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Title: The coexistence of *Escherichia coli* serotype O157:H7 and its specific bacteriophage in continuous culture

Running title: COEXISTENCE OF *E. COLI* O157:H7 AND BACTERIOPHAGE

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ABSTRACT

For the development of phage therapy, systematic understanding mechanisms of bacteriophage resistance will be required. We describe a new strain of *Escherichia coli* O157:H7, named Mu^L, which stably co-exists with the O157:H7-specific lytic bacteriophage PP01. Chemostat cultures of *E. coli* O157:H7 infected with PP01 showed unchanging cell concentration, but phage concentrations which increased by $\sim 10^8$ PFU mL⁻¹. However, the latent period, burst size, and growth rate of Mu^L were the same as in a PP01-susceptible strain. The binding rate of PP01 to the cell surface was diminished 8.5-fold in Mu^L. By observation of the binding of fluorescently labeled O157:H7-specific phage to individual Mu^L cells, we found that clonal Mu^L cultures were heterogeneous in their ability to bind bacteriophage. 15% of the Mu^L population was completely resistant to PP01 infection. Mu^L also coexisted with bacteriophages unrelated to PP01. Broad-range phage resistance by clonal heterogeneity represents a new class of bacteria-phage interactions.

INTRODUCTION

Bacteriophages target their host bacterium with high specificity and do not affect other bacteria. Additionally, even after administration, virulent bacteriophages multiply inside and eventually lyse their host cell, releasing progeny that can again destroy more bacteria cells [1-6]. Both of these features are attractive therapeutically, and the concept of using phages to kill pathogenic bacteria, or “phage therapy” has recently been extensively reviewed [1, 2, 7-9]. However, obstacles remain in the development of effective, well-characterized, and safe phage therapeutics. These include the difficulty of the delivery and persistence of bacteriophage in vivo, and the emergence of phage-resistant bacteria.

Enterohemorrhagic *Escherichia coli* (EHEC) infection is a disease whose symptoms can include bloody diarrhea, and hemolytic uremic syndrome. The serotype responsible for most EHEC infections is *E. coli* O157:H7 [10]. Antibiotic-resistant strains of *E. coli* O157:H7 have been isolated [11]. Toward development of bacteriophage-based controls of this pathogen, our laboratory has studied lytic bacteriophages of *E. coli* O157:H7 and the mechanisms by which phage-resistant mutants appear. We previously isolated the O157-specific T-even lytic bacteriophage PP01 from porcine feces and found that the receptor for this phage is the *E. coli* outer membrane protein OmpC [12]. As a simple laboratory model of the mammalian digestive tract, we operated a continuous co-culture of this phage with *E. coli* O157:H7. From this culture a phage-tolerant mutant, Mu^L, which unlike its parent strain, formed mucoid colonies on LB agar, was isolated [13]. This strain had the unusual ability to co-exist with bacteriophage PP01. That is, in batch culture, the number of bacteria increased along with the number of bacteriophage. OmpC expression level in Mu^L was unchanged relative to the parent strain, and lipopolysaccharide (LPS) expression patterns were also similar [13]. Simple understanding of this unusual phage-resistant phenotype was thus elusive. However, if phage therapy is to be a viable treatment for *E. coli*

O157:H7 and other diseases, the mechanisms by which mutant strains evade phage-induced lysis must be systematically understood. Toward such an understanding, in the present study we further characterize the strain Mu^L and show that it represents a new class of bacteria-phage interaction.

MATERIALS AND METHODS

Bacteria strains and bacteriophages. The non-pathogenic *E. coli* O157:H7 strain ATCC43888 (wild-type) was used as a host for O157-specific bacteriophages. This strain was isolated from human feces but does not produce Shiga-like toxins 1 or 2 because of a lack of genes for these toxins. A mutant strain derived from ATCC43888, called Mu^L, was isolated from a continuous co-culture of bacteriophage and the wild-type strain because unlike the wild-type strain, Mu^L forms mucoid colonies on Luria-Bertani (LB) agar [13].

The bacteriophage PP01 was originally isolated from porcine feces. It is a T2-like phage, lytic to *E. coli* O157:H7 but not to other *E. coli* strains [12].

In some experiments, the O157-specific bacteriophages SP15 and PP17, isolated from activated sludge and porcine feces, respectively, were used. These phages have been studied by transmission electron microscopy (TEM) and have λ- and T4-like morphologies, respectively.

Bacteriophage PP01-SOC/GFP, a fluorescent derivative of PP01, was used to microscopically examine the phage-binding capability of single *E. coli* O157:H7 cells. PP01-SOC/GFP expresses green fluorescent protein (GFP) as a fusion to the small outer capsid (Soc) protein, the primary structural protein of the phage capsid [14]. PP01-SOC/GFP lysates were prepared by at 28 °C and phage was concentrated by 35,000 g ultracentrifugation for 40 min at 4 °C. Precipitated phage was resuspended overnight in SM buffer [15] to a final concentration of 10¹⁰ plaque forming units (PFU) mL⁻¹.

Coincubation of bacteria and phage in batch and continuous culture. For batch culture, overnight liquid LB cultures of bacteria were diluted to about 10^7 CFU mL⁻¹ in fresh LB medium and incubated 37 °C at 120 rpm for one hour. Bacteriophage was then added to the culture at a multiplicity of infection (MOI) of 2. Bacterial growth or lysis was monitored by measuring the culture turbidity ($A_{600\text{nm}}$) and the phage concentration (plaque assay).

Continuous culture was performed aerobically as described in [13]. Briefly, a peristaltic pump was used to move LB medium at 37 °C through a stirred Erlenmeyer flask at a dilution rate of 0.33 hr⁻¹. Bacteria were cultured for 24 hrs prior to phage inoculation, in order to allow cell populations to reach equilibrium. At time zero, the culture was inoculated with PP01 bacteriophage at an MOI of 0.01.

Single-step growth curves. Single-step growth curves of PP01 on wild-type hosts or on Mu^L hosts were measured according to Carlson [16]. Briefly, exponentially growing cultures were infected with phage at an MOI of 0.01, in LB medium at 37 °C. After 4 min, phage binding was stopped by 100-fold dilution of the culture. The phage titer in the culture was measured as a function of time.

Binding of phage to bacterial surface. Overnight LB cultures of the relevant *E. coli* strain were diluted to 10^8 CFU mL⁻¹ in fresh LB medium and incubated at 37 °C at 120 rpm for one hour. The relevant bacteriophage was added to a final concentration of 2×10^5 PFU mL⁻¹ and the culture vortexed briefly. At periodic intervals, samples of the culture were withdrawn and centrifuged at 11,000 g for 1 min at 4 °C. The supernatant containing unbound bacteriophage was diluted in SM buffer and titered for phage.

Culture heterogeneity. Exponentially growing cultures of either wild-type or Mu^L cells growing at 37 °C in LB broth were infected with PP01 at an MOI of 100 and incubated for at 28 °C. After 30 min, samples of the cultures were plated onto LB agar and colonies counted after 18 hours

incubation. The ratio of CFUs in infected cultures to CFUs in uninfected control cultures represents the fraction of the culture populations that is lysis-resistant.

For microscopic observation of phage binding to individual cells, the phage PP01-SOC/GFP ($\sim 2 \times 10^9$ PFU mL⁻¹) was infected to exponentially growing Mu^L or wild-type *E. coli* O157:H7 cells ($\sim 10^8$ CFU mL⁻¹) at a 10:1 volume ratio (MOI=1000) and the mixture was incubated for 30 min at 28 °C. Cells were washed once with phosphate-buffered saline (PBS), and resuspended in a small volume of PBS for microscopic observation. PP01-SOC/GFP bound to the surface of *E. coli* O157:H7 cells was observed with an epifluorescence microscope (Olympus BX-60, Japan) equipped with a digital camera. Exposure times were 0.04 sec for light-field images and 2 sec for fluorescent images.

Electron microscopy. For transmission electron microscopy (TEM), overnight LB cultures of *E. coli* O157:H7 were centrifuged and washed two times with a dilute Ringer's solution (2.2 g NaCl, 0.075 g KCl, and 0.082 g CaCl₂ per L), and resuspended in 0.1 volumes of the dilute Ringer's solution. Colloidon-coated copper grids were cleaned by flash ionization *in vacuo*, and contacted to the cell suspension for 3 min. The grids were negatively stained in 2% w/v phosphotungstic acid (pH 6.8) for 3 min, air dried, and mounted in a Hitachi H-7500 TEM microscope operating at 100,000 V.

For scanning electron microscopy (SEM), overnight LB cultures were first fixed with glutaraldehyde (1.3% v/v in 0.67M sodium phosphate pH 6.5) for 1 hr at 4°C. Following mild centrifugation (400 x g for 10 min at 4 °C), cells were washed in glutaraldehyde-free phosphate buffer and fixed again in 1.3% w/v osmium tetroxide. Cells were dehydrated in 30%, 70%, 90%, and finally 99% ethanol and dried in a supercritical carbon dioxide dryer (HSP-2, Hitachi Co., Ltd., Japan). Palladium-and-platinum sputtered cells were observed by electron microscopy with an S4700 FE-SEM (Hitachi Co., Ltd., Japan) operating at 10,000 V.

Antibody agglutination. Exponentially growing cultures of *E. coli* O157:H7 cells in LB medium at 37 °C were mixed with one volume of peroxidase-labeled goat antibody against *E. coli* O157:H7 whole cells (0.1 mg mL⁻¹, Kirkegaard & Perry, Gaithersburg, MD) and incubated for 30 min at room temperature. This antibody reacts with purified O157 antigen in Western blots. Cell suspensions were observed by optical microscopy for the presence of cell aggregates.

Extracellular polysaccharide quantification. Extracellular polysaccharide (EPS) was purified via cold acetone precipitation [17]. Briefly, wild-type or Mu^L cells were grown overnight in 1 L of LB broth at 37 °C and harvested by centrifugation (48,000 g for 10 min at 4°C). Cells were washed twice in PBS and resuspended in 1% w/v phenol in water with vigorous shaking to break up all clumps (300 rpm for 15 min). The phenol suspension was centrifuged at 16,000 g for 5 hr at 4°C and the supernatant was slowly poured into 1.5 volumes of cold (-70°C) acetone. The resulting precipitate was spooled around a Pasteur pipette, dried by immersion in cold acetone (5 min) and in air (20 min). This material was dissolved in 0.15 M sodium chloride in 0.02 % sodium azide overnight at room temperature. This purified EPS was analyzed by standard chromogenic assays for uronic acid [18], and hexose [19].

RESULTS

Coexistence of *E. coli* O157:H7 strain Mu^L and bacteriophage PP01. The Mu^L mutant was originally isolated from a continuous culture of PP01 phage and wild-type *E. coli* O157:H7 ATCC43888 by virtue of its distinct mucoid colony formation on LB agar. Initial characterization showed that phage and bacteria could increase together in batch culture [13]. This behavior was further elucidated by continuous culture of the Mu^L mutant with PP01 phage (Fig. 1.) No significant decrease in cell concentration was observed at all in the Mu^L continuous culture (Fig. 1). In the wild-type culture, after 10 hrs mutant bacterial strains, resistant to PP01 and with different

colony morphologies, were detected [13], but with the Mu^{L} culture, all colonies were highly mucoid throughout over 80 hrs of culture.

Thus, the equilibrium population of Mu^{L} cells did not change in response to exposure to lytic bacteriophage, despite rapid proliferation of the bacteriophage. This result cannot be explained by traditional ecological models [20], [21], [22] and has not been observed experimentally, in *E. coli* O157:H7 or in other bacteria-phage systems.

Phage-bacteria interaction parameters. The number of progeny phage released per infected cell (burst size), the delay from phage infection to host lysis (latent period), the growth rate of the bacterial host, and the binding rate of phages to the host cell surface are the principal parameters responsible for modulating bacteria-phage populations dynamics [20-21]. To further characterize the coexistence phenotype, we measured these parameters for PP01 growing on wild-type and Mu^{L} cells. Figure 2 shows the result of a single-step growth experiment for the two strains. In this experiment phage is infected to bacteria at high concentrations (10^9 CFU mL^{-1} and 10^7 PFU mL^{-1}) and phage begins to bind to the bacterial surface. PP01 growing on the wild-type strain had a burst size of 14 ± 2 . On the Mu^{L} strain it was 20 ± 4 . These values are not significantly different (two-sided *t* test, $P = 0.12$). The measured values for the latent period were 29 min and 35 min for wild-type and Mu^{L} , respectively. This difference was statistically significant ($P = 1 \times 10^{-6}$) but also relatively small (21% of the mean).

The growth rate of the bacterial host is known to strongly affect the burst size and latent period of a bacteriophage [22, 24]. Although statistically significant ($P = 3 \times 10^{-4}$), the difference in specific growth rates of the wild-type strain (1.59 hr^{-1}) and the Mu^{L} strain (1.49 hr^{-1}) was only 6% (data not shown).

The binding rate of bacteriophage to the cell surface determines the rate of cell infection [25]. The pseudo-first order binding constant was $1.7 \times 10^{-8} \text{ mL min}^{-1}$ for the wild-type strain and $2.0 \times$

10^{-9} for the Mu^{L} strain (Fig. 3). Unlike the other measured parameters (Fig. 2), the binding rate was widely different in the two strains, by a factor of 8.5. However, we had previously determined that the expression of the phage receptor protein, OmpC, was similar in the two strains [13]. The lipopolysaccharide profiles of the two strains were also similar [13], so the biochemical reason for the difference in phage binding was not immediately apparent.

Coexistence of unrelated phages with Mu^{L} . The virulent coliphages SP15 and PP17 were isolated separately from PP01 and do not use OmpC as their receptor (see Materials and Methods). When infected to exponentially growing cultures of *E. coli* O157:H7 at an MOI of 2, both these phages were highly lytic to the wild-type bacteria (Fig. 4a), causing nearly total lysis within 2 hrs of infection. After 9 hours of incubation, in the PP17-infected culture a resistant strain of bacteria appeared, but only after several hours in which growth stopped completely. Phage concentrations increased by a nearly 1000-fold in the wild-type culture in the initial 3 hr after infection (data not shown). In contrast, in Mu^{L} , these phages did not completely lyse the *E. coli* O157:H7 cells (Fig. 4B). Mu^{L} cells continued to grow even after infected with bacteriophage, although at a slower rate compared to uninfected cultures. Nevertheless, both of PP15 and SP15 were able to propagate themselves on the Mu^{L} strain, with the concentration of each increasing by nearly 1000-fold over the course of the experiment, just as in the wild-type case. Like PP01, the phages SP15 and PP17 can replicate in Mu^{L} hosts synchronously with Mu^{L} bacterial growth. Thus, the coexistence phenotype of Mu^{L} is not phage-specific, and is independent of the biochemical recognition of the phage receptor by the phage.

Heterogeneity of pure cultures of the Mu^{L} strain. Although Mu^{L} was able to grow in the presence of SP15 and PP17, growth was hampered relative to uninfected cultures (Fig. 4a). In the case of SP15, a small but incomplete fall in culture turbidity was observed. These results suggested that a subset of the Mu^{L} population was sensitive to lysis by these phages. To test this possibility,

the phage-binding abilities of wild-type and Mu^{L} strains were observed at the single-cell level by using O157-specific phage PP01-SOC/GFP. This phage is a derivative of PP01 engineered to express GFP on the phage capsid (see Materials and Methods). Observation by epifluorescent microscopy revealed significant differences in patterns of phage binding (Fig 5A). All cells of the wild-type strain glowed, indicating that high quantities of PP01-GFP/SOC phage had bound to the cell surface.

On the other hand, in Mu^{L} , some cells did not glow under epifluorescent illumination and were not visible in the dark-field epifluorescence photomicrographs (red circle in Fig. 5A 4), and therefore had not bound PP01-SOC/GFP (red circles in Fig. 5A 3 and 4). Thus in the Mu^{L} strain, a fraction of cells was resistant to phage infection by PP01-SOC/GFP, and a distinct fraction was susceptible (Figs. 2, 3, 4B). This shows that the PP01-SOC/GFP phages were heterogeneously distributed on the surface of single Mu^{L} cells, compared to the wild-type.

The fraction of completely resistant Mu^{L} cells was quantified by infecting cultures of wild-type or Mu^{L} bacteria with PP01, SP15, or PP17 at an MOI of 100 for 0.5 hrs, followed by plating and colony counting (Fig. 5B). In the wild-type strain less than 10^{-3} cells was resistant to any of the three phages tested. In contrast, in Mu^{L} cultures on the order of 10^{-1} cells were resistant to the bacteriophages. To ensure that this trait stably persisted in the Mu^{L} strain, single colonies isolated from PP01-infected Mu^{L} cultures were re-cultured and re-infected with PP01. Single-colony isolation and re-infection with PP01 was repeated a total of five times. The phage resistance after the fifth cycle of reinfection (Mu^{L} -5 in Fig. 5B) was similar, for each phage, as in the original Mu^{L} infection. Thus, approximately 15% of cells in the Mu^{L} population were completely resistant to PP01 infection. A slightly lesser percentage, ~10%, was completely resistant to SP15 and PP17. Thus, colonies arising from PP01-resistant cells produced both sensitive and resistant cells on re-culturing, even after multiple rounds of re-infection. The fraction of resistant cells did

not appreciably change from bacterial generation to generation. This conclusively demonstrates that susceptible Mu^{L} and resistant Mu^{L} cells share the same genotypic background.

Antibody agglutination. Incubation of whole *E. coli* O157:H7 cells of the wild-type strain with anti-O157:H7 antibody resulted in formation of large aggregations of cells in less than 30 minutes. In contrast, this antibody did not agglutinate the Mu^{L} strain within this time (data not shown). The same antibody was used in Western blotting to show that expression of the O157 antigen in Mu^{L} is identical to the wild-type [13].

EPS production. Negative-stain TEM revealed several major differences in cell morphology. Wild-type cells (Fig. 6a) had morphology typical of *E. coli* O157:H7, with a cylindrical cell shape and a prominent flagellar H7 antigen. The concentration of negatively-charged phosphotungstate stain around the wild-type cells was visible as a dark halo around the cell-substrate interface. In Mu^{L} cells (Fig. 6b), the number and length of visible flagella was drastically decreased, although not completely absent. Additionally, phosphotungstate did not appear to concentrate around the cells. Instead, a thick layer of an extracellular polymeric substance was visible on some cells. In Fig. 6b this layer was several hundreds of nanometers thick on the shown cell, but in other cells it was not observed at all. To confirm that this material was a polysaccharide, EPS from the two strains was purified (see Materials and Methods). The purified material was assayed for neutral hexose and uronic acid (Table 1). Uronic acid was not found in either the wild-type extract or the Mu^{L} extract. This result was unexpected because the EPS produced by other *E. coli* O157:H7 strains is colanic acid, a polysaccharide in part comprised of galacturonic acid [17]. However, the level of neutral hexose was twice as high in the Mu^{L} isolate, indicating increased production of some form of polysaccharide (Table 1).

The aggregate results suggest that a likely reason for the clonal heterogeneity is elevated EPS secretion in general in the Mu^{L} strain. In particular, a subset of the Mu^{L} population seems to

be surrounded with an EPS layer thick enough to block adsorption of all bacteriophage, and this blockage endows the Mu^L strain with a tolerance to a wide range of bacteriophages.

For the realization of phage-based therapies, a detailed understanding of the long-term interactions between lytic phages and their hosts will be required. In this study, it was demonstrated that pathogenic serotypes of *E. coli* can evolve to tolerate phage by mutations that affect the heterogeneity of even clonal populations' interaction with bacteriophages. This class of bacteria-bacteriophage interactions will need to be addressed by successful phage therapeutics.

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Figure Legends

Figure 1: Continuous culture of the O157-specific, lytic bacteriophage PP01 with phage-tolerant derivative strain Mu^L. Bacteria (open triangle) were first allowed to reach equilibrium density in LB medium at 37 °C and a dilution rate of 0.33 hr⁻¹. At time zero PP01 phage (closed triangle) was added to culture at a MOI of 0.01. The Mu^L parental strain ATC43888 does not show this behavior [13].

Figure 2: Single step growth of PP01 phage on *E. coli* O157:H7 wild-type strain ATCC43888 (open squares) and Mu^L (closed squares) in LB medium at 37 °C. The lines are best fits of the data to the error function Erf(x).

Figure 3: Binding of PP01 bacteriophage to exponentially growing *E. coli* O157:H7 to ATCC43888 (wild-type, open squares) or to phage-tolerant Mu^L (closed squares) at a multiplicity of infection of 0.01, in LB medium at 37 °C.

Figure 4: Coexistence of *E. coli* O157:H7 strain Mu^L (A) with bacteriophages SP15 and PP17, which are highly lytic to the wild-type *E. coli* ATCC43888 strain (B). At time $t=1$ hr, exponentially growing *E. coli* O157:H7 cultures in LB medium at 37 °C were infected at a multiplicity of infection of 2 with either SP15 (closed squares), PP17 (closed triangles), or uninfected (open circles).

Figure 5: Heterogeneity of pure cultures of *E. coli* O157:H7 strain Mu^L. (A) Bacteriophage PP01-SOC/GFP was incubated for 10 minutes at MOI 1000 with wild-type (1, 2) and Mu^L (3, 4) cells. Light-field phase-contrast photomicrographs (1, 3) show all cells in the viewing field, but epifluorescent photomicrographs (2, 4) show only cells to which PP01 had bound. Circles mark

Mu^L cells invisible in the fluorescent image. (B) Exponentially growing cells were infected with phage at an MOI of 100 for 0.5 hr, and plated on LB agar. The fraction of colonies surviving is shown. Mu^L-5 indicates a Mu^L descendant that had been subjected to 5 cycles of PP01 infection and single colony isolation. Error bars show the standard error in the mean for four replicates.

Figure 6: Electron micrographs of *E. coli* O157:H7 strain ATCC43888 (wild-type, a, c) and phage-tolerant strain Mu^L (b, d). (a and b) Cells were negatively stained with phosphotungstic acid and observed directly by TEM at 20,000× magnification. (c and d) Cells were fixed with glutaraldehyde and osmium-tetroxide were coated with platinum and observed by SEM at 50,000

TABLE 1. Composition of purified EPS from *E. coli* O157:H7.

<i>E. coli</i> O157:H7 Strain	Neutral hexose ($\mu\text{g} / 10^9 \text{ CFU}$)	Uronic acid ($\mu\text{g} / 10^9 \text{ CFU}$)
ATCC43888	3.3	0.09
Mu^L	6.4	0.07