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Influence of Plasmid pO157 on *Escherichia coli* O157:H7 Sakai Biofilm Formation▽

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The role of plasmid pO157 in biofilm formation was investigated using wild-type and pO157-cured *Escherichia coli* O157:H7 Sakai. Compared to the wild type, the biofilm formed by the pO157-cured mutant produced fewer extracellular carbohydrates, had lower viscosity, and did not give rise to colony morphology variants that hyperadhered to solid surfaces.

Enterohemorrhagic *Escherichia coli* serotype O157:H7 is a major food-borne pathogen causing hemorrhagic colitis and the hemolytic-uremic syndrome (17). Many *E. coli* O157:H7 outbreaks have been associated with contaminated undercooked ground beef, vegetables, fruits, and sprouts (20, 31). One of the largest disease outbreaks occurred in Sakai City, Japan, in 1996 with nearly 8,000 confirmed cases. The *E. coli* isolate responsible for this outbreak, referred to as "Sakai," is one of the best-characterized isolates and one of only three O157 strains for which the genome has been fully sequenced (8, 16). Because of its importance as a human pathogen and its characterization, Sakai was the focus of this investigation.

There is significant phenotypic diversity among *E. coli* O157:H7 strains, including the ability to form biofilm. Previous studies show that certain *E. coli* O157:H7 strains form biofilm on various surfaces, and biofilm on food or food-processing surfaces can serve as a source or vehicle of contamination that may result in human infection (6, 18, 25). Biofilm is an organized and structured community of microorganisms that attaches to solid surfaces and contains cells embedded in an extracellular polymer matrix (4, 26). Exopolysaccharide (EPS) is a major component of the biofilm matrix and is required for the development of characteristic biofilm architecture (5, 29). Bacteria gain a variety of advantages from biofilm formation that include attachment, colonization, and protection from adverse environments (4, 11).

E. coli O157:H7 carries a 92-kb virulence plasmid (pO157) encoding a number of putative virulence determinants, including *ehxA*, *etpC* to *etpO*, *espP*, *katP*, *toxB*, *ecf*, and *stcE* (31). However, the biological role of pO157 is not fully understood, and only 19 genes among the 100 open reading frames (ORFs) in pO157 have been characterized (2, 15). Our previous work indicates that pO157 is a colonization factor in cattle and may regulate several chromosomal genes (14, 24, 31).

To investigate the role of pO157 in biofilm formation, we characterized the biofilm of wild-type *E. coli* O157:H7 strain Sakai and an isogenic pO157-cured Sakai (Sakai-Cu). Both strains were kindly provided by C. Sasakawa (University of Tokyo). Sakai-Cu was generated using a plasmid incompatibility method (27). This method is not prone to secondary mutations and requires minimal passage in laboratory medium. The mini-R plasmid pK2368, harboring a chloramphenicol (CM) resistance gene and being in the same plasmid incompatibility group as pO157, was introduced into wild-type Sakai by transformation. Transformants were isolated on LB agar containing CM and selected for loss of pO157 by agarose gel electrophoresis analysis. CM-resistant transformants were cured of pKP2368 by subculturing in LB broth without CM. The absence of pO157 was confirmed by Southern blot hybridization with a pO157-specific gene probe (derived from *ecf1*), and chromosomal DNA integrity was confirmed by pulsed-field gel electrophoresis (data not shown).

Because *E. coli* O157:H7 strains are generally not strong biofilm producers, the condition most conducive to biofilm production, a fluorometric flow cell method, was used to compare separately grown Sakai and Sakai-CU (3). The biofilm cultivation systems consisted of seven parts: (i) medium reservoir, (ii) multichannel pump (205S; Watson Marlow, United Kingdom), (iii) bubble trap (BioSurface Technologies Co., Bozeman, MT), (iv) flow cell, (v) outflow reservoir, (vi) air pump (DrsFosterSmith, Rhinelander, WI), and (vii) flow meter (Gilmont, BC Group, St. Louis, MO). The flow cell was constructed from two rectangular acrylic plates that were 104 by 48 mm. Sidewalls (62 by 26 by 5 mm) were glued to the top plate to form an elongated hexagonal growth chamber. There were 56- by 20-mm square openings in the top and bottom rectangular plates that were sealed with 60- by 24-mm glass slides (Fisher, Pittsburgh, PA). The upper and lower plates were assembled with screws and sealed using a microseal B film (MJ Research, Waltham, MA). The flow cell volume was about 10.4 ml, the medium flow rate was 10.5 ml/h, and the hydraulic retention time was 1 h. Under these conditions, the linear surface velocity was about 80 mm/h at the center of the flow cell. The biofilm was grown with BGM2 medium (21). To prepare the inoculums, Sakai and Sakai-Cu were grown at 37°C in BGM2 medium to mid-exponential phase, and cells were harvested by centrifugation and resuspended in 0.85% NaCl. One hundred μl of the resuspended cell solution was inoculated from the effluent side of flow cells through a long stainless steel needle (Fisher, Pittsburgh, PA). The cells were incubated for at least 3 h without

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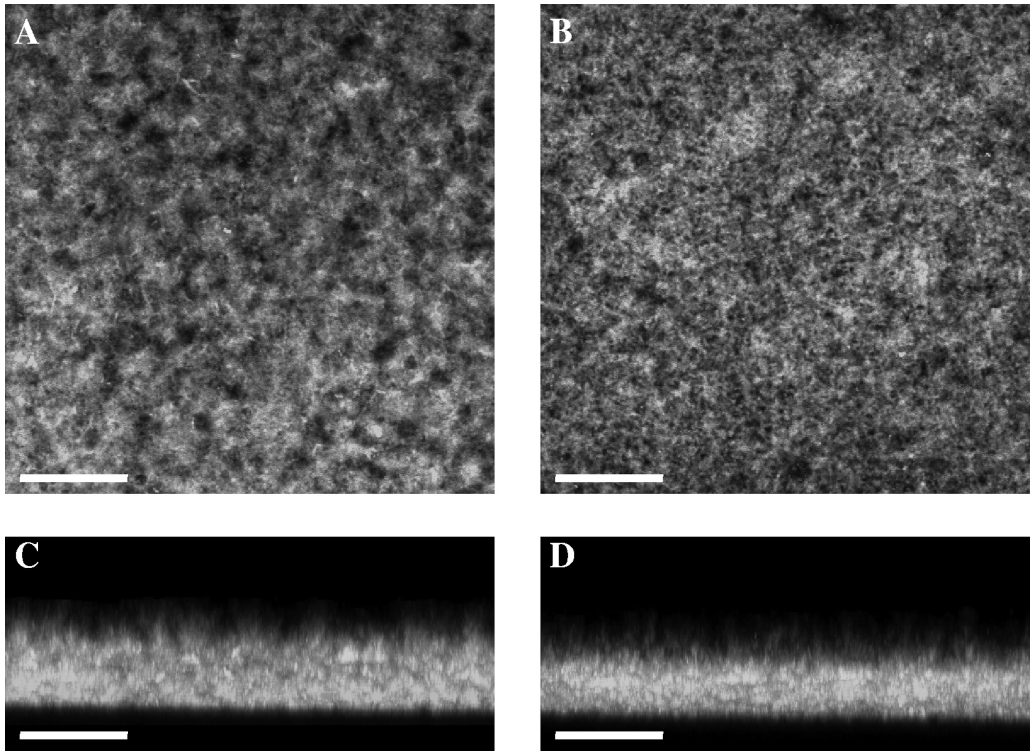

FIG. 1. Wild-type Sakai (A and C) or Sakai-Cu (B and D) biofilms after 3 days of incubation. Both strains were grown at 30°C in an individual flow cell apparatus. The biofilm was stained with WGA-Alexa Fluor 488 and examined by confocal microscopy. Representative overhead (A and B) or sagittal (C and D) images are shown and were generated using the deconvolution software. Bar, 50 μm.

supplying fresh medium, and then fresh medium was supplied to the biofilm cultivation system at 30°C.

At various times, the resulting biofilms were stained with a green fluorescent dye, wheat germ agglutinin (WGA)- Alexa Fluor 488 (Invitrogen, Carlsbad, CA), and analyzed using the Olympus FluoView confocal laser scanning microscopy system (Olympus, Tokyo, Japan). Using the Olympus FluoView software program, version 1.7b, for analysis, the fluorescence intensities of Sakai and Sakai-Cu biofilm matrices were each analyzed from >20 three-dimensional-complexity images. Fluorescence was greater for Sakai than for Sakai-Cu, with average values of 2,448 ± 668 and 2,022 ± 619, respectively (Student's t test; $P < 0.05$). Overhead images from the Sakai-Cu strain biofilm revealed more-compact cell clusters than images from wild-type Sakai (Fig. 1A and B). Comparisons of images taken sideways indicated that the Sakai-Cu biofilms were not as thick as those of wild-type Sakai (Fig. 1C and D), and typical ratios were consistently 9:11, respectively ($P < 0.05$). A previous study demonstrated that the biofilm of a *wcaF*::*can* mutant of *E. coli* K-12, which is deficient in EPS production, lacked depth and complex architecture (5). Sakai-Cu showed a similar but less dramatic phenomenon. These observations indicated that pO157 influenced biofilm formation and architecture.

To quantitatively compare Sakai and Sakai-Cu biofilms, the contents of each flow cell apparatus were collected at various times and analyzed for bacterial cell number, viscosity, and EPS production. Biofilms were harvested by a standard technique that preserves cell numbers and minimizes viscosity changes (9). Briefly, floating cells in the biofilm were carefully collected with a pipet, and the remaining cells were scraped from the flow cell apparatus with sterilized applicator sticks. Biofilm samples were collected on days 1, 3, 5, 8, and 12, and measurements were means ± standard deviations (SD) of at least triplicate measurements from separately grown biofilms. There was no significant difference in bacterial number (CFU/ml) from Sakai and Sakai-Cu biofilms at any of the times measured (data not shown). A Cannon-Fenske routine viscometer (Size 100; Cannon Instrument Co., Pennsylvania) was used to determine biofilm viscosity. The conversion constant was 0.015 cSt/s (mm^2/s^2), and viscosities were measured according to the manufacturer's instructions. Briefly, the viscometer was aligned vertically in the holder, and the sample was charged into the viscometer tube until the sample reached the "F" mark in the tube. A suction bulb was used to draw the sample slightly above mark "E." The sample was allowed to flow freely, and the efflux time was measured as the time for the meniscus to pass from mark "E" to mark "F." Measurements were repeated at least six times, and the kinematic viscosity in mm^2/s (cSt) of the samples was calculated by multiplying the efflux time in seconds by the viscometer constant. The viscosity of Sakai biofilm was dramatically increased after 8 days ($P < 0.001$), while there was no significant change in the viscosities of Sakai-Cu biofilms through day 12 (Fig. 2).

Bacterial EPS are associated with attachment to both inanimate surfaces and host cells (29). EPS can be categorized as extracellular carbohydrate complexes (ECC) that are loosely associated with cells and easily removed, referred to as slime (fraction I), or ECC that are closely associated with cells and removed

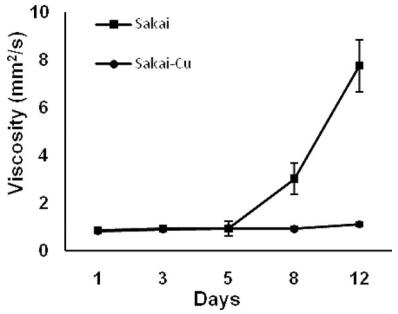

FIG. 2. Comparison of Sakai and Sakai-Cu biofilm viscosity. Three or four separately grown biofilms were each harvested on the days indicated, and viscosity was measured using a Cannon-Fenske Routine viscometer.

only after heat treatment, referred to as capsule (fraction II) (22). No significant difference in ECC was observed until days eight and 12, when the level of total ECC produced from Sakai biofilms was significantly higher than that from the Sakai-Cu biofilms ($P < 0.05$) (Fig. 3). Also, by days eight and 12, levels of Sakai ECC fraction I, representing primarily secreted slime carbohydrates, were 5 and 10 times higher than Sakai-Cu ECC fraction I, respectively. These results correlated with the results of increased viscosity in Sakai biofilm samples that had aged for 8 or 12 days.

Interestingly, during biofilm sampling, two colony morphology variants were isolated that are referred to here as sticky and mucoid. These variants were found only in wild-type Sakai biofilms that had aged for ≥8 days and were not found in Sakai-Cu biofilms even after screening of 10^4 colonies and even among biofilms aged for 18 days. The percentages of sticky and mucoid variants in Sakai biofilms ranged from 5 to 30% and 0 to 5%, respectively. The differences in colony morphology were readily distinguished, as shown in Fig. 4. The sticky variant was raised in elevation and shinier than the Sakai parent strain but was not difference in size. When single bacterial colonies grown on agar plates were touched with a sterilized toothpick and that toothpick was gently lifted up, the colonies

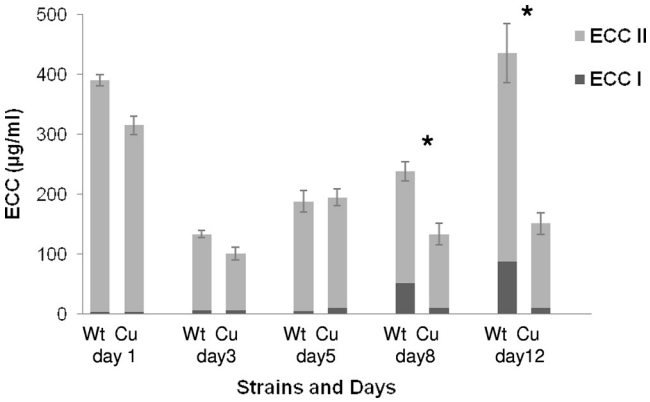

FIG. 3. Comparison of Sakai and Sakai-Cu biofilm extracellular carbohydrate (ECC) production. ECC I was collected from cells by centrifugation, and ECC II was collected by centrifugation after heat treatment on each indicated day. Bar height represents total ECC production from each biofilm sample. The proportion of total ECC that was either ECC I (dark gray) or ECC II (light gray) is shown. Asterisks indicate significant differences between wild-type Sakai (Wt) and Sakai-Cu (Cu); day 8, $P < 0.05$; day 12, $P < 0.001$.

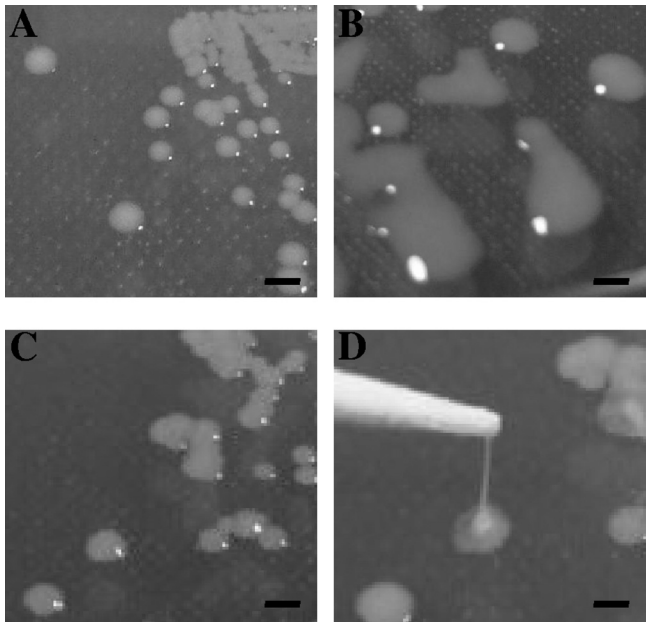

FIG. 4. Colony morphologies of wild-type, mucoid, and sticky variants. The wild-type *E. coli* O157:H7 Sakai strain formed small, flat, and nonsticky colonies on LB agar (A). The mucoid variant formed irregular, large, shiny, mucoid, convex, and nonsticky colonies (B). The sticky variant formed small, slightly raised, and sticky colonies (C). The sticky variant adheres to a toothpick touched to the colony surface (D). Bar, 1 cm.

had a hyperadherence phenotype and elongated to approximately 1 cm between the plate and the toothpick. This phenomenon was unique to the sticky colony variants and was not observed among colonies of the parent Sakai strain (Fig. 4D). The mucoid colony variants were convex in elevation and shiny in texture, had irregular colony shapes, and were larger than the Sakai parent strain but were not hyperadherent. The motility of variants was determined using 0.3% soft agar, and both sticky and mucoid variants exhibited 30- to 90%-reduced motility compared to the parent Sakai strain (data not shown). The characteristics of both sticky and mucoid variants were inherited, and the variant characteristics were maintained in laboratory subculture through 15 generations.

It is known that mutation is a powerful mechanism of adaptation when bacteria are faced with environmental change (1). Like other bacterial variants, the sticky and mucoid phenotypic biofilm variants may provide a survival advantage in specific niches (10, 19). *Pseudomonas aeruginosa* is a well-known biofilm model, and colony morphology variants are a common biofilm-related phenomenon. Both reduced-motility and hyperadherence variants have been described (10) and have characteristics similar to those of the *E. coli* O157:H7 biofilm variants described here. However, unlike the *P. aeruginosa* biofilm variants, the sticky and mucoid Sakai variants were not smaller, rougher, or more wrinkled than the parent colony.

Although it is possible that the changes measured in biofilm formation and the generation of hyperadherent variants were not due to the plasmid, it is highly unlikely. The method of plasmid curing by incompatibility is gentle and is not prone to secondary mutation. A powerful and common approach to address possible secondary mutations is complementation;

however, it was not used here because reintroduction of the plasmid requires the manipulation of a very large piece of DNA (92 kb) and the procedure itself is likely to introduce mutation. Also, reintroduction of the large 92-kb pO157 plasmid would require antibiotic resistance for efficient selection, and this may influence biofilm formation.

Many regulatory mechanisms are involved in biofilm formation (7, 12, 13, 28, 30, 32). Among those mechanisms, the relationship between biofilm formation and acid resistance is well known. Biofilm formation is upregulated after the deletion of the *gad* or *hde* gene, which allows bacteria to survive under acidic conditions (12). Previously we showed that an isogenic pO157-cured strain of *E. coli* O157:H7, ATCC 43894, enhanced acid resistance through increased expression of Gad (14). Similarly, Sakai-Cu has enhanced acid resistance compared to wild-type Sakai (data not shown and J. Y. Lim, B. Hong, H. Sheng, S. Shringi, R. Kaul, and C. J. Hovde, submitted for publication). The link between increased acid resistance and reduced biofilm formation, reduced ESP production, reduced viscosity, and lack of colony morphology variants was not explored here. Comparisons of biofilm formation were not made between these two strains because neither wild-type *E. coli* O157:H7 ATCC 43894 nor its plasmid-cured strain form significant biofilm under the laboratory conditions tested (data not shown).

Two pO157-cured *E. coli* O157 strains (ATCC 43894 and Sakai) do not colonize cattle as well as their wild-type counterpart (14, 24). The mechanism for this difference may be related to pO157 encoding a set of putative type II secretion genes, *etpC* to *etpM*, *etpO*, and *etpS*, and these *etp* genes may be associated with protein secretion required for efficient adherence (23). Tatsuno et al. reported that the *toxB* gene encoded on pO157 is required for the full epithelial cell adherence phenotype (27). These results may relate to the defect of Sakai-Cu in biofilm formation.

In conclusion, this is the first report that pO157 affects biofilm formation of *E. coli* O157:H7 Sakai through increased EPS production and generation of hyperadherent variants. Further study of biofilm formation under a variety of conditions and comparisons of Sakai with other *E. coli* O157:H7 strains will be important for understanding the relationship between biofilm formation and *E. coli* O157:H7 virulence and survival on foods and in the farm environment.

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