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Fate of Extracellular DNA in the Production of Fertilizers from Source-Separated Urine

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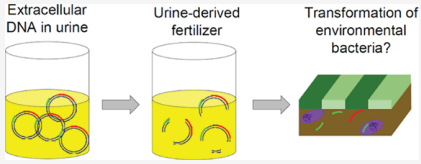
Supporting Information

ABSTRACT: The practice of urine source-separation for fertilizer production necessitates an understanding of the presence and impact of extracellular DNA in the urine. This study examines the fate of plasmid DNA carrying ampicillin and tetracycline resistance genes in aged urine, including its ability to be taken up and expressed by competent bacteria. Plasmid DNA incubated in aged urine resulted in a >2 log loss of bacterial transformation efficiency in *Acinetobacter baylyi* within 24 h. The concentration of ampicillin and tetracycline resistance genes, as measured with quantitative polymerase chain reaction, did not correspond with the observed transformation loss. When the plasmid DNA was incubated in aged urine that had been filtered (0.22 μm) or heated (75 °C), the transformation efficiencies were more stable than when the plasmids were incubated in unfiltered and unheated aged urine. Gel electrophoresis results indicated that plasmid linearization by materials larger than 100 kDa in the aged urine caused the observed transformation efficiency decreases. The results of this study suggest that extracellular DNA released into aged urine poses a low potential for the spread of antibiotic resistance genes to bacteria once it is released to the environment.



INTRODUCTION

Urine contains the majority of nitrogen and phosphorus found in domestic wastewater.[1] Collecting this nitrogen and phosphorus from urine before it is diluted in the wastewater stream can offset energy-intensive fertilizer production processes,[2] reduce the need for energy-intensive nutrient removal processes at wastewater treatment plants,[3,4] and prevent nutrient discharges that generate harmful algal blooms and eutrophication.[5] Furthermore, trace organic chemical contaminants that are present at high concentrations in urine can be removed more efficiently from urine than wastewater. These multiple environmental benefits make urine separation and reprocessing a promising option for nutrient recovery.

Source-separated urine can be converted to fertilizer through multiple methods, with the simplest being urine storage in a sealed container for weeks to months.[6] When stored, the urea in "fresh" urine is hydrolyzed by urease enzymes. This causes substantial increases in ammonia concentrations and pH values and shifts in microbial communities.[7] Although these conditions kill many of the bacteria present in the urine, hydrolyzed urine still contains culturable and metabolically active bacteria after months of aging.[7] Pasteurization is another common approach for preparing urine-derived fertilizers, with the major goal of inactivating any human pathogens present in the urine.

Urinary tract bacterial infections are commonly treated with antibiotics, and this can lead to an enrichment of antimicrobial resistant organisms[8] and antimicrobial resistance genes (ARGs)[9] in urine. When microorganisms in urine are inactivated and lysed, their nucleic acids are released. Consequently, ARGs can be present as extracellular DNA in source-separated urine even after the microorganisms that carry them are inactivated. When the aged urine is applied as a fertilizer, the DNA containing resistance genes is released to the environment. In the environment, the DNA may be available to transform environmental bacteria, resulting in the spread of antibiotic resistance.[11,12] In terms of the fate of extracellular DNA in urine, the concentrations of viral dsDNA genomes measured with quantitative polymerase chain reaction (qPCR) were stable for several weeks in aged urine despite the fact that the virus capsids had disassembled.[10]

PCR-based methods are commonly employed to quantify the intracellular and extracellular resistance genes that are present in environmental samples.[13−17] These measurements, however, do not assess the potential of the genes to be transferred to other organisms. As the field moves toward translating the measured ARG concentrations to posed risk, the transfer potential of genes needs to be assessed. When a solution of plasmids containing ARGs was treated with UV_{254}, the first-order reaction rate constants of the ARGs, as measured by qPCR, were 2−7× larger than the rate constants of the same ARGs measured with transformation efficiency assays.[18] In other words, when qPCR is employed to measure the degradation of ARGs, it does not accurately relay reductions in the risk of new organisms attaining resistance.

Based on the observed stability of DNA for several weeks in urine[7,9] and the reports of antibiotic resistance genes in urine detected with qPCR,[9] we sought to better understand the fate of extracellular DNA in urine-derived fertilizers and its

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potential to be transferred and expressed by competent bacteria in the environment. We employed plasmid DNA as a model for extracellular DNA because it results in high transformation efficiencies in competent bacteria and because plasmid-mediated resistance has been detected in urine.[48,49] In our experiments, plasmid DNA containing two antibiotic resistance genes (tetracycline resistance, tet_A and ampicillin resistance, bla_{TEM-1}) was incubated in aged, filtered, and heated urine, and then the ability of the plasmids to transform *Acinetobacter baylyi* was evaluated. We used *A. baylyi* as a model organism because it is common in soils, is highly competent for natural transformation,[19,20] and is relevant for opportunistic infections.[21] The relatively high transformation rates of this system allowed us to measure up to a 99% loss in transformation efficiency of *A. baylyi* and thus a 99% decrease in the expression of the tetracycline and ampicillin resistance. As plasmids lost their ability to transform *A. baylyi*, we measured the gene concentration with qPCR and plasmid conformation with gel electrophoresis. The results of this study provide insight into how the conditions of hydrolyzed urine impact extracellular DNA and ultimately reduce the risk of antibiotic resistance gene transfer.

MATERIALS AND METHODS

Urine Collection and Characterization. The urine used in the experiments was collected from over 100 female and male donors in Vermont. The collected urine (>100 L) was stored at room temperature in a sealed plastic container between 12 and 16 months. Each "batch" of hydrolyzed urine used in a set of experiments was from the same volume of collected urine but had been aged for a unique period of time. The total ammonia nitrogen levels were measured with the standard method 4500-NH_3.[22] The measured ammonia concentrations (5400 ± 400 mg N/L ammonia; standard deviation for $n \geq 3$) were consistent with the concentrations reported previously for hydrolyzed, filtered, and heated urine.[23,24]

General Experimental Approach. Plasmid DNA fate was tested in a number of experimental solutions, including aged urine, aged urine that had been filtered through 0.22 μm membrane filters (PES, Dot Scientific), aged urine that had been filtered through centrifugal ultrafilters with 100 kDa pores (Amicon Ultra centrifugal filter units, Millipore-Sigma), and aged urine that had been filtered through 0.22 μm membrane filters, and heated to 75 °C in a water bath for 10 min. Positive control samples were included in every experiment and consisted of the plasmid DNA incubated in nuclease-free water. Our model DNA molecule, plasmid pWH1266, was spiked into each experimental solution after the solution had been aged, filtered, heated, and so forth at a final plasmid DNA concentration of 7−8 ng/μL. This plasmid concentration is well above the background DNA levels in the aged urine (0.18 ng/μL), as measured with a Qubit 2.0 fluorometer (Invitrogen). The concentration was selected to facilitate optimum ratios of plasmid DNA to competent cells in the subsequent transformation assays.[18] The spiked solutions were incubated in the dark at room temperature for the duration of the experiments. Aliquots were collected immediately after the aged urine sample or nuclease-free water control sample was mixed with the plasmid DNA ($t = 0$) and after incubation with the plasmid DNA at room temperature between 10 and 48 h. The pWH1266 plasmid in the aliquots was tested for transformation efficiency in *A. baylyi*, for gene concentration by qPCR, and for plasmid DNA conformation by gel electrophoresis. The aliquots collected for qPCR and gel electrophoresis were frozen and stored at −20 °C until they were analyzed. The aliquots collected for transformation efficiency measurements were assayed immediately.

Model Transformation System. We selected an experimental transformation system that can achieve high detectable transformation rates and thus represents a worst case scenario for bacterial transformation when urine-derived fertilizers with extracellular ARGs are applied to the environment. This system was used previously by our group to study the impact of UV_{254} on bacterial transformation by extracellular DNA.[18] *A. baylyi* strain AC811, which is a derivative of the Acinetobacter sp. *Strain BD413*,[20,25] is a naturally competent bacterium that is transformed by pWH1266. The pWH1266 plasmid (8.9 kb) is a construct of plasmids pBR322 and pWH1277 (Figure S1). pBR322 is an *Escherichia coli* plasmid that includes both tetracycline (tet_A) and ampicillin (bla_{TEM-1}) resistance genes (Figure S1).[26−28] pWH1277 is an *Acinetobacter calcoaceticus* plasmid whose sequence was not available prior to this study. We therefore used the primer walking method with Sanger sequencing to obtain the sequence of the pWH1277 region in pWH1266 (see Supporting Information). Dr. Chuanwi Xi at the University of Michigan provided the transformation system, including plasmid pWH1266, the *E. coli* strain TOP10 that was used to propagate the plasmid, and the *A. baylyi* strain that was transformed in the experiments.

Plasmid Extraction. Plasmid pWH1266 DNA was propagated in the *E. coli* strain TOP10. Frozen cultures of *E. coli* were inoculated on LB broth (Lennox) media agar with tetracycline (10 μg/mL) overnight.[18] A single colony was selected and inoculated in liquid LB media with tetracycline (10 μg/mL). *E. coli* was grown overnight at 37 °C with shaking (180 rpm). The following morning, the cultures were concentrated 2× by centrifuging two 30 mL overnight cultures (total 60 mL) and resuspending the pellet in 30 mL of LB media. The plasmid DNA was extracted using QIAprep Plasmid Spin mini-prep kits as per the manufacturer's instructions (Qiagen). The DNA concentrations in the plasmid extract (~50 to 100 ng/μL) were measured with a Nanodrop 1000 spectrophotometer.

Transformation Assays. Transformation assay conditions, including the ratio of plasmid DNA to competent cells, the point in the *A. baylyi* growth curve when the plasmids were added, and the incubation time necessary for the plasmid to transform the bacteria, were optimized and reported in our previous study.[18] In summary, the frozen aliquots of *A. baylyi* were inoculated on LB media agar and incubated overnight at 30 °C. A single colony was selected from the agar plate, inoculated in liquid LB media, and incubated at 30 °C with shaking (240 rpm) until it reached the stationary phase (16−18 h). The cultures were diluted 10× in LB media and incubated at 30 °C with shaking (240 rpm). After 2 h, the bacteria reached the early exponential phase and were competent for transformation experiments. At this point, a 480 μL aliquot of competent cells was combined with 20 μL of the experimental solution (e.g., pWH1266 incubated in aged urine) in culture tubes, resulting in a final bacteria concentration of ~10^8 cells/mL and plasmid concentration of 0.4 ng/uL. The mixture was incubated for 24 h at 30 °C with shaking (240 rpm). These growth conditions were consistent throughout all experiments. Following incubation, the sample was serially diluted and plated on both LB media

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Table 1. Primers for pWH1266[a]

gene	length of amplicon (bps)	forward primer (5′ to 3′)	reverse primer (5′ to 3′)
tet_A	1191	CGTGTATGAAATCTAACAATGCGCT[18]	CCATTCAGGTCGAGGTGGC[18]
bla_{TEM-1}	861	TTACCAATGCTTAATCAGTGAGGC[18]	ATGAGTATTCAACATTTCCGTGTCG[18]
ori	901	AGGCGGTAATACGGTTATCCAC[a]	GAGATAGGTGCCTCACTGATTAAG[a]

[a]Designed using NCBI Primer Blast.

agar and selective LB media agar that contained either tetracycline (10 μg/mL) or ampicillin (100 μg/mL). The colonies on the agar plates were counted after overnight growth at 30 °C. Transformation efficiencies (N) represented the number of colonies that grew on LB media with antibiotics divided by the number of colonies that grew on nonselective LB media. The transformation efficiencies measured with plasmids that had been incubated in samples for a specific amount of time were typically normalized by the transformation efficiencies at time = 0 (N/N_0). The initial transformation efficiencies in the aliquots collected immediately after the plasmid DNA was added to the hydrolyzed urine or water controls ($t = 0$) were in the range of 6.3×10^{-7} to 1.2×10^{-5}. With these initial transformation efficiencies, we were able to observe 2 $\log_{10}$ (i.e., 99%) loss of transformation efficiency through the experiments before reaching the assay detection limits. The transformation assay detection limit for each set of experiments was equal to one transformant on the selective media divided by the number of viable cells grown on the nonselective media.

Every experiment included negative control samples, which consisted of the *A. baylyi* cells without the addition of plasmid DNA plated on the selective media. No growth was observed on these plates, demonstrating that the bacteria did not exhibit background resistance to tetracycline and ampicillin. The initial transformation efficiencies immediately after the plasmids were spiked into nuclease-free water were not statistically different than the efficiencies immediately after the plasmids were spiked into urine (*tet*: $p = 0.66$, -0.097 $\log_{10}$; *amp*: $p = 0.67$, -0.054 $\log_{10}$; $N = 6$); the constituents in urine therefore did not have a major impact on the initial transformation efficiencies. Additional control experiments of urine plated on selective media exhibited no bacterial growth during the duration of the experiment (<48 h).

Gel Electrophoresis. The conformation and integrity of plasmid DNA were evaluated by gel electrophoresis. The control samples consisting of plasmid DNA in nuclease-free water and experimental samples (10 μL each) were thawed, mixed with 6× blue loading dye (Promega), and then loaded onto 0.5% agarose (Biorad) gels diluted in 1× TAE buffer (Biorad). The Biorad PowerPac Basic gel electrophoresis system was run at 40 V for 80−120 min. The DNA bands were visualized with SYBR safe DNA gel stain (Life Technologies), and the molecular mass of the plasmid DNA was confirmed with GeneRuler 1 kb DNA ladders (ThermoFisher Scientific).

qPCR Measurements. The concentrations of pWH1266 regions were measured throughout the experiments with qPCR. Three regions of the pWH1266 plasmid were quantified, namely the tet_A gene (1191 bps), bla_{TEM-1} gene (861 bps), and a region including the origin of replication (*ori*) (901 bps). Primers were designed to cover the entire resistance gene sequences and the entire origin of replication sequence (Figure S1). Primers for the tet_A and bla_{TEM-1} genes were designed previously (Table 1).[18] Primers for the *ori* gene were designed using the NCBI Primer-Blast tool (Table 1). qPCR was conducted with a Mastercycler RealPlex 2 system (Eppendorf, Hamburg, Germany) with Fast EvaGreen qPCR Master Mix (Biotium). Standards used to develop qPCR standard curves consisted of purified pWH1266 plasmids that were quantified (as ng/uL) with a Qubit 2.0 fluorometer (Invitrogen), and the mass concentrations were converted to plasmid or gene copy concentrations based on the molecular mass of the plasmid and Avogadro's constant. The qPCR standard curves were conducted in duplicate, with the concentrations ranging from 10^3 to 10^8 gene copies/mL. The 10 μL standard and sample reactions included 5 μL of 2× Biotium Fast EvaGreen Master Mix, 0.05 μL of each forward and reverse 100 μM primers, 0.13 μL of 50 mg/mL bovine serum albumin, 3.77 μL nuclease-free water, and 1 μL of DNA template (0.5−5 ng). All qPCR standard curve R^2 values were greater than 0.99 (Supporting Information Table S1). The qPCR assay efficiencies ranged from 65 to 90% (Supporting Information Table S1), which are typical for long-amplicon qPCR.[18,46,47] All sample readings were within the standard curve.

Data Analyses. Statistical analyses were completed in GraphPad Prism software. We employed Student's t test to compare the plasmid transformation efficiencies between different sets of samples (e.g., before and after incubation times in aged urine). In each case, we report p values at the 95% confidence level (e.g., p_{tet}), the effect sizes as the $\log_{10}$-fold change in the means, and the number of replicate experiments conducted (N).

RESULTS AND DISCUSSION

Plasmid DNA Stability in Aged Urine Measured with Transformation Assays. Plasmid DNA incubated in aged urine was assayed for its ability to transform *A. baylyi* cells. The transformation efficiencies did not change significantly after the plasmid DNA had incubated for 10 h in the aged urine (*tet*: $p = 0.16$, $+0.28$ $\log_{10}$; *amp*: $p = 0.50$, $+0.061$ $\log_{10}$; $N = 3$; Figure 1). We note that the lack of statistical difference in this case may be partly because of the small number of

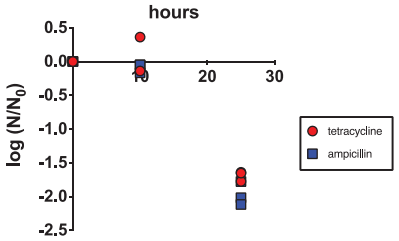

Figure 1. Transformation efficiency relative to the initial efficiency for tetracycline resistance (circle) and ampicillin resistance (square) of the pWH1266 plasmid in hydrolyzed urine ($N = 3$ experiments per time point). This urine had been aged for 12 months prior to the initiation of the experiment. The positive control transformation efficiency of the pWH1266 plasmid in nuclease-free water (not shown) did not change over the duration of the experiment.

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experimental replicates and partly because of the limitations in transformation assay sensitivity. After 24 h of incubation in aged urine, the plasmid transformation efficiencies did decrease by 1.7 $\log_{10}$ (p_{tet} = 0.021; p_{amp} = 0.0003; Figure 1). No statistical difference of transformation efficiencies was observed between the two resistance genes at this time point (p = 0.35; N = 3). The positive controls of plasmid DNA in nuclease-free water indicated no loss in transformation over time. These results suggest that extracellular DNA released in aged urine loses its ability to be acquired by other bacteria after 1 day of storage; therefore, extracellular ARGs released in urine as it ages are unlikely to be transferred to bacteria in the environment when the urine is applied as a fertilizer.

Plasmid Gene Stability in Aged Urine Measured with qPCR. We hypothesized that the decrease in ARG transformation efficiency was due to modifications in the ARG DNA and that the damage was detectable with qPCR. Control experiments confirmed that tetracycline and ampicillin resistance genes were not detectable in the aged urine samples before plasmid pWH1266 was added. After 24 h in aged urine, the concentrations of tetracycline and ampicillin resistance genes and the origin of replication gene on the plasmid, as measured by qPCR assays that covered the entire gene sequences, increased slightly (tet_A: p = 0.0066, +0.18 $\log_{10}$%; $bla_{TEM\text{-}1}$: p = 0.0008, +0.17 $\log_{10}$; *ori*: p = 0.037, 0.12 $\log_{10}$; N = 3; Figure S2). In these same samples, the transformation efficiencies had decreased by 1.7 $\log_{10}$ for both tetracycline and ampicillin after 24 h. We therefore conclude that damage within the genes and detectable with qPCR was not the cause of the observed decreases in transformation efficiencies.

We next assessed if the inactivation of the plasmid transformation efficiency was due to the damage that was distributed across the plasmid and detectable with qPCR. As mentioned above, the three plasmid DNA regions measured by qPCR did not decrease in concentration following 24 h of incubation in aged urine despite the 2 $\log_{10}$ (i.e. 99%) reduction of tetracycline and ampicillin transformation efficiencies. Combined, these three genes cover 33% of the total plasmid DNA sequence. If the modifications that caused the 99% decrease in transformation efficiencies after incubating for 24 h were normally distributed across the plasmid and detectable by qPCR, the corresponding decrease in gene concentration can be estimated based on the size of the gene relative to the entire plasmid size. Specifically, if only 1% of the plasmids are unmodified, we would expect that 46% of the tet_A genes are modified, 36% of the $bla_{TEM\text{-}1}$ genes are modified, and 37% of the *ori* genes are modified (calculations described in the Supporting Information). qPCR sensitivity tests confirmed that these decreases in gene concentrations were detectable with our qPCR assays (Supporting Information). Taken together, these results suggest that either the inactivating modifications in the plasmid DNA are not normally distributed across the entire plasmid sequence and are located outside of the tet_A, $bla_{TEM\text{-}1}$, and *ori* genes (i.e., in the 67% of the plasmid sequence not assayed in our qPCR assays) or that the polymerase enzyme used in qPCR does not detect the DNA modifications that inactivate the plasmid.

Plasmid DNA Conformation in Aged Urine Measured with Gel Electrophoresis. We evaluated conformation changes in plasmid DNA exposed to aged urine by gel electrophoresis over the same timescale studied in the transformation and qPCR experiments. If the observed transformation losses were due to plasmid DNA modifications that partially or fully linearized the supercoiled structure, the conformation changes should be detectable with gel electrophoresis. As shown in Figure 2, the supercoiled plasmid band

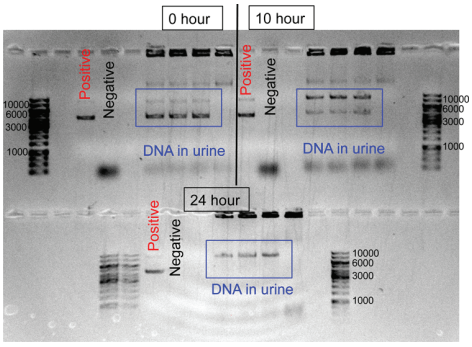

Figure 2. Plasmid DNA as it is incubated in unfiltered, aged urine. Gel electrophoresis shows supercoiled plasmid pWH1266 in positive controls and no plasmid in the negative controls. In aged urine, the plasmid is supercoiled at time = 0 h and begins to linearize after incubation for 10 h. This urine in this experiment had been aged for 12 months prior to the initiation of the experiment.

was visible in the aged urine samples immediately after the plasmid was added (0 h samples). Following 10 h of incubation in the aged urine, the 5 kbp band lightened, and a band representing the plasmid in a linear form appeared (~8.9 kbp). Following 24 h of incubation in aged urine, the supercoiled plasmid band disappeared, and the band at 8.9 kbp was the only band remaining. Based on these results, we conclude that the plasmid DNA linearized over the same timeframe that the plasmid transformation efficiency was reduced by 99%. Indeed, linearization of plasmids significantly reduces transformation efficiencies in some bacteria, including *A. baylyi*.[18] Combined with the qPCR and transformation results, the gel electrophoresis data suggest that plasmid DNA cleavage and linearization inhibited plasmid transformation and that the plasmid DNA cleavage was not detected with our qPCR assays.

Impact of Aged Urine Filtration and Heat Treatment on Plasmid DNA Fate. In our prior work on the fate of polyomavirus in aged urine, virus inactivation was prevented when the aged urine was filtered through a 0.22 μm filter prior to virus addition;[10] we therefore proposed that the virus inactivation in aged urine was due to microbial activity. To test if a similar phenomenon was taking place with extracellular plasmid DNA, we spiked the plasmid into the aged urine after it was filtered through a 0.22 μm membrane filter. In contrast to the unfiltered urine where transformation efficiencies were below detection limits within 24 h, plasmids spiked in the filtered urine experienced 0.90 $\log_{10}$ (tetracycline) and 0.50 $\log_{10}$ (ampicillin) decreases in transformation efficiencies (p_{tet} < 0.0001; p_{amp} = 0.027; N = 3; Figure 3). After 48 h, 1.5 $\log_{10}$ (tetracycline) and 2.0 $\log_{10}$ (ampicillin) decreases in transformation efficiencies were observed (p_{tet} < 0.0001; p_{amp} = 0.0067; N = 3; Figure 3).

To further define the size of the material that was affecting plasmid integrity, we incubated plasmids in aged urine that had been filtered through a 100 kDa ultrafilter. We did not detect statistical changes in transformation efficiencies following incubation for 24 h (*tet*: p = 0.13, +0.13 $\log_{10}$; *amp*: p = 0.088, +0.30 $\log_{10}$; N = 3; Figure 3) or 48 h (*tet*: p = 0.75,

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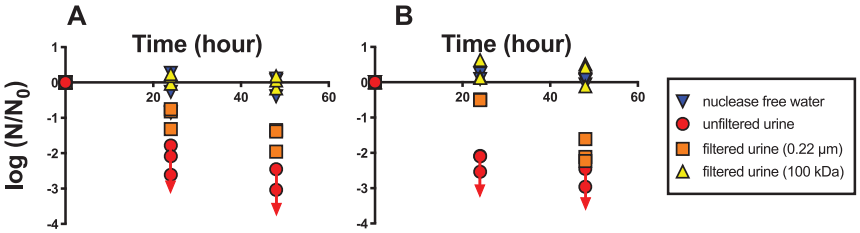

Figure 3. Transformation efficiencies for (A) tetracycline resistance and (B) ampicillin resistance as the plasmids were incubated in nuclease-free water (positive control), unfiltered aged urine, and filtered, aged urine. Transformation efficiencies at levels below the assay detection limits for unfiltered urine are depicted with red arrows. Note that the transformation efficiency detection limits varied between urine replicates (N = 3). This urine had been aged for 14 months prior to the initiation of the experiment.

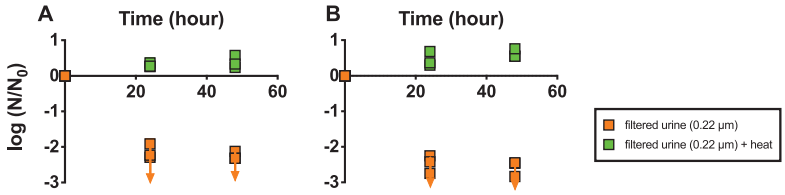

Figure 4. Impact of urine heat treatment on plasmid transformation efficiencies. Transformation efficiencies were measured for (A) tetracycline resistance and (B) ampicillin resistance after the plasmids were added and incubated in filtered urine with and without heat treatment. Experiments were conducted in triplicate (N = 3). Transformation efficiencies below the assay detection limits for filtered urine are depicted by orange arrows. This urine had been aged for 15 months prior to the initiation of the experiment. Positive control transformation efficiency of the pWH1266 plasmid in nuclease-free water (not shown) did not change over the duration of the experiment.

+0.028 $\log_{10}$; *amp*: p = 0.17, +0.30 $\log_{10}$; N = 3; Figure 3). Together, the results from the filtration experiments suggest that a fraction of the material in aged urine that is responsible for the decrease in plasmid DNA transformation efficiencies is larger than 0.22 μm and all of the material responsible for the decrease in plasmid DNA transformation efficiencies is larger than 100 kDa. Unlike DNA, RNA undergoes transesterification by soluble species in hydrolyzed urine, including NH_3 and OH^-.[29] Specifically, the 3′,5′-phosphodiester bonds in RNA are susceptible to base-catalyzed transesterification because of the 2′-hydroxyl group on the ribose rings.

In soil, plasmid DNA degradation and the loss of transformation ability have been attributed to microorganisms.[30] Bacteria, for example, can metabolize DNA for microbial growth[31] and produce enzymes that disrupt DNA.[32,33] Here, removing material greater than 0.22 μm impacted the inactivation of the plasmid in the aged urine. Although most bacteria are larger than 0.22 μm, some are small enough to pass through 0.22 μm pores.[34] Extracellular nucleases are naturally excreted from bacteria and can remain active after the bacteria are removed.[33] Nuclease enzymes can vary in size (~20 up to 400 kDa), structure, and function and exhibit a range of sensitivities to elevated temperatures and pH values.[35−39]

To assess the potential role of small bacteria or extracellular enzymes in the hydrolyzed urine and to also assess the impact of pasteurization on DNA fate in hydrolyzed urine, we heated the urine that had been filtered through membranes with 0.22 μm pores to 75 °C for 10 min and let it cool prior to adding the plasmid DNA. This temperature treatment should inactivate the remaining bacteria and inhibit nucleases in the aged, filtered urine[39] but does not impact the ammonia concentration or pH.[7] In the heat-treated urine, we observed slight increases in transformation efficiencies after the plasmid had incubated in the urine for 48 h (*tet*: p = 0.019, +0.37 $\log_{10}$; *amp*: p = 0.0009, +0.59 $\log_{10}$; N = 3; Figure 4). This is in stark contrast to plasmids incubated in the same aged urine that had only been filtered through membranes with 0.22 μm pores; in these samples, the transformation efficiencies decreased below the assay detection limits after incubating for 24 h. In addition to indicating that bacteria or extracellular enzymes are responsible for the degradation of the plasmid DNA in hydrolyzed urine, these results also suggest that DNA released into heat-treated urine will degrade slower than DNA released into urine that has not been heat-treated. We note that the decrease in transformation efficiencies at specific incubation times differed from one batch of source-separated urine to another (e.g., urine samples filtered through 0.22 μm in Figure 3 vs that in Figure 4). We did not observe these variations in the replicate experiments conducted in the same batch of hydrolyzed urine (i.e., aged for the same amount of time). We attribute this to different bacterial communities or enzyme contents in different urine batches and discuss this in more detail below.

In agreement with the transformation experiments, qPCR analysis demonstrated that the tet_A and $bla_{TEM\text{-}1}$ gene concentrations remained stable in aged urine that was filtered and heated but decreased in concentration in the same filtered sample that was not heated after incubating for 48 h (Figure S3). Likewise, gel electrophoresis showed that the supercoiled plasmid was stable throughout the 48 h of incubation in the heat-treated urine and was unstable in the same sample that was not heated (Figure 5).

Because of the observed impacts of heating and filtering aged urine, we conclude that the plasmid DNA lost its ability to transform bacteria after incubation in aged urine because of microbial or biochemical processes. The processes could be either because of the intact bacterial cells that pass through 0.22 μm pores or because of the extracellular nucleases that are greater than 100 kDa.

Variations in Different Hydrolyzed Urine Samples. We observed differences in the extent of plasmid trans-

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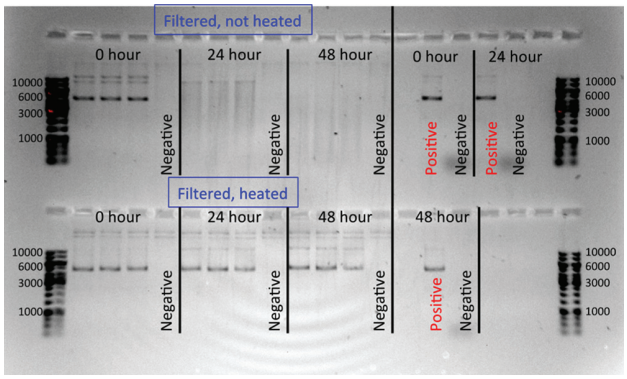

Figure 5. Plasmid DNA as it is incubated in filtered and heat-treated urine. The plasmid retains its supercoiled configuration when incubated in aged urine that was filtered and heated (bottom row), but the supercoiled band disappears when incubated in aged and filtered urine that was not heated (top row). Positive controls (plasmid DNA in nuclease-free water) and negative controls (no plasmid DNA in LB media) are presented to the right of the full black line. Negative controls to the left of the full black line consist of no plasmid DNA added to the respective aged urine samples. This urine had been aged for 15 months prior to the initiation of the experiment.

formation loss in urine samples that had been aged for different periods of time. For example, the transformation efficiency losses after the plasmids were incubated for 24 h in aged and filtered urine (orange squares) were 0.90 $\log_{10}$ (tetracycline) and 0.50 $\log_{10}$ (ampicillin) in experiments conducted with one urine batch (Figure 3) and greater than 2 $\log_{10}$ for both tetracycline and ampicillin in another urine batch (Figure 4). We did not observe these same variations when replicate experiments were conducted on the same urine batch. These results suggest that the observed differences were due to the variability of the batches and not due to variability in the experimental measurements. In our previous study, we observed similar variability in the inactivation kinetics of polyomavirus in different hydrolyzed urine batches.[10] These differences in the inactivation kinetics of both DNA plasmids and polyomaviruses in different hydrolyzed urine samples are likely because of composition differences across the batches of hydrolyzed urine, including differences in the microbial communities and activities.[7] For this reason, we compared untreated and treated samples (e.g., filtered vs unfiltered, heated vs unheated) from the same urine batch and did not pool results from different urine batches. Replicates were always conducted in the same urine batch.

DNA Degradation in Environmental Samples. In our previous work, a 900 bp region of the BK polyomavirus dsDNA genome (5.1 kbp) was monitored with qPCR in hydrolyzed urine, and remained intact for more than 3 weeks despite the rapid loss of virus infectivity.[10] The loss of BK polyomavirus infectivity in hydrolyzed urine was attributed to protein disassembly. The viral dsDNA remained stable even after the capsid disassembled. In this study with extracellular plasmid DNA, we observed minor decreases in the qPCR ARG target concentrations relative to the decreases in transformation efficiencies of our model plasmid when incubated in hydrolyzed urine for 24−48 h. Consequently, the plasmid conformation changes and minor DNA modifications (i.e., site-specific cleavage) appear to drive the loss of plasmid transformation efficiency in the hydrolyzed urine.

For context, in activated sludge, plasmid DNA concentrations decreased below the PCR and electrophoresis assay detection limits within 6 h of incubation, based on a PCR assay targeting 1042 bps.[40] The authors attributed the rapid loss of PCR signal to both endo- and exonuclease activity in wastewater.[40] In groundwater, extracellular DNA binds to the aquifer material and thus remains stable and retains its ability to transform bacteria.[41] Likewise, the plasmid DNA added to soil microcosms remains stable for long periods of time because of adsorption to soil particles.[30,41−44] As observed here for different batches of source-separated urine, DNA plasmid degradation rates in soil differ from sample to sample.[45]

In summary, we demonstrated that the extracellular DNA released in hydrolyzed urine rapidly loses its ability to transform bacteria and that the degradation of the DNA is driven by microorganisms or extracellular enzymes. In practice, urine-diverting systems collect urine over time and the urine is then aged for weeks or months. Under these conditions, extracellular DNA in the hydrolyzed urine is unlikely to pose a threat of spreading plasmid-associated antibiotic resistance. Our results demonstrate that the methods used to evaluate DNA persistence can result in seemingly contradictory results. In truth, molecular methods and functional assays together provide a more complete story of DNA fate than when the methods are used independently.

In light of these results, it is important to note the limitations of this study and recommend future research directions. We employed the model *A. baylyi* transformation system to track the loss of natural transformation efficiency of plasmid DNA. It is possible that other extracellular DNA molecules are impacted differently by microbial or biochemical activity in hydrolyzed urine and that factors such as plasmid size and sequence impact the DNA susceptibility. Likewise, other bacterial species may have different tolerances for modifications in the plasmid DNA. Future work should therefore expand to additional transformation models, as well as incorporate a range of fresh and treated urine samples.

This study focused on transformation to better understand the risks of extracellular ARG transfer to environmental microorganisms. Transduction via bacteriophages and conjugation via viable antibiotic resistant bacteria may also be important horizontal gene transfer mechanisms for ARGs in hydrolyzed urine, and these mechanisms should also be studied. We should note that because of the dynamic range of transformation assays, our plasmid concentrations in urine were much higher than the background extracellular DNA concentrations in source-separated urine. Consequently, we assumed that DNA degradation at these high concentrations is representative of the degradation that occurs at the background concentrations. Finally, the specific microorganisms or enzymes that are responsible for DNA degradation in the hydrolyzed urine have not yet been identified, and this should be further explored.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b04263.

Sensitivity of qPCR analyses; qPCR efficiencies and R^2 values; gene copy concentrations in hydrolyzed, filtered (0.22 μm), and filtered and heated urine; and plasmid map and sequence (PDF)

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Notes

The authors declare no competing financial interest.

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