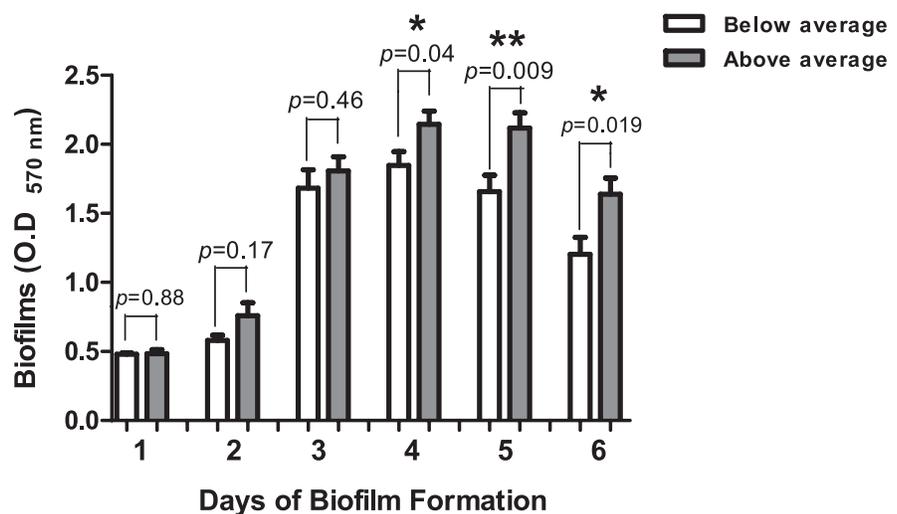
contamination in the food industry is cell transfer from contact surfaces to food products via direct contact. In the present study, we investigated the potential contribution of biofilm formation and sanitizer resistance to HEP beef trim contamination by *E. coli* O157:H7. We first evaluated the biofilm-forming ability of the HEP and control strains on materials commonly used in the meat industry, and the results are in agreement with our previous data *(17)*, obtained on 96-well polystyrene plates using CV staining; the HEP strains have higher biofilm-forming ability than the control strains.

A previous study *(20)* showed that high disinfectant MICs of *E. coli* strains were associated with the presence of disinfectant-resistant genes, especially the *qac* and *sugE(p)* genes. Because the HEP strains exhibited significantly higher QAC and ClO$_2$ MICs (Table 1) than the control strains, we investigated the distribution of the 10 known QAC-resistant genes and their potential association with the high sanitizer MICs of the HEP strains. The five chromosome-encoded QAC-resistant genes were found in all HEP and control strains, but none of the MGE-encoded resistant genes was detected; thus, the presence of these

resistant genes is not the determining factor for the higher QAC and ClO$_2$ MICs of the HEP strains. Our results are also in agreement with the recent findings *(20)* that the chromosome-encoded QAC-resistant genes are commonly present in 77.2 to 100% of the *E. coli* strains isolated from retail meats in the United States but the other MGE-encoded resistance genes are much less prevalent. Several studies *(3, 10, 14, 20)* have shown that these chromosome-encoded efflux pump genes could induce QAC resistance but do not confer cross-resistance to antimicrobials, whereas the presence of the MGE-encoded QAC-resistant genes, which frequently coexist with various antimicrobial resistant genes, is often associated with resistance to multiple antimicrobial classes. This is in agreement with our present study, which identified a very low prevalence of antimicrobial resistance in all the examined strains along with the absence of the MGE-encoded QAC-resistant genes.

The pO157 plasmid is a 92-kb virulence plasmid that encodes a number of putative virulence determinants in *E. coli* O157:H7. Several genes located on the pO157 plasmid have been found to be important for biofilm formation, bacterial adherence to colonic epithelial cells, and in vivo

FIGURE 4. *Postsanitization recovery growth of* E. coli *O157:H7 strains classified as "above average" (>2) or "below average" (<2) based on their pO157 plasmid copy numbers. QAC and ClO$_2$ solutions were prepared in sterile distilled water and used at recommended final concentrations of 300 and 50 ppm, respectively. Biofilms formed by the* E. coli *O157:H7 strains on 96-well polystyrene plates for 3 days were treated with each sanitizer, and the bacterial recovery growth after sanitization in Dey-Engley broth was measured using absorbance values obtained at 433 nm; n = 4. Error bars represent the mean ± standard deviation. Statistical analysis was performed using unpaired t tests. P values less than 0.05 were considered statistically significant. *, P < 0.05.*
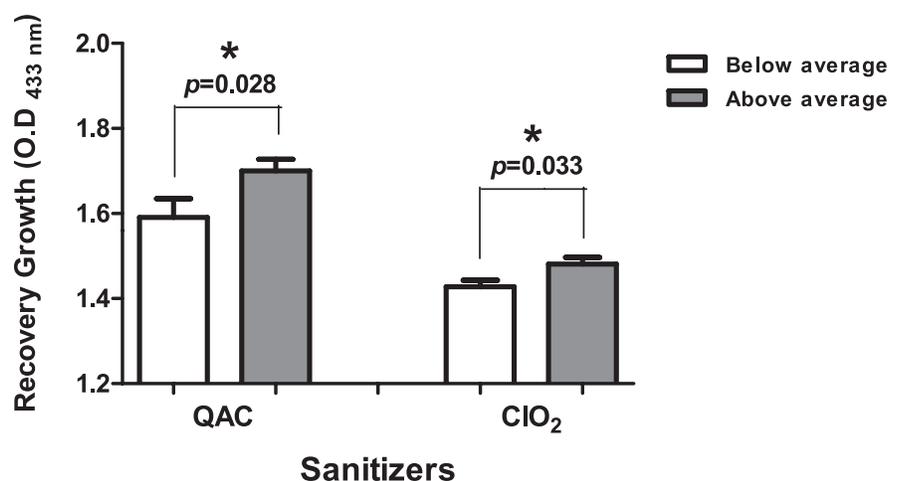
TABLE 3. *MIC distribution for the examined strains grouped by pO157 plasmid copy number*[a]

pO157 copy number group	No. (%) of strains with the MIC at a QAC concn (ppm) of:				P
	0.75	1.5	3	6	
Below average		3 (9)	16 (48)	14 (43)	
Above average	2 (3)	1 (2)	16 (27)	40 (68)	0.031

pO157 copy number group	No. (%) of strains with the MIC at a ClO_2 concn (ppm) of:						P
	1.5	3	6	12.5	25	50	
Below average	1 (3)		1 (3)	2 (6)	4 (12)	25 (76)	
Above average		1 (2)	1 (2)	2 (4)	10 (16)	45 (76)	0.67

[a] Strains were classified as "above average" (>2) or "below average" (<2) based on the number of pO157 plasmid copies. MIC tests were conducted using the microdilution method in cation-adjusted Mueller-Hinton broth on sterile 96-well polystyrene plates. Statistical analysis of the frequency distributions was performed using the Pearson chi-square statistic. P values less than 0.05 were considered statistically significant.

bovine colonization *(4, 5)*. To investigate the potential involvement of the pO157 plasmid in the significant difference in biofilm-forming ability between the HEP and control strains, we measured and compared the copy numbers of the plasmid in the strains. We found an average of two copies of the plasmid per genome in our present study, which is within the same range as the recent determination *(7, 19)* that two pO157 plasmids per chromosome is the average for *E. coli* O157:H7 strains and across the enterohemorrhagic *E. coli* isolates. Our results further indicate that the HEP strains overall retain a higher number of copies of the pO157 plasmid than the control strains and that the plasmid copy number is stable after multiple passages of cell growth.

Our previous study *(17)* indicated that the HEP strains have a stronger mature biofilm-forming ability than the control strains; however, bacterial EPS was not the determining factor for this observation because we observed no difference in EPS expression between the HEP and control stains. In addition, numerous investigations relevant to our present study have reported the involvement in biofilm formation of multiple genes encoded on the pO157 plasmid as well as the requirement of the plasmid for *E. coli* O157:H7 colonization in cattle *(5, 6, 11)*. Our current study further confirms that the strong mature biofilm formation by *E. coli* O157:H7 strains, observed on 96-well plates using CV staining in our previous study *(17)*, correlates with the high plasmid copy number that the strains harbor (Fig. 3). However, we observed no statistical difference (data not shown) between the high and low plasmid copy number groups in biofilm development on the PVC and SS surfaces, even though we observed similar trends in the results indicating that more of the strains with high plasmid copy numbers developed stronger biofilms on the chips than strains with low plasmid copy numbers.

Caution should be taken when interpreting and comparing these results because the technical and accuracy limitations of each experimental method might affect the observations. For example, CV staining is based on the assumption that all the biofilm cells are completely and equally stained by the dye. However, for the strong biofilm formers with multiple layers of bacterial cells, CV diffusion through the three-dimensional biofilm architectures is not as efficient and homogeneous as for the weak biofilm formers. In addition, the accurate quantification of biofilms using the colony enumeration method is even more difficult to achieve because the biofilms adhere to the chip surface and the harvested biofilm cells are most likely aggregating at various levels; so, the effectiveness of vortexing with glass beads to remove the biofilms from the surfaces and to disrupt cell-cell aggregates will vary depending on the degree of tightness of the cell–chip surface adhesions and cell-cell adhesions. The incomplete removal of the biofilms from the chip surface and the incomplete disruption of the cell aggregates, which are more likely in strong biofilm formers, will result in lower colony enumeration results than the actual numbers of cells in the biofilms on the surfaces; this will in turn affect any significance that might have been observed using a statistical analysis of more accurate data. Because it is not feasible to evaluate or measure the degree of tightness of cell adhesion and aggregation, which is apparently not the same for all bacterial strains, the limitations of the experimental method and the inaccuracy of colony enumeration data are not avoidable.

The pO157 plasmid is highly conserved in *E. coli* O157:H7 and has been identified in 99% of clinical isolates, but the role of this plasmid in the capability of the bacteria to survive under stressful and adverse circumstances is largely unidentified. Available results have suggested that the plasmid encodes critical virulence factors required for the optimal survival and persistence of the bacteria in the environment and in the host *(6)*. A previous study *(4)* has shown that a wild-type *E. coli* O157:H7 strain was able to outcompete its ΔpO157 mutant strain (which completely lacks the plasmid) in an in vitro cell competition assay, suggesting that genes encoded on the pO157 plasmid could affect the metabolic activity and growth capability of the bacteria. In the present study, our sanitizer MIC data indicate that the HEP strains were significantly less susceptible to QAC and ClO_2 than were the control strains (Table 1), but we observed no difference in the presence of the disinfectant-resistant genes. Our data analysis further suggests that the high pO157 plasmid copy number might play a role in low sanitizer susceptibility (Table 3), although

the level of the influence of the plasmid on the susceptibility to the bacterial sanitizer might also be affected by the different microbial inactivation mechanisms employed by the various sanitizers.

Our previous study (17) observed that the 3-day biofilms on 96-well plates produced by the HEP strains had a stronger resistance to and higher recovery growth after QAC and ClO_2 treatments than the biofilms produced by the control strains, even though the biofilm formations produced by the HEP and control strains on day 3 showed no significant differences. Our current study further indicates that the strong survival and recovery capability was positively correlated to the strains' high pO157 plasmid copy number, even though the biofilm development of the high and low plasmid copy number groups showed no significant differences (Figs. 3 and 4). This also confirms our previous observation that the high sanitization tolerance and recovery capability of E. coli O157:H7 are not solely dependent on biofilm mass development or bacterial EPS expression (16) but might also be related to other regulation mechanisms influenced by the presence of the pO157 plasmid. Available results (6) have suggested that the effects of the pO157 plasmid on bacterial metabolism, colonization, and survival capability might occur either directly through increased gene expression on the pO157 plasmid or indirectly via the regulation of chromosomal gene expressions by the presence of the plasmid. This regulation mechanism, whether directly correlated with gene expression on the pO157 plasmid or acting indirectly via chromosomal gene regulations, requires further investigations.

In summary, our data show that, compared with the control strains, E. coli O157:H7 strains isolated from HEPs had a stronger biofilm-forming ability on materials commonly used in the meat industry and a lower susceptibility to common sanitizers. The HEP strains also harbored higher copy numbers of the pO157 plasmid, which are positively correlated to stronger mature biofilm formation and to a higher survival and recovery growth capability after sanitization. Taken together, it is reasonable to speculate that the pO157 plasmid may play important roles in the phenotypes of biofilm formation and sanitizer resistance in certain E. coli O157:H7 strains that have a higher potential of causing HEPs in the meat plants.

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