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Letter

Beta-lactam-Induced Outer Membrane Alteration Confers *E. coli* a Fortuitous Competitive Advantage through Cross-Resistance to Bacteriophages

Pengxiao Zuo, Pingfeng Yu,* and Pedro J. J. Alvarez*

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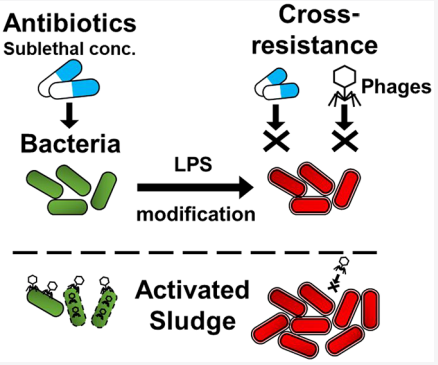
ABSTRACT: Bacteriophages play an important role in controlling bacteria populations; yet, little is known about their differential effects on antibiotic resistant bacteria (ARB) proliferation. Here, we report that beta-lactam resistance may fortuitously confer phage resistance as a critical factor for enhanced ARB proliferation. Following sublethal exposure to amoxicillin, *Escherichia coli* experienced lipopolysaccharides (LPS) modifications (corroborated by FTIR) and became cross-resistant to various phages that adsorb to receptors on LPS as the first infection step. Although, wildtype cells' LPS adsorbed 39 ± 8.7% of T3 phages, cross-resistant cells' LPS only adsorbed 4.1 ± 2.3%. We demonstrate the relevance of this phenomenon using activated sludge microcosms. Cross-resistant *E. coli* experienced a negligible decrease from 6.27 ± 0.07 to 6.07 ± 0.18 log CFU/mL after 5 days, whereas the wildtype decayed from 6.11 ± 0.13 to 5.29 ± 0.02 log CFU/mL. Furthermore, cross-resistant *E. coli* (but not wildtype) proliferated in Luria broth-fed microcosms. We also show a fitness cost associated with amoxicillin resistance; however, due to acquired phage resistance, cross-resistant *E. coli* had greater fitness than the wildtype. Overall, this study demonstrates that antibiotics can alter interactions between phages and bacteria, resulting in an overlooked competitive advantage for antibiotic-resistance propagation.



INTRODUCTION

Overcoming antibiotic resistance to protect global health is one of the greatest challenges of the 21st century. This requires improved fundamental understanding of conditions that enhance the growth and dissemination of antibiotic resistant bacteria (ARB) in the environment.[1] Chemical and physical factors that enhance the propagation of ARB and their antibiotic resistance genes (ARGs) have been widely investigated, including the presence of antibiotics at sublethal levels,[2] of heavy metals that coselect for resistance conferred by efflux pumps,[3] and of clay minerals that adsorb and stabilize extracellular ARGs, hindering their degradation by nucleases.[4] However, biological stressors have received relatively limited attention.

One of the most important biological stressors for bacteria in the environment is predation by phages, which are the most abundant biological entities on the planet.[5] Phages are viruses that only infect bacteria and can account for ∼20% daily turnover of bacterial mass.[6,7] Phages can select for bacteria without efflux pumps (which disrupt phage receptors) that are more sensitive to antibiotics[8] or work synergistically with antibiotics to kill bacteria.[9,10] However, potential antagonistic interactions associated with bacterial exposure to both antibiotics and phages, and cross-resistance implications, have been overlooked.

Antibiotic resistance may emerge not only from mechanisms coded by ARGs (e.g., efflux pumps or enzymatic degradation of beta-lactams[11]) but also from alterations of bacteria surface chemistry. This includes loss of transport proteins to limit uptake of antibiotics[12] and lipopolysaccharides (LPS) modifications to prevent their initial binding.[13−15] Some of these cell surface structures may also serve as phage receptors,[16] and their alteration can hinder phage adsorption, preventing infection.[17−19] Thus, phage resistance could also be obtained fortuitously due to antibiotic-induced bacterial surface alterations that modify phage receptors, and it is important to investigate whether cross-resistance develops easily and provides a competitive advantage for ARB propagation, even in the absence of selective pressure by antibiotics.

Wastewater treatment plants (WWTPs) may be breeding grounds and point sources for ARB discharge into the environment,[20] and the secondary effluent is likely to contain both antibiotics[21,22] and phages.[23,24] Thus, we used activated sludge microcosms to assess potential development of cross-

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resistance and its implication on ARB proliferation. We chose *E. coli* (a common bacterium in this environment) as a representative ARB. Amoxicillin was used since it is one of the most prescribed antibiotics. Furthermore, similar to other beta-lactams, the mode of action of amoxicillin involves passing through the outer cell membrane to hinder peptidoglycan cross-linking, which makes resistance through cell membrane permeability alterations possible. Wildtype *E. coli* were treated with amoxicillin to select for resistant phenotypes, which were then tested for phage resistance, and their growth in activated sludge microcosms were compared to wildtype.

MATERIALS AND METHODS

Bacteria Strains and Phage Stocks. Cross-resistant *E. coli* K-12 was isolated from wildtype *E. coli* K-12 (ATCC 10798) after two-day exposure to 5 mg/L of amoxicillin on day 1 (below the MIC of 8 mg/L) followed by 10 mg/L on day 2. This selected for amoxicillin-resistant mutants, which were also fortuitously resistant to three phages. Following the manufacturer's instructions, ARG $bla_{\text{NDM-1}}$ was inserted into *E. coli* BL21 (Bioline BIO-85035) to obtain a phage-susceptible ARB. This served as an additional control to verify that the competitive advantage of cross-resistant mutants in activated sludge microcosms was primarily due to their ability to resist phage infection rather than antibiotic resistance. Bacteria stocks were grown in Luria–Bertani Broth (LB) (BD-Difco) at 37 °C. Resistance was verified by determining minimal inhibitory concentration of amoxicillin, which is about 8 mg/L for wildtype *E. coli* K-12, 50 mg/L for cross-resistant *E. coli* K-12, and 200 mg/L for *E. coli* BL21 with $bla_{\text{NDM-1}}$. To explore response variability and demonstrate that amoxicillin resistance fortuitously confers resistance to multiple phages, different families of phages were used in this study. These include *Podoviridae* T3 (ATCC 110303-B3), *Myoviridae* T4 (ATCC 110303-B4), and a wild sludge phage (WSP) isolated from activated sludge.[25]

Bacteria Enumeration. Viable *E. coli* concentration was determined using a plate assay to count colony forming units (CFU),[26] which was correlated to optical density ($OD_{600\ nm}$) (Figure S1). Optical density was measured with a plate reader (Tecan Infinite 200 Pro) set at 37 °C with measurements taken every 30 min for 20 h. *E. coli* concentration in activated sludge was determined by RT-qPCR with the *E. coli*-specific *uspA* gene.[25] A standard curve was developed between cycle time and bacteria concentration (Figure S2).

Activated Sludge Microcosm Tests. Activated sludge with an indigenous *E. coli* background concentration of about 4.34 log CFU/mL was collected from a local WWTP in Houston, Texas. Three 25 mL activated sludge microcosm sets were prepared in triplicate, one for each type of *E. coli* strain added (to reach ~6 log CFU/mL). The control set was amended with wildtype *E. coli* K-12. One additional control set (amended with *E. coli* BL21, which is solely resistant to the antibiotic, Figure S3) was used to verify that cross-resistant mutants had a competitive advantage primarily due to their ability to resist phage infection. Every day for 5 days 1.25 mL of sludge was removed for qPCR analysis and replaced with 1.25 mL of minimal broth Davis (BD-Difco).

Bacterial Cell Surface analysis. Bacterial suspensions (10 mL) were centrifuged at 8000 rpm for 10 min, and the pellet was resuspended in a SM buffer[25] (1 mL) and centrifuged again at 15,000 rpm for 2 min. The pellet was freeze-dried overnight with liquid nitrogen and a Millrock benchtop freeze-dryer. FTIR spectra of the pellet was obtained using a Nicolet FITR infrared microscope.

Phage adsorption to bacteria and LPS extracts was investigated to elucidate the potential mechanism for phage resistance. Briefly, phages suspended in SM buffer were added to bacteria suspensions (wildtype or cross-resistant *E. coli* K-12) using a MOI of 0.0001 at 37 °C while constantly shaking for 10 min.[27] Adsorbed phages were removed by centrifugation at 15,000 rpm for 1 min with 5% v/v chloroform, and the free phages in the supernatant were counted by a plaque assay plated on a wildtype *E. coli* K12 lawn.[28] For phage–LPS adsorption tests, LPS was extracted from ~10^9 cells using an iNtRON biotechnology LPS extraction kit. Adsorption tests were repeated with the same phage concentrations, but bacteria were replaced with extracted LPS.

Statistical Analysis. Experiments were run as independent biological triplicates to characterize response variability, and error bars represent ± 1 standard deviation from the mean of independent triplicates. A Student's *t*-test (two-tailed, unpaired, assuming equal variance) was used to determine if differences in bacterial concentration, decay rates, and phage adsorption capacity were significant (i.e., $p < 0.05$).

RESULTS AND DISCUSSION

Exposure to Low Levels of Beta-lactams Develops Phage Resistant *E. coli*. Wildtype *E. coli* K-12 is susceptible to coliphages T3 and T4 and to phage WSP. In contrast, the amoxicillin-induced beta-lactam-resistant K-12 mutant was fortuitously resistant to these three phages (Figure 1). These cells achieved exponential growth on the same time scale as wildtype *E. coli* even in the presence of antibiotics, indicating that cross-resistance was inherited. When grown without

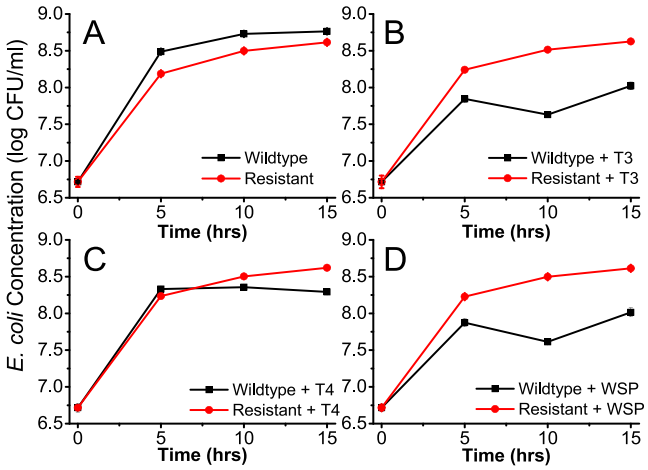

Figure 1. Beta lactam-resistant *E. coli* fortuitously developed resistance to coliphages T3 and T4 and to a wild phage from activated sludge (WSP). Wildtype *E. coli* K12 is susceptible to all three phages as shown by lower final concentrations when the phages are added separately (B, C, and D) than in the unamended control (A). Each triplicate well contained 190 μL LB (pH 7) and was inoculated with 10 μL of overnight cultures of wildtype or resistant *E. coli* K12. Phage titers were 2 × 10^5 PFU/mL, and 1 μL was added to the wells. Optical density was measured for 15 h at 37 °C and converted into log CFU/mL using the standard curve in Figure S1. Error bars represent ± 1 standard deviation from the mean of independent triplicates. Differences in final concentration between wildtype and resistant *E. coli* K-12 in panels A–D were significant ($p < 0.05$).

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phages or antibiotics, the final population of wildtype *E. coli* (8.76 ± 0.004 log CFU/mL) was higher ($p < 0.05$) than that of cross-resistant *E. coli* (8.61 ± 0.002 log CFU/mL), indicating a common fitness cost associated with resistance.[29] Coliphages T3 and T4 and phage WSP could suppress growth of the wildtype but not of the cross-resistant *E. coli* despite the latter only having prior exposure to amoxicillin (Figure 1). Cross-resistance was not observed with different treatment sequences; when added simultaneously, beta-lactams did not protect the wildtype from phages, ruling out antagonism as a potential defense mechanism. When wildtype *E. coli* was first exposed to and became resistant to phage T3, it did not become beta-lactam resistant (Figure S4). Thus, only beta-lactam was able to induce cross-resistance, but not when added simultaneously with phages, which apparently overwhelms *E. coli* and hinders development of resistance.

Beta-lactams inhibit penicillin-binding proteins (PBPs) responsible for peptidoglycan cross-linking in the cell wall of both Gram-positive and -negative bacteria.[30] In Gram-negative bacteria, beta-lactams must first pass the outer membrane to reach the PBPs. Thus, resistance can arise from modification of LPS,[14] possibly by disrupting trimerization and biogenesis of OmpF porins, which require formation of porin-LPS complexes.[31,32] This would decrease OmpF-facilitated permeation of beta-lactams.[33,34] Furthermore, phage adsorption depends on the presence of specific phage receptors on the outer membrane.[35] Because some beta-lactams and phages both interact with the outer membrane, alterations endowing beta-lactam resistance may fortuitously hinder phage adsorption by altering these phage receptors, resulting in cross-resistance. LPS modifications may provide resistance to numerous phages since LPS is the main receptor on Gram-negative bacteria[16] and is targeted by multiple phages.[36] In theory, this mechanism could also apply to Gram-positive bacteria, which can modify wall teichoic acids[37]—a known phage receptor[36]—to become antibiotic resistance.

The observed cross-resistance phenomenon seems dependent on very specific combinations of phages and antibiotics, since many antibiotics may induce resistance mechanisms unrelated to phage adsorption.[38] Nevertheless, beta-lactams, particularly amoxicillin, are among the most prescribed antibiotics,[39] and all phages require adsorption as the essential first step for infection.[35] Because phages T3 and T4 are both model coliphages isolated from sewage[40] and phage WSP was isolated from activated sludge, resistance to these phages may provide a significant advantage for ARB in WWTPs. Therefore, it is important to discern how cross-resistance affects ARB proliferation and decay in activated sludge.[20]

Cross-Resistant *E. coli* Had a Competitive Advantage over Wildtype in Activated Sludge Due to Decreased Susceptibility to Phages. When added to activated sludge microcosms, cross-resistant *E. coli* decreased marginally from an initial concentration of 6.27 ± 0.07 to 6.07 ± 0.18 log CFU/mL in 5 days with a decay rate of −0.013 day^{-1} (Figure 2). The wildtype decreased faster ($p < 0.05$), from 6.11 ± 0.13 to 5.29 ± 0.02 log CFU/mL at a decay rate of −0.028 day^{-1}, and antibiotic resistant (but not phage-resistant, Figure S3) *E. coli* with bla_{NDM-1} (NDM-1) decreased from 6.22 ± 0.05 to 4.74 ± 0.18 log CFU/mL with a decay rate of −0.082 day^{-1}. The ARG bla_{NDM-1} encodes a beta-lactamase that degrades beta-lactam antibiotics without altering the outer membrane and hindering phage infections. NDM-1 decayed faster than the wildtype (−0.082 vs −0.028 day^{-1}) (Figure 2). The greater

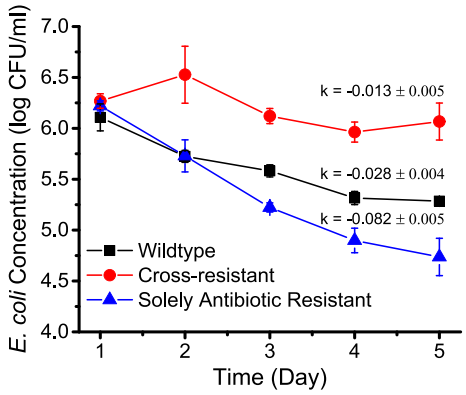

Figure 2. Cross-resistant *E. coli* K12 have a slower decay rate than wildtype and *E. coli* BL21 harboring the bla_{NDM-1} gene (solely resistant to antibiotics) in activated sludge. *E. coli* was added to activated sludge to a concentration of about 6 log CFU/mL. Initial concentration of pre-existing *E. coli* in activated sludge was 4.34 ± 0.24 log CFU/mL. 5% v/v of the microcosm was replaced with Davis minimal broth daily, and RT-qPCR was performed targeting the *E. coli*-specific *uspA* gene. Experiments were run as independent triplicates at room temperature with constant shaking at 300 rpm. Error bars represent ± 1 standard deviation from the mean of independent triplicates. Differences in final concentration between the three groups were significant ($p < 0.05$).

fitness of cross-resistant *E. coli* can be attributed to phage resistance, which is an important advantage in activated sludge[41,42] where phage concentrations are typically about 10^9 mL^{-1}, the highest of any measured environment.[43]

Cross-resistance may also enhance ARB proliferation under favorable growth conditions. This is illustrated in activated sludge microcosms fed nutrient-rich LB. Cross-resistant *E. coli* K-12 grew from 7.77 ± 0.04 to 8.22 ± 0.06 log CFU/mL in 5 days, whereas the wildtype decayed from 7.76 ± 0.04 to 7.48 ± 0.04 log CFU/mL (Figure 3).

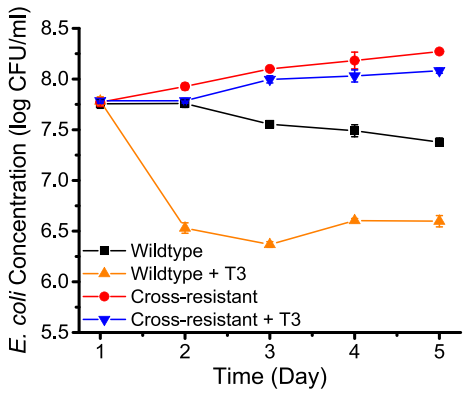

Figure 3. Cross-resistant *E. coli* has a competitive growth advantage compared to wildtype in activated sludge microcosms. *E. coli* was added at about 8 log CFU/mL, and phage T3 was spiked in some microcosms at MOI = 1. Initial concentration of pre-existing *E. coli* in the microcosms was 4.34 ± 0.24 log CFU/mL. 5% v/v of the microcosm was replaced with LB daily, and RT-qPCR was performed to target the *E. coli*-specific *uspA* gene. Experiments were run as independent triplicates at room temperature with constant shaking at 300 rpm. Error bars represent ± 1 standard deviation from the mean of independent triplicates. Differences in final concentration between the four groups were significant ($p < 0.05$).

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Cell surface alterations could be caused by mutation of membrane structural or regulation genes not associated with ARGs.[15,44,45] Thus, cross-resistant bacteria could be under-detected contributors to antibiotic resistance, which is typically assessed per ARGs analysis. However, such outer membrane alterations may contribute to antibiotic resistance propagation. For example, antibiotics taken orally can cause significant shifts in the human gut microbiota and resistome.[46] This could induce adaptive changes including LPS modifications.[45,47] Some resistant bacteria may become fortuitously cross-resistant to phages, representing an important competitive advantage in WWTPs and beyond. Higher survival of cross-resistant bacteria increases the likelihood of ARB discharge in secondary effluent or land-applied biosolids, which is conducive to augmenting the environmental resistome.

Cross-Resistant *E. coli* Exhibit LPS Modifications That Contribute to General Phage Resistance through Decreased Adsorption. Phage adsorption is the essential first step for successful infection and is determined by the presence of phage receptors on the bacteria surface. These receptors could be various surface structures such as LPS or outer membrane proteins,[35] and their alterations would hinder phage adsorption. Thus, differences in phage adsorption between wildtype and amoxicillin-resistant *E. coli* were assessed to test this hypothesis. Wildtype cells adsorbed 34.5 ± 8.5% of free T3 phages and 34.0 ± 1.63% of free T4 phages, whereas the cross-resistant cells only adsorbed 8.6 ± 8.5% of free T3 phages and 16.5 ± 3.63% of free T4 phages ($p < 0.05$) (Figure 4). Previous studies report that adsorption of phages T3 and

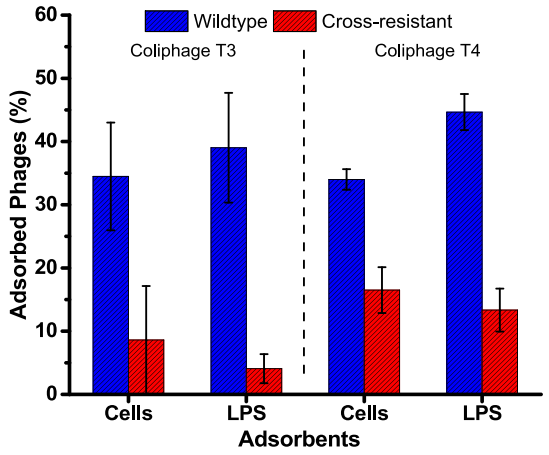

Figure 4. Cross-resistant *E. coli* K12 cells and extracted lipid polysaccharide (LPS) adsorb less phages than their wildtype counterparts ($p < 0.05$). Phages T3 and T4 were added to bacteria cultures in an exponential phase (MOI = 0.0001) at 37 °C for 10 min under constant shaking. Samples were plated on wildtype *E. coli* K12 to enumerate phage plaques. The experiment was repeated using the same amount of phages but with bacteria replaced by LPS extracted from ~10^9 cells. Error bars represent ± 1 standard deviation from the mean of independent triplicate tests.

T4 can be prevented by modification of their common phage receptor, the LPS.[18] To determine whether that was the case here, adsorption tests were repeated using extracted LPS instead of live cells as adsorbent. Wildtype cells' LPS adsorbed 39 ± 8.7% of free T3 phages and 22.33 ± 1.43% of free T4 phages, whereas the cross-resistant cells' LPS could only adsorb 4.1 ± 2.3% of free T3 phages and 6.67 ± 1.70% of free T4 phages ($p < 0.05$) (Figure 4).

FTIR data corroborate altered surface chemistry associated with cross-resistance (Figure 5). A weaker absorption band in

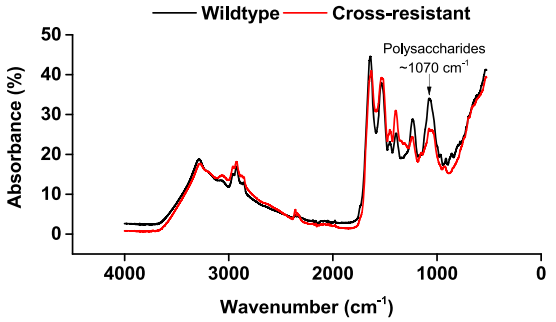

Figure 5. FTIR spectra show differences in the outer membrane composition between wildtype and cross-resistant *E. coli*. Most notably, cross-resistant cells have a weaker band in the region of the C−O−C stretching vibration (1140−1000 cm^{-1}) of polysaccharides, indicative of LPS alteration.[48,49] Differences in LPS between wildtype and cross-resistant cells were confirmed by SDS-PAGE (Figure S5).

the region around 1140−1000 cm^{-1} (corresponding to C−O−C stretching vibrations of polysaccharides)[48,49] was detected for cross-resistant *E. coli* relative to the wildtype. Differences in LPS were also observed using SDS-PAGE (Figure S5). Thus, converging lines indicate that LPS modifications induced by antibiotics incidentally conferred phage resistance.

Overall, the finding that some antibiotics can induce bacterial LPS alterations, which provides fortuitous phage resistance, highlights an important overlooked (cross-resistance) competitive advantage for ARB of high environmental relevance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.0c00318.

Standard curves of bacterial enumeration, bacterial growth curves under different treatments, and SDS-PAGE gel and silver staining of LPS (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Pingfeng Yu − *Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States*; Email: pingfeng.yu@rice.edu

Pedro J. J. Alvarez − *Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States*; orcid.org/0000-0002-6725-7199; Email: alvarez@rice.edu

Author

Pengxiao Zuo − *Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.estlett.0c00318

Notes

The authors declare no competing financial interest.

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