

# Coculture of *Escherichia coli* O157:H7 with a Nonpathogenic *E. coli* Strain Increases Toxin Production and Virulence in a Germfree Mouse Model

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*Escherichia coli* O157:H7 is a notorious foodborne pathogen due to its low infectious dose and the disease symptoms it causes, which include bloody diarrhea and severe abdominal cramps. In some cases, the disease progresses to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), due to the expression of one or more Shiga toxins (Stx). Isoforms of Stx, including Stx2a, are encoded within temperate prophages. In the presence of certain antibiotics, phage induction occurs, which also increases the expression of toxin genes. Additionally, increased Stx2 accumulation has been reported when O157:H7 was cocultured with phage-susceptible nonpathogenic *E. coli*. This study characterized an *E. coli* O157:H7 strain, designated PA2, that belongs to the hypervirulent clade 8 cluster. Stx2a levels after ciprofloxacin induction were lower for PA2 than for the prototypical outbreak strains Sakai and EDL933. However, during coculture with the nonpathogenic strain *E. coli* C600, PA2 produced Stx2a levels that were 2- to 12-fold higher than those observed during coculture with EDL933 and Sakai, respectively. Germfree mice colonized by PA2 and C600 showed greater kidney damage, increased Stx2a accumulation in feces, and more visible signs of disease than mice given PA2 or C600 alone. These data suggest one mechanism by which microorganisms associated with the colonic microbiota could enhance the virulence of *E. coli* O157:H7, particularly a subset of clade 8 strains.

*Escherichia coli* can be either nonpathogenic, as exemplified by the bacteria associated with the normal human gut microflora, or pathogenic, classified as intestinal or extraintestinal (1). The intestinal pathogenic *E. coli* group is further subdivided into six pathotypes, among which enterohemorrhagic *E. coli* (EHEC) is a causative agent of bloody diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS). Shiga toxins (Stx) are one of the defining virulence factors of *E. coli* O157:H7 (2, 3). The *stx* genes are carried within lambdoid prophages downstream of the promoter for late gene expression. The toxin is released after phage-induced lysis of the bacterial cell (4). Shiga toxins are approximately 70-kDa proteins, consisting of an enzymatically active A subunit and a pentameric B subunit. The A subunit is an *N*-glycosidase and inactivates the 60S ribosomal subunit. This leads to inhibition of protein synthesis in the eukaryotic cell (5). There are two antigenically distinct Stx proteins, designated Stx1 and Stx2 (6). Despite having an amino acid identity of 57%, mouse and primate studies have shown that Stx2 causes more severe disease outcomes than Stx1 (7, 8). Several allelic variants of Stx2 have been described, based on the amino acid differences in the A and B subunits. The most common forms of Stx2 expressed by *E. coli* O157:H7 human isolates are Stx2a and Stx2c (9, 10).

Since its first report in 1983 (11), the serotype O157:H7 rapidly became a notorious foodborne pathogen due to its severe disease outcomes and an infectious dose of less than 100 CFU (12). Cattle are the main reservoir, and transmission to humans often occurs through contaminated food and water, or sometimes person-to-person contact (13). The best-studied O157:H7 strains are Sakai and EDL933, which were isolated from early outbreaks and were the first to be fully sequenced (14, 15). Sakai was implicated in an outbreak associated with radish sprouts and caused illness in over 9,000 children in Sakai city, Japan (16). EDL933, on the other

hand, was linked to consumption of tainted hamburgers in Michigan (11).

Octamer-based genome scanning (OBGS) (17), lineage-specific polymorphism assay (LSPA) (18), and single nucleotide polymorphism (SNP)-based methods (19) have successfully classified *E. coli* O157:H7 strains into informative subgroups. OBGS and LSPA were used to define three lineages. Lineage I is associated with strains expressing higher levels of Stx2 (20) that are common human isolates in the United States (21), while lineage II strains are more likely to be of bovine origin and express lower Stx2 levels than the other two lineages (17, 20). The third lineage described is designated I/II (22). A SNP-based method was also used to subtype *E. coli* O157:H7 strains into nine clades (19). The strains clustering within clade 8 demonstrate increased virulence potential (19), as patients presenting HUS symptoms were more likely to be infected with strains from this group. A larger study confirmed this observation, noting that clade 6 and clade 8 strains

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TABLE 1 Strains used in the study

Strain	Relevant characteristics	Reference
<i>E. coli</i> O157:H7		
Sakai	<i>stx</i> <sub>1</sub> <i>stx</i> <sub>2a</sub> ; clade 1, lineage I	14
EDL933	<i>stx</i> <sub>1</sub> <i>stx</i> <sub>2a</sub> ; clade 3, lineage I	15
PA2	<i>stx</i> <sub>2a</sub> ; clade 8, lineage I/II	9
PA3	<i>stx</i> <sub>2a</sub> <i>stx</i> <sub>2c</sub> ; clade 8, lineage I/II	9
PA8	<i>stx</i> <sub>2a</sub> ; clade 8, lineage I/II	9
PA9	<i>stx</i> <sub>2a</sub> ; clade 8, lineage I/II	9
PA13	<i>stx</i> <sub>2a</sub> <i>stx</i> <sub>2c</sub> ; clade 8, lineage I/II	9
PA19	<i>stx</i> <sub>2a</sub> ; clade 8, lineage I/II	9
PA24	<i>stx</i> <sub>1</sub> ; clade 8, lineage I/II	9
PA25	<i>stx</i> <sub>2a</sub> ; clade 8, lineage I/II	9
PA28	<i>stx</i> <sub>2a</sub> <i>stx</i> <sub>2c</sub> ; clade 8, lineage I/II	9
Other <i>E. coli</i>		
C600	K-12 derivative	43
JM109	Host for plaque assays	44

were more frequently associated with HUS than were strains from other clades (23).

Stx2 expression requires induction of a *stx*<sub>2</sub>-bearing prophage (4), many of which adsorb to the essential outer membrane protein BamA (24), and is enhanced by DNA-damaging agents, including antibiotics, UV light, and H<sub>2</sub>O<sub>2</sub> (25–27). Increased phage and Stx2 accumulation also occurs when *E. coli* O157:H7 is cocultured with phage-sensitive *E. coli* strains (28). The model proposed by Gamage et al. suggested that a lytic infection of these strains by phage that are spontaneously released by *E. coli* O157:H7 increases the number of phage particles, as well as Stx2 levels. This model was developed using a single strain of *E. coli* O157:H7, although *stx*<sub>2</sub>-bearing phage are known to be highly polymorphic (29–31). We hypothesized here that strain-specific differences between these phage would impact the level of toxin amplification observed.

## MATERIALS AND METHODS

**Strains and culture conditions.** The strains and plasmids used in the study are listed in Table 1. The *E. coli* O157:H7 strains with “PA” designations were human isolates obtained from the Pennsylvania Department of Health and were characterized previously (9). The bacteria were routinely propagated in Luria-Bertani (LB) broth at 37°C, while culture stocks were maintained at –80°C in 10% glycerol. For coinubation assays, modified LB broth supplemented with calcium chloride (CaCl<sub>2</sub>) at a final concentration of 10 mM was used.

**Induction and supernatant collection.** *E. coli* O157:H7 strains were grown overnight and diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.05 using LB broth. For phage induction, ciprofloxacin was added to the LB broth to a final concentration of 45 ng/ml. The growth curve for both the induced and uninduced cultures was profiled for 8 h, with samples collected every 2 h. The OD<sub>600</sub> was measured using a DU730 spectrophotometer (Beckman Coulter, Pasadena, CA), while phage and Stx2 levels were measured for each collected sample as described below.

**Stx2 quantification using receptor ELISA.** For Stx2 quantification, samples were treated with 6 mg/ml of polymyxin B (PMB) and incubated at 37°C for 5 min to lyse cells and release intracellular Stx2. The PMB-treated samples were centrifuged at 4,000 × g for 10 min, and the collected supernatant was either used immediately or stored at –80°C. Receptor enzyme-linked immunosorbent assay (RELISA) uses ceramide trihexoside (CTH), a Gb3 analogue, as a capture for Stx (32). The 96-well polystyrene microtiter strip plates (Thermo Scientific, Waltham, MA) were coated with 2.5 μg of CTH (Matreya Biosciences, Pleasant Gap, PA). The

plates were incubated at 4°C overnight with blocking buffer composed of 4% bovine serum albumin (Sigma-Aldrich, St. Louis MO) in 0.01 M phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST). Diluted or undiluted samples were dispensed in triplicate and placed on a shaking incubator at room temperature (RT) for 1 h. Monoclonal mouse anti-Stx2 (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes the A subunit, was added to a final concentration of 0.1 μg/ml and incubated at room temperature for 1 h. After 5 washes with PBST, goat anti-mouse IgG peroxidase conjugate was added to a final concentration of 0.1 μg/ml and incubated at RT for 1 h. Subsequently, 100 μl of 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine) (Thermo Fisher, Waltham, MA) was added to each well, and the plates were incubated for 10 min at room temperature. Finally, 100 μl of stop solution (2 M H<sub>2</sub>SO<sub>4</sub>) was added, and the OD<sub>450</sub> was measured. Wells containing supernatant from *E. coli* O157:H7 strain PA24 (9), which produces only Stx1, served as a negative control. With each run, a 2-fold serially diluted Stx2a of known concentration was used to construct a standard curve. This standard was made by filter sterilizing supernatant from 30 ml of ciprofloxacin-induced *E. coli* O157:H7 strain PA11, followed by quantification using recombinant Stx2a (BEI Resources, Manassas, VA) to make a standard curve. Aliquots of the supernatant (300 μl) were individually frozen at –80°C, and typical concentrations were 20 μg Stx2a/ml. Each aliquot was thawed only once. Total protein was measured using the Bradford reagent (Amresco, Solon, OH).

**Plaque assay.** A double-overlay agar method for measuring plaques was adapted from Islam et al. (33). Phage from induced and uninduced cultures were harvested as described in that publication. The supernatant was centrifuged at 4,000 × g for 10 min, and serial dilutions were made in SM buffer (0.1 M NaCl, 50 mM Tris-Cl, 8 mM MgSO<sub>4</sub>, and 0.01% gelatin). The indicator strain JM109 (200 μl) was added to 100 μl of phage and mixed in modified LB agar-soft agar (0.75% agar). The soft-agar mixture was overlaid on modified LB agar and incubated at 42°C to allow overexpression of the phage receptor *bamA* gene (24). Plaques were counted after 16 h of incubation.

**Coinubation experiments.** The coinubation assays were developed based on experiments by Gamage et al. (28). Briefly, overnight cultures of *E. coli* O157:H7 and C600 were diluted in LB broth to an OD<sub>600</sub> of 0.03. For control experiments, O157:H7 or C600 was present as a monoculture, while coculture setups contained both O157:H7 and C600 diluted in LB broth to a final OD<sub>600</sub> of 0.03 each. The 6-well plates (BD Biosciences Inc., Franklin Lakes, NJ) containing modified LB agar served as a base for the modified LB broth, on which 1 ml of monoculture or coculture setup was overlaid and incubated at 37°C. The *E. coli* O157:H7 strains PA2, Sakai, and EDL933 were either incubated as monocultures or cocultured with C600. Samples for enumerating cell density and Stx2 levels were harvested every 3 h after the 6-h time point. Viable-cell counts were measured by plating onto Sorbitol MacConkey agar (SMaC), a differential medium where O157:H7 appears as white colonies and C600 appears as red colonies. Stx2 levels were quantified using the RELISA described above. To test Stx2 accumulation when other clade 8 strains were cocultured with C600, *E. coli* O157:H7 strains from the collection previously characterized by Hartzell et al. (9) were chosen. PA2, PA8, PA9, PA19, and PA25, which carry *stx*<sub>2a</sub> only, and PA3, PA13, and PA28, which carry both the *stx*<sub>2a</sub> and *stx*<sub>2c</sub> alleles, were tested. The monoculture and coinubation samples were harvested after 16 h of incubation, and the cell counts from three biological repeats were enumerated from 10<sup>8</sup> dilutions. For the clade 8 coinubation, the cell counts were reported as the percentage of C600 in the total *E. coli* population.

**Mucus assays.** Three Angus cows and one Jersey-Holstein mix were slaughtered at the Penn State Meat Laboratory or Rising Spring Mills Meat Co. (Rising Spring Mills, PA), and 3 feet from the rectum and distal colon were sequestered to collect mucus. The mucus was autoclaved, and approximately 2.5 ml was added to each well in a 6-well polystyrene plate (BD Biosciences Inc., Franklin Lakes, NJ). The inoculum was prepared by resuspending overnight cultures in PBS to wash off residual medium from

the overnight culture. Dilution was done in PBS to a final OD<sub>600</sub> of 0.03. The monocultures and cocultures of PA2, EDL933, and C600 were assembled as described above. A negative control with mucus in PBS was also included. Growth was profiled on SMaC every 3 h until 16 h. After 16 h, the mucus was collected in Eppendorf tubes and vortexed at maximum speed for 1 min to homogenize the mucus. Subsequently, centrifugation was done for 15 min at 4,000 × g to collect the supernatant. ELISA was done using the Premier EHEC ELISA kit (Meridian Biosciences Inc., Cincinnati, OH), which detects both Stx1 and Stx2.

**Animal experiments.** Male and female germfree Swiss-Webster mice 3 to 5 weeks of age were raised in the University of Michigan germfree colony. They were housed in soft-sided bubble isolators and fed autoclaved water and laboratory chow *ad libitum*. The mice were infected orally with ~10<sup>6</sup> CFU of LB medium-cultured bacteria. Following inoculation, the mice were placed in sterile microisolator cages within the germfree isolators. The microisolator cages were then aseptically removed from the isolators and kept in a laminar flow hood for the duration of the experiment. Throughout the experiment, the mice received sterile food, water, and bedding to maintain germfree conditions, except for the infecting *E. coli* strains. All animal experiments were conducted with the approval of the University of Michigan Animal Care and Use Committee.

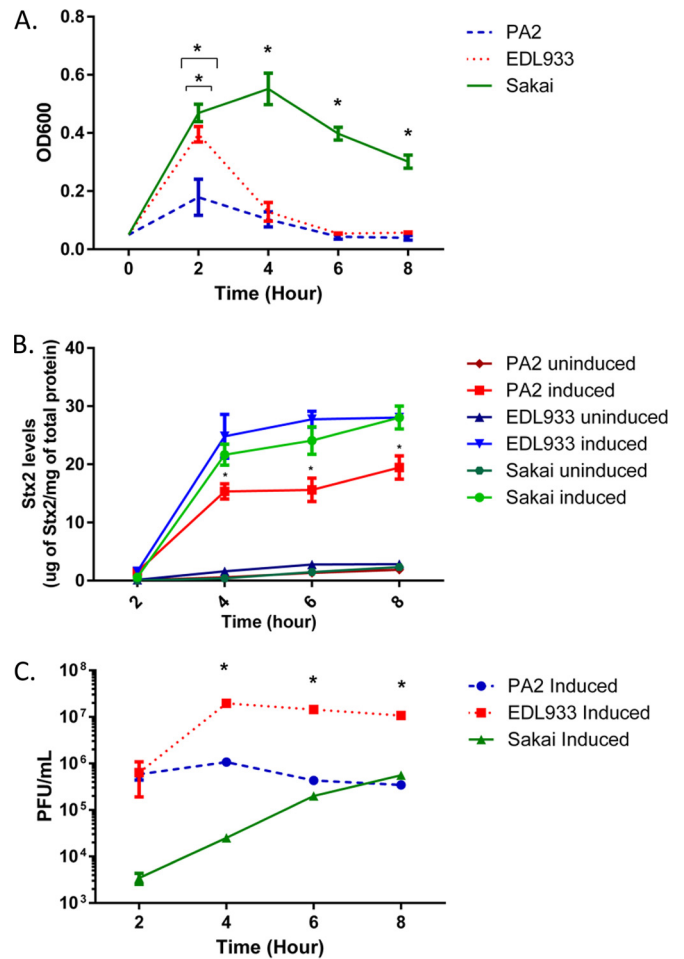
The mice were given one of three inocula: C600 alone, PA2 alone, or C600 followed by PA2 1 week later. They were weighed prior to C600 inoculation, prior to PA2 inoculation, and just prior to euthanasia 6 days after PA2 inoculation. The mice were evaluated daily for evidence of illness (dehydration, ruffled coat, or reluctance to move) and were euthanized 6 days after PA2 inoculation or when they became moribund. Prior to euthanasia, evidence of illness was recorded, and at necropsy, samples were collected for bacterial culture, Stx2 ELISA, and histologic examination. For bacterial culture, samples of the cecal contents were weighed, serially diluted in sterile LB broth, and cultured on SMaC plates. For quantification of Stx2, the cecal content was stored at -20°C until evaluation with a Premier EHEC ELISA kit. For histologic examination, samples of the right and left kidneys were bisected and emersion fixed in 10% neutral buffered formalin. The colon was removed and fixed in its entirety. Tissues were paraffin embedded, and 5-μm sections were stained with hematoxylin and eosin and scored for histologic lesions.

**Histologic scoring.** Acute renal tubular necrosis was scored as previously described (34). Briefly, scores were as follows: 0, no necrosis; 1, occasional dilated tubules with cellular debris; 2, necrotic tubules in most histologic fields; 3, necrotic tubules in all fields with minimal normal cortex present. Colon necrosis and neutrophilic infiltration were scored as a weighted average. For this, each 200× microscopic field was scored separately for the presence of epithelial necrosis or neutrophils in the lamina propria. Necrosis was scored as follows: 0, no necrotic cells; 1, scattered necrotic epithelial cells; 2, many necrotic cells in the surface epithelium and most glands and scattered necrotic cells in the lumen; 3, severe necrosis of most of the surface epithelium extending into many glands with rafts of necrotic epithelial cells in the lumen. Neutrophils were scored as follows: 0, none; 1, scattered neutrophils in the lamina propria; 2, occasional clusters or prominent infiltration of neutrophils between glands; 3, sheets of neutrophils infiltrating much of the superficial lamina propria. Each field was scored separately, and a weighted average was calculated by dividing the number of fields with each score by the score and adding the result.

**Data analysis.** Microsoft Excel was used to calculate the mean, standard deviation, and standard error, while GraphPad Prism 8 software was used to calculate *P* values.

## RESULTS

**Ciprofloxacin induces less Stx2a production in PA2 than in EDL933 and Sakai.** We began by comparing the kinetics of induction, Stx2a, and phage production between *E. coli* O157:H7 strains EDL933, Sakai, and PA2. The last is a clade 8 strain previously characterized in our laboratory (9) for which we have the full



**FIG 1** Growth, Stx2a accumulation, and phage production by ciprofloxacin-induced and uninduced strains. (A and B) Overnight cultures of *E. coli* O157:H7 strains PA2, EDL933, and Sakai were inoculated into LB medium supplemented to 45 ng/ml ciprofloxacin, and phage induction was visualized by the optical density. (A) At hour 2, PA2 and EDL933 showed significantly lower OD<sub>600</sub> values ( $P < 0.0001$  and  $P = 0.005$ , respectively) than Sakai. At hour 4, Sakai showed a significantly higher OD<sub>600</sub> ( $P < 0.0001$ ) than EDL933 and PA2. Asterisks highlight data points where statistical significance was observed. (B) Stx2a accumulation in the same experiment. The Stx2a levels were normalized to total protein. The error bars represent standard errors of the mean (SEM) from three biological repeats, and the asterisks indicate that PA2 produced less Stx2a than both EDL933 and Sakai between hour 4 and hour 8 ( $P < 0.005$ ). (C) Overnight cultures of *E. coli* O157:H7 strains PA2, EDL933, and Sakai were inoculated into fresh media supplemented to 45 ng/ml of ciprofloxacin, and plaque assays were performed at the indicated time points. The error bars represent SEM from three biological repeats, and the asterisks indicate significantly more ( $P < 0.0001$ ) plaques were produced by EDL933 than by PA2 and Sakai at hours 4 through 8.

genome sequence (GenBank accession no. [NZ\\_AOEL000000000](https://www.ncbi.nlm.nih.gov/nuccore/NZ_AOEL000000000)). Initial sequence analysis indicated the *stx*<sub>2a</sub>-bearing phage from PA2 is distinct from those found in the EDL933 and Sakai genomes (unpublished data). Strains PA2, EDL933, and Sakai did not show significant differences in their growth in LB medium in the absence of antibiotics (data not shown). However, the growth profiles were different when the lytic cycle was induced by growth in subinhibitory concentrations of ciprofloxacin (Fig. 1A). Both PA2 and EDL933 reached their peak OD<sub>600</sub>s at hour 2, although this maximum was approximately 2-fold lower for PA2 than for

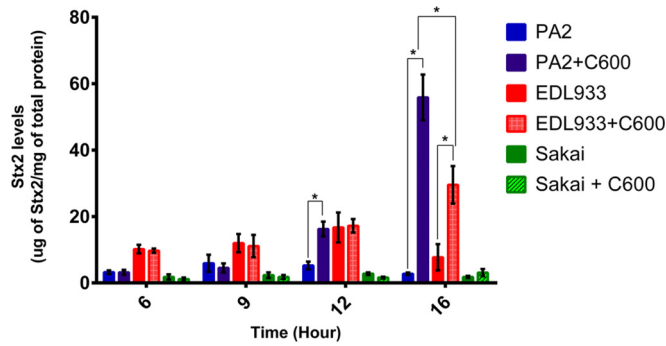


FIG 2 Stx2a levels after coinoculation with C600. *E. coli* O157:H7 strains were incubated statically at 37°C, either alone or with C600. Samples were harvested and assayed for Stx2a. The error bars indicate SEM from three biological repeats, and the asterisks indicate significant ( $P < 0.05$ ) differences between samples.

EDL933. Sakai, on the other hand, reached its highest  $OD_{600}$  at hour 4, followed by a steady decline in turbidity. An increase in toxin production was observed over the time course, and as expected, the induced samples produced significantly higher ( $P < 0.0001$ ) Stx2a levels than their uninduced counterparts (Fig. 1B). Among the induced samples, Sakai produced significantly less ( $P < 0.005$ ) Stx2a than EDL933 only at the hour 4 time point. PA2 consistently produced less Stx2a than both EDL933 and Sakai from hour 4 until hour 8 ( $P < 0.005$ ). EDL933 produced significantly more ( $P < 0.0001$ ) plaques than PA2 and Sakai at hours 4 through 8 (Fig. 1C).

**Coinoculation with *E. coli* strain C600 amplifies Stx2a in PA2 and EDL933 in vitro.** We investigated the strain-specific differences in Stx2a levels when the three O157:H7 strains were individually cocultured with C600. There was no difference in Stx2a levels between the monoculture and coculture samples of all three strains between hours 6 and 9; however, from hour 12 onward, a significant increase in Stx2a levels was noted for the PA2 coculture setup (Fig. 2). At hour 16, both PA2 and EDL933 had significantly higher Stx2a levels in the coculture samples than O157:H7 monoculture controls. On the other hand, Stx2a levels in the Sakai monoculture and the coculture with C600 were statistically indistinguishable. A significantly higher ( $P < 0.05$ ) amplification was reported for PA2 cocultures than for monoculture controls, with the difference being 20.3 ( $\pm 1.2$ )-fold compared to EDL933 and Sakai, which showed 2.7 ( $\pm 0.4$ )- and 1.6 ( $\pm 0.4$ )-fold differences, respectively. Cell counts of PA2, EDL933, Sakai, and C600 were enumerated in monoculture and coculture to profile the population dynamics during the course of coinoculation (Fig. 3). When PA2 and C600 were cocultured, a sharp reduction in C600 cell counts was observed between hours 9 and 16 (Fig. 3A). Similarly, the C600 cell counts in the EDL933-plus-C600 coculture were consistently lower than when C600 was present alone (Fig. 3B). In contrast, coculture with Sakai did not significantly reduce the colony counts of C600 at any time (Fig. 3C).

We also developed an *ex vivo* model to investigate whether toxin amplification seen during PA2-plus-C600 coculture was repeatable in environments other than laboratory media. In autoclaved bovine rectal mucus, there was a reproducible increase in Shiga toxin production in coculture compared to the monoculture controls. The increases in total toxin production comparing

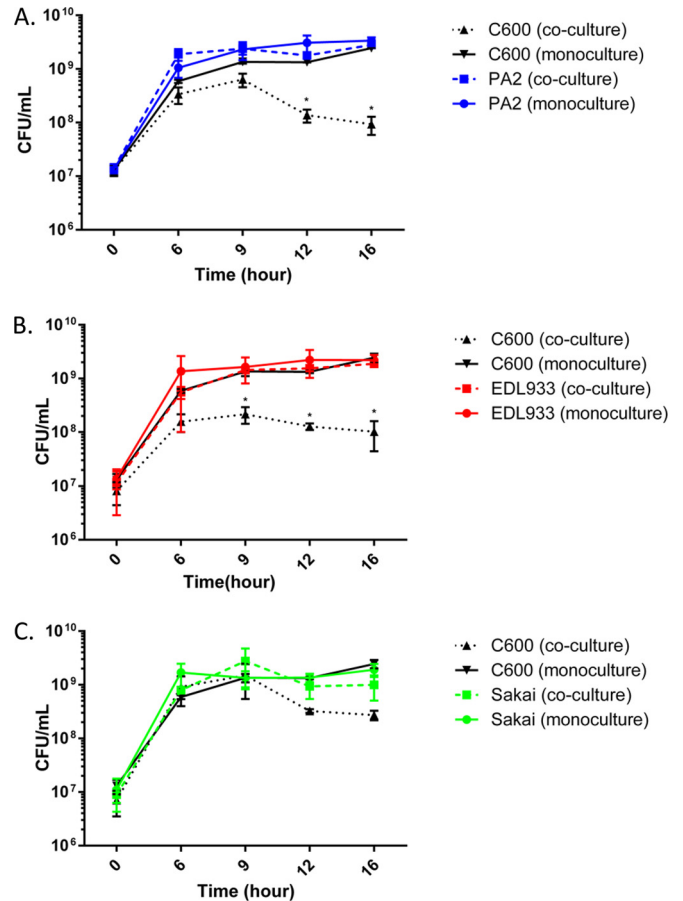
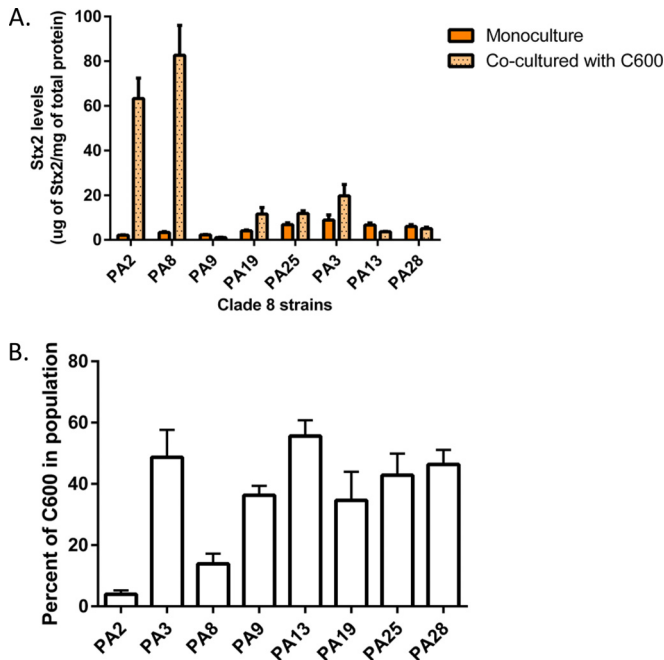


FIG 3 Population dynamics in cocultured samples. *E. coli* O157:H7 strains were statically incubated at 37°C, either alone or with C600. Samples were harvested every 3 h from 6 h onward and plated onto sorbitol MacConkey agar. On this medium, C600 grows as red colonies and O157:H7 as white colonies. The error bars represent SEM from three biological repeats. The C600 coinoculation samples marked with asterisks are significantly lower than the C600 monoculture control ( $P < 0.01$ ).

coculture to monoculture experiments were 4.3 ( $\pm 1.2$ )-fold (1 standard deviation) for PA2, and 2.2 ( $\pm 0.5$ )-fold for EDL933. As seen in laboratory media, growth suppression of C600 was observed when the strain was cocultured with either *E. coli* O157:H7 strain (Fig. 4A and B).

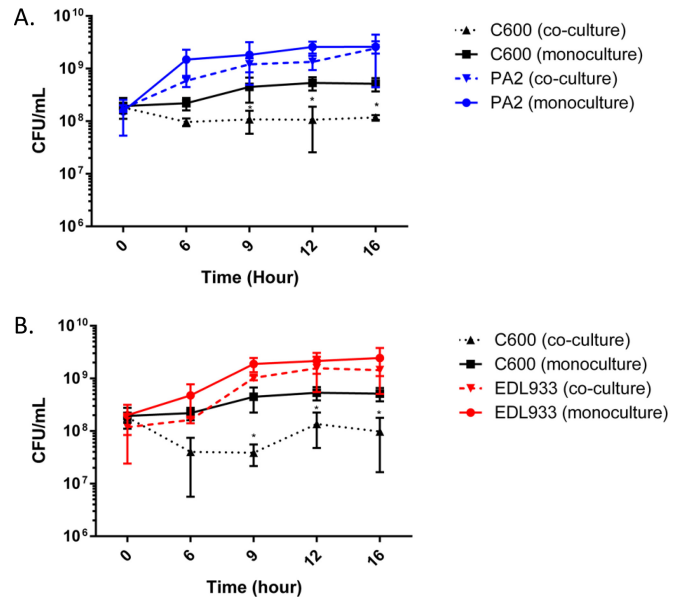
**Toxin amplification occurs with a limited number of clade 8 strains.** Since cocultures of PA2 and C600 showed a drastic increase in Stx2a levels compared to PA2 alone, we next hypothesized that other clade 8 strains would also amplify Stx2a. We chose eight clade 8 strains from our previously characterized collection (9). PA2 carries only the *stx<sub>2a</sub>* gene; therefore, we selected four other clade 8 strains that also carry *stx<sub>2a</sub>* only, namely, PA8, PA9, PA19, and PA25. Three other strains, PA3, PA13, and PA28, carrying both *stx<sub>2a</sub>* and *stx<sub>2c</sub>*, were also chosen. Stx2 quantification demonstrated that PA8 cocultured with C600 also increased Stx2 levels by 24.1 ( $\pm 2.5$ )-fold compared to monoculture controls (Fig. 5A). Other strains, including PA3, PA19, and PA25, showed increased Stx2 levels by 2.3 ( $\pm 0.1$ )-, 2.7 ( $\pm 1.1$ )-, and 1.9 ( $\pm 0.5$ )-fold, although the numbers were not statistically significant compared to the *E. coli* O157:H7 monoculture controls. Cell counts were quantified for *E. coli* O157:H7 and C600 at the end of the



**FIG 4** Stx2 levels and percentages of C600 after co-cultivation of clade 8 strains with C600. Clade 8 strains carrying genes for *stx*<sub>2</sub> (PA2, PA8, PA9, PA19, and PA25) or *stx*<sub>2</sub> and *stx*<sub>2c</sub> (PA3, PA13, and PA28) were cocultured with C600 for 16 h. (A) Stx2 levels quantified from the cocultured samples demonstrated that PA2 and PA8 significantly ( $P < 0.01$ ) amplified Stx2a in comparison to monoculture controls. (B) The cell count for C600 relative to that for O157:H7 showed that during coculture, PA8 reduced the number of C600 colonies to the same extent as PA2. The error bars indicate SEM.

coculture experiments. Both PA2 and PA8 outgrew *E. coli* C600, reducing the nonpathogenic strain from the initial 50% of the total bacterial population to 4.0% ( $\pm 0.1.3\%$ ) and 14.0% ( $\pm 3.3\%$ ), respectively (Fig. 5B). Therefore, only a minority of clade 8 strains show the same toxin amplification phenotype observed with cocultured PA2 and C600.

**Increased lethality and Stx2a levels in germfree mice cocultured with C600.** To investigate whether toxin amplification could be observed in an *in vivo* system, we used a germfree mouse model (34). Germfree mice precolonized with C600 were infected with PA2 and euthanized after 6 days, while mice inoculated with PA2 and C600 alone served as controls. When infected alone, PA2 (Fig. 6A) and C600 (data not shown) colonized mice to approximately  $10^9$  and  $10^{10}$  CFU/g of cecal contents, respectively, after 6 days, although in the same period, coculture counts of C600 were reduced to approximately  $2 \times 10^8$  CFU/g (Fig. 6B). Mice precolonized with C600 6 days prior to PA2 infection appeared sicker and showed signs of becoming moribund (Table 2). The PA2-inoculated mice, on the other hand, appeared healthy at the time of being euthanized. Consistent with the observations *in vitro*, the Stx2a levels recovered from the feces of PA2-plus-C600-infected mice were significantly higher than those from mice infected with PA2 alone on both day 1 ( $P < 0.05$ ) and day 6 ( $P < 0.005$ ) (Fig. 7). Moreover, the histopathological data demonstrated more extensive acute tubular necrosis (ATN) in the kidneys of mice infected with PA2 plus C600 than in those infected with PA2 alone (Fig. 8A to G). Infection with *E. coli* O157:H7 caused weight loss, colon epithelial cell necrosis, and neutrophilic colitis compared to mice



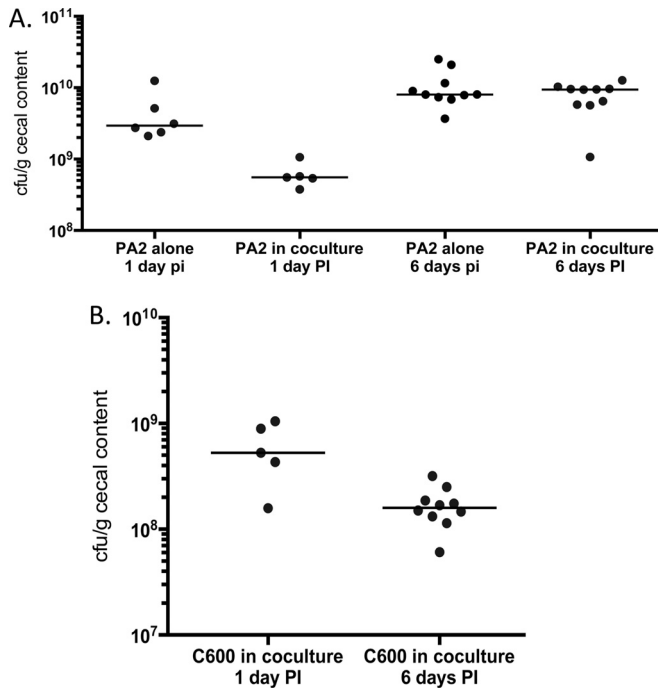
**FIG 5** Cell counts in the *ex vivo* mucus model. Shown is the time course of PA2 and C600 (A) and EDL933 with C600 (B) cell counts when grown as cocultures or monocultures. The cultures were grown for 16 h total, and colony counts were measured by plating onto sorbitol MacConkey agar. On this medium, O157:H7 colonies are white, while C600 colonies are red. The asterisks indicate time points where C600 counts under coculture conditions were lower ( $P < 0.01$ ) than *E. coli* O157:H7 counts. The error bars indicate SEM.

infected with C600 ( $P < 0.05$ ), but scores were statistically indistinguishable between mice given PA2 alone and those coinoculated with PA2 and C600 (Fig. 8H and I).

## DISCUSSION

Previous studies described how Stx2a production increases when O157:H7 is cocultured with phage-susceptible *E. coli* (28, 35). Our work augmented the understanding of this phenotype by demonstrating that the level of amplification depends upon the strain of *E. coli* O157:H7 used. Moreover, we identified clade 8 O157:H7 strains that produce low Stx2a levels compared to EDL933 and Sakai (Fig. 2 and 5A) but show a drastic increase in Stx2a accumulation when cocultured with the nonpathogenic *E. coli* strain C600 (Fig. 3 and 5A).

We used the prototypical strains Sakai and EDL933 to characterize the phenotypes of PA2 and other clade 8 *E. coli* O157:H7 strains. The first two were the first O157:H7 strains to be fully sequenced (14, 15). Sakai is a lineage I, clade 1 strain carrying both *stx*<sub>1</sub> and *stx*<sub>2a</sub> and was responsible for an outbreak that affected over 9,000 children in Sakai city, Japan. EDL933 is a clade 3, lineage I strain that also carries *stx*<sub>1</sub> and *stx*<sub>2</sub>. It was implicated in the multistate hamburger outbreak in 1982 that caused at least 47 illnesses (11). Studies on Stx2 expression showed that human clinical isolates belonging to lineage I or I/II produce higher Stx2 levels than cattle-derived lineage II isolates (17, 20). Further classifying the strains based on SNP typing identified a subset called clade 8, which is associated with higher incidence of HUS than other clades (19, 23). An example of a large outbreak caused by a clade 8 strain is the multistate spinach outbreak in 2006, with 205 cases and 3 casualties reported (36). It resulted in hospitalizations in over 60% of cases and a 13% HUS rate (19). The isolates PA2 and PA8 belong to a clade 8, lineage I/II strain and carry only *stx*<sub>2a</sub>.



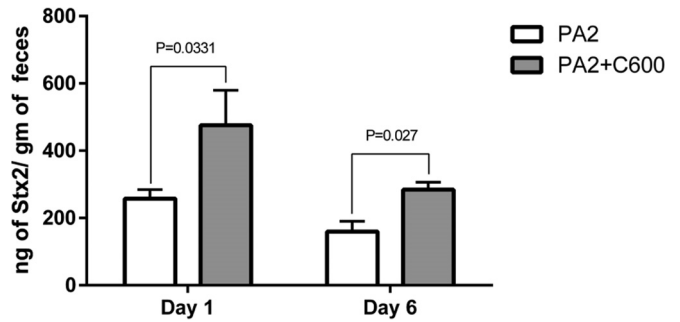
**FIG 6** Colonization of germfree mice. Mice were orally inoculated with approximately 10<sup>6</sup> CFU/ml of PA2 in the monoculture control. In the coculture experiment, germfree mice were precolonized with C600, followed by inoculation with PA2 7 days later. Fecal cell counts for PA2 (A) and C600 (B) are reported for day 1 and day 6 postinoculation (pi or PI) with strain PA2. The medians are indicated by the horizontal lines. The detection limit for plate counts was approximately 100 to 1,000 CFU/ml.

According to the model proposed by Gamage et al., spontaneously induced phage infect susceptible strains of *E. coli*, leading to lysis and enhanced Stx<sub>2a</sub> production (28). We initially hypothesized that *E. coli* O157:H7 strains that were high Stx<sub>2a</sub> producers in the presence of ciprofloxacin (Fig. 2) would also be the highest producers when cocultured with C600. However, we were surprised to see that the lowest-Stx<sub>2a</sub>-producing strain, PA2, showed the highest toxin levels in coculture experiments (Fig. 3). Bacterial cell count data showed reduced numbers of the susceptible host when cocultured with PA2, further supporting the assumption that phage-mediated lysis of C600 was occurring (Fig. 4A). Moreover, we found that addition of supernatants from ciprofloxacin-induced PA2 cultures to *E. coli* C600 resulted in Stx<sub>2a</sub> production (data not shown). This leads to the conclusion that the fold amplification observed after coculture with C600 is not dependent on the basal Stx<sub>2a</sub> levels expressed by O157:H7. Toxin amplification could also be recapitulated when *E. coli* O157:H7 strains were cocultured with C600 in an *ex vivo* model using sterile bovine rectal mucus (data not shown). The fold amplification was less

**TABLE 2** Qualitative observation of disease in germfree mice 6 days postinfection with *E. coli* O157:H7 PA2 with and without *E. coli* C600

<i>E. coli</i> inoculum	No. healthy	No. sick	No. moribund/dead
PA2 only	11 <sup>a</sup>	0	0
PA2 + C600	2	5	3

<sup>a</sup> The PA2-plus-C600-infected mice had a higher morbidity and mortality rate than mice infected with PA2 alone ( $P < 0.05$  by chi-square test).



**FIG 7** Stx<sub>2a</sub> levels in feces of germfree mice. Stx<sub>2a</sub> levels were quantified, using ELISA, from the feces of PA2- and the PA2-plus-C600-infected mice on day 1 and day 6 postinoculation. The error bars represent SEM from different mice.

than that observed in laboratory media but provided further support for pursuing *in vivo* studies. We suggest that the amplification differences observed may be driven by polymorphisms between the *stx*<sub>2a</sub>-converting phage. It was noted previously that genes within the early regulation and replication regions differ between these phage in EDL933 and Sakai (29). The sequencing of the phage genomes from PA2 and PA8 (unpublished data) revealed that the *stx*<sub>2a</sub>-converting phage differ only by the position of an IS629 element, and both are related to the *stx*<sub>2a</sub>-converting phage VT2\_phi272 (31). VT2\_phi272 is more closely related to a phage from *E. coli* O104:H4 than it is to the one from EDL933 and Sakai (31, 37).

Gamage et al. (35) also tested whether the toxin amplification phenotype could be recapitulated in streptomycin-treated mice and obtained encouraging although inconclusive results. The study showed that 8 of 36 stool samples from mice precolonized with a phage-susceptible commensal *E. coli* strain prior to inoculation with *E. coli* O157:H7 had Shiga toxin levels above the limit of detection. Mice given the *E. coli* O157:H7 strain alone were more commonly toxin positive (17 of 36 stool samples). However, toxin quantifications were similar between the two groups. As pathogen colonization was approximately 50-fold less in cocultured mice, this suggested that the commensal may enhance toxin production (35). In our study, we used the established germfree mouse model (34) to limit the inherent variability when a complex microbiota is present. Our results clearly demonstrate that *E. coli* C600 adversely affects disease outcome in this model. As hypothesized, the Stx<sub>2a</sub> levels observed *in vivo* when PA2 was cocolonized with C600 was significantly higher than with PA2 alone (Fig. 7). In parallel with elevated Stx<sub>2a</sub>, cocolonized mice showed more severe signs of disease and were more likely to die than the mice inoculated with PA2 alone (Table 2). In addition, the histopathological data showed more extensive renal tubular necrosis in the PA2-plus-C600 mice than in the mice colonized by PA2 alone.

Given the dramatic *in vitro* and *in vivo* data related to strain PA2; the known link between Stx<sub>2a</sub> and development of severe outcomes, such as HUS (2, 3); and reports that strains of clade 8 are particularly virulent (19, 23), we tested whether the Stx<sub>2a</sub> amplification could be generalized to other clade 8 strains. We included strains that produce Stx<sub>2a</sub> only and strains that produce both Stx<sub>2a</sub> and Stx<sub>2c</sub>, as these are the two toxin genotypes most correlated with HUS development (23). We observed that not all clade 8 strains show increased Stx<sub>2a</sub> production during coculture



an increase. It is noteworthy, though, that the first two studies quantified Stx2 after ciprofloxacin induction while the third study did not induce the phage during coculture. Regardless, these studies and the current report highlight the complex interactions affecting Stx2 production when Shiga toxin 2-producing *E. coli* interacts with other gut microorganisms.

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