# Tetracycline Resistance Gene Maintenance under Varying Bacterial Growth Rate, Substrate and Oxygen Availability, and Tetracycline Concentration

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**S** *Supporting Information*

**ABSTRACT:** Neither amplification nor attenuation of antibiotic resistance genes (ARG) in the environment are well understood processes. Here, we report on continuous culture and batch experiments to determine how tetracycline (TC), aerobic vs anaerobic conditions, bacterial growth rate, and medium richness affect the maintenance of plasmid-borne TC resistance ($Tet^R$) genes. The response of *E. coli* (a model resistant strain excreted by farm animals) versus *Pseudomonas aeruginosa* (a model bacterium that could serve as a reservoir for ARGs in the environment) were compared to gain insight into response variability. Complete loss of the $Tet^R$ RP1 plasmid (56 kb) occurred for *P. aeruginosa* in the absence of TC, and faster loss was observed in continuous culture at higher growth rates. In contrast, *E. coli* retained its smaller pSC101 plasmid (9.3 kb) after 500 generations without TC (albeit at lower levels, with ratios of resistance to 16S rDNA genes decreasing by about 2-fold). A higher rate of ARG loss was observed in *P. aeruginosa* when grown in minimal growth medium (M9) than in richer Luria broth. Faster ARG loss occurred in *E. coli* under anaerobic (fermentative) conditions than under aerobic conditions. Thus, in these two model strains it was observed that conditions that ease the metabolic burden of plasmid reproduction (e.g., higher substrate and $O_2$ availability) enhanced resistance plasmid maintenance; such conditions (in the presence of residual antibiotics) may be conducive to the establishment and preservation of ARG reservoirs in the environment. These results underscore the need to consider antibiotic concentrations, redox conditions, and substrate availability in efforts to evaluate ARG propagation and natural attenuation.
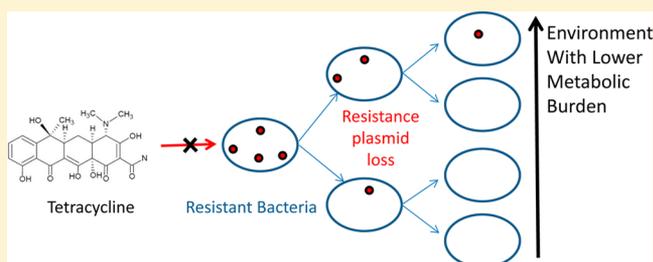
## INTRODUCTION

Recent studies have shown that antibiotic resistant bacteria and associated resistance genes are widespread in a multitude of natural environments.[1−6] The propagation of antibiotic resistance has in part been linked to the increasing use of antimicrobial agents in animal agriculture, which favors resistant bacteria that can migrate and proliferate in aquatic and soil systems.[7−10] Feces from confined animal feeding operations (CAFOs), which are often used as soil amendments (i.e., manure), are also potential sources of environmental contamination by antibiotics and the associated ARGs. The propagation of ARGs is currently a major concern to public health due to the increased occurrence of clinical infections with resistant strains[11,12] as well as foods contaminated with antibiotic resistant bacteria.[13] However, little is known about how environmental factors influence the proliferation (or loss) of antibiotic resistance in microbial populations, which hinders our ability to develop strategies or technologies to attenuate the growth of ARG reservoirs in the environment.

Exposure to high antibiotic concentrations (e.g., up to 100 mg/L) has been reported to select for antibiotic resistant plasmid-bearing cells,[14] increase ARG copy numbers in resistant strains,[8] and influence which ARGs are harbored within bacterial hosts.[15] Conversely, the absence of the antibiotic (as well other chemical stressors for which ARGs may offer resistance, such as heavy metals) usually results in resistance plasmid curing (i.e., complete loss of the plasmid), possibly due to the metabolic burden of carrying an unneeded plasmid.[14,16] Nevertheless, curing is not always observed, and plasmid persistence has been shown to be enhanced by efficient replication and by partitioning and/or plasmid addiction systems.[17−19]

Research on the effects of the growth medium and bacterial growth rate on plasmid stability has yielded different results that probably reflect differences in host/plasmid specific interactions.[20] Some studies observed higher plasmid stability in rich media,[21,22] while others reported higher stability with minimal medium.[23] Although bacterial growth rates are influenced by nutrient availability and are thus dependent on the growth medium composition, such confounding effects can be avoided by using continuous culture (chemostats) to allow independent control of the growth rate. However, continuous culture studies have not reached consistent results on the effects of growth rate on resistance plasmid stability, with increased plasmid loss reported for both high[24,25] and low growth rates.[26,27]

Some of these discrepancies have been attributed to differences in experimental variables, including criteria used to determine plasmid maintenance (e.g., growth on antibiotic enriched agar versus $\beta$-lactamase activity), growth medium composition, and host bacteria and plasmid characteristics (e.g., plasmid size, copy numbers and presence of sequences such as the overlapping *stbD* and *stbE* genes which may contribute to plasmid segregation stability,[28] and the 28bp psi site that may act as a target site for recombinase enzymes,[29] both mechanisms that may enhance plasmid maintenance). To our knowledge, there has been no comparative study to quantify the effects of growth medium composition, redox conditions, microbial growth rate, and concentration of the antibiotic on the dynamics of antibiotic resistance plasmids for environmentally significant bacteria.

This study seeks to discern commonalities about how different ARG plasmid/host systems respond to environmental variables that influence cell fitness and the associated metabolic burden of plasmid reproduction and maintenance. We compare the stability of plasmid-borne tetracycline resistance (Tet[R]) genes in an enteric bacterium (*Escherichia coli*), which could be excreted by farm animals,[30] versus an indigenous soil microorganism (*Pseudomonas aeruginosa*), which could serve as a reservoir for ARG maintenance and propagation in the environment, as well as a potential human pathogen. Specifically, the concentration and relative abundance of two different Tet[R] genes (*tet*(A) in the RP1 plasmid in *P. putida*, and *tet*(C) in the pSC101 plasmid in *E. coli*) were monitored to assess resistance maintenance and loss dynamics as a function of bacterial growth rate, medium richness, presence of tetracycline, and aerobic versus anaerobic conditions.

## ■ MATERIALS AND METHODS

**General Approach.** Batch growth experiments were conducted to investigate resistance gene maintenance over many bacterial generations (approximately 500 generations) in the presence/absence of tetracycline (TC) or dissolved oxygen, as well as the effect of the growth medium richness. Cells were grown to stationary phase before dilution into fresh medium to simulate nutrient limiting conditions induced by the stationary phase[31] that may be representative of conditions downstream of the nutrient rich surroundings of agricultural facilities impacted by antibiotic contamination.

Continuous growth (chemostat) experiments were also conducted to study the stability of Tet[R] genes in the two resistant strains as a function of the antibiotic dosing treatment and microbial growth rate. The use of continuous culture allowed maintenance of the microbial cultures at steady state, excepting the confounding effects of metabolic changes caused by transitioning from exponential to stationary to decay growth phases, while facilitating precise control of the specific microbial growth rate ($\mu$) and antibiotic concentration through adjustments to the continuous culture dilution rate ($D$).[32]

Real time quantitative PCR (qPCR) was used for antibiotic resistance gene monitoring. This allowed for sensitive detection of the targeted genes and the quantification of their relative abundance, while eliminating the bias associated with cell culturing techniques.

**Serial Batch Experiments.** Two pure Tet[R] strain cultures were investigated: an enteric *E. coli* c600 carrying the pSC101 plasmid (9.3 kb) that contains the *tet*(C) resistance gene (ATCC#37032), and the indigenous soil strain *Pseudomonas aeruginosa* PU21 (NCTC#50183) carrying the RP1 plasmid (56 kb) that contains the *tet*(A) determinant (NCTC050076). Both plasmids have low copy numbers (between 4 and 10 copies) and both harbor a partitioning (*par*) locus that stabilizes plasmid inheritance in dividing cell populations.[33−37]

The initial cultures, grown in LB medium and supplemented with 50 mg L$^{-1}$ TC (T3383, Sigma Co) to enhance plasmid maintenance within the host bacteria,[7] were grown overnight and then used to seed the batch reactors. Subsequent cultures were continually grown in LB medium, both in the presence of TC (TC+, 50 mg·L$^{-1}$) and absence of TC (TC−, 0 mg·L$^{-1}$) as a control for the effect of antibiotic treatment on resistance plasmid maintenance. Overnight cultures were harvested, and samples were analyzed for OD$_{600}$ (Ultrospec 2100 pro spectrophotometer, Amersham Biosciences) to calculate the number of bacterial generations ($n$) between each sampling period, assuming reproduction by binary fission as follows

$$OD_{final} = OD_{initial} \times 2^n, \text{ thus}$$

$$n = \frac{\log\left(\frac{OD_{final}}{OD_{initial}}\right)}{\log 2}$$

Aliquots of the harvested cultures were used to seed subsequent batch reactors (TC+ and TC−) for the duration of the experiment (approximately 500 generations for *E. coli*, and 120 generations for *P. aeruginosa*).

*P. aeruginosa* grows well both in minimal medium M9 and in rich LB medium, and was selected for a comparative study of the effect of growth medium richness on resistance plasmid loss. Thus, additional *P. aeruginosa* cultures were grown in M9 without TC for about 80 bacterial generations.

*E. coli* is a facultative bacterium and was selected for an additional comparative study of the effect of redox conditions. These batch experiments were conducted to determine if an anaerobic environment, as compared to an aerobic environment, would diminish the energy harvesting capabilities within the cell and thus accelerate the loss of the resistance plasmid. *E. coli* c600 was grown in 50-mL Falcon tubes both in the presence of TC (TC+, 15 mg·L$^{-1}$) and absence of TC (TC−, 0 mg·L$^{-1}$) on LB medium under aerobic and anaerobic (fermentative) conditions. Resazurin (Sigma-Aldrich, USA) at 0.001% was used as an oxygen indicator in both the aerobic and anaerobic cultures to ensure there was no oxygen present in the anaerobic cultures. Anaerobic cultures were prepared in an anaerobic chamber (COY Laboratory Products Inc.) using equilibrated LB medium in a mixed gas atmosphere (10% CO$_2$, 10% H$_2$, 80% N$_2$). To preclude oxygen diffusion into the growth medium the tubes were capped tightly prior to transfer to a temperature controlled incubator (New Brunswick G25).

Aliquots from overnight cultures grown at 37 °C were used to seed subsequent reactors under aerobic and anaerobic conditions for each growth condition respectively. $OD_{600}$ of the overnight cultures was measured to determine the number of bacterial generations between each sampling period. Cultures were then harvested for DNA extraction and NADH/NAD⁺ analysis.

**NADH/NAD⁺ Measurement.** NADH/NAD⁺ analysis was performed using EnzyChrom NADH/NAD⁺ Assay Kit (ECND-100) following manufacturer's protocol except for the addition of a 10 s sonication step (Sonic Ruptor 250 Omni International) with samples on ice at 50% power, to ensure cell lysis.[38]

**Continuous Culture (Chemostat) Experiments.** Three 250-mL Pyrex bottles equipped with 3-hole Teflon caps were used as the chemostat reactors and operated on magnetic stir plates (Fisher Scientific). The growth medium (LB) was delivered from a 10-L pyrex reservoir via a Masterflex L/S peristaltic pump (Cole Parmer Co.) in perfluoroalkoxy (PFA) tubing (Cole Parmer Co.) at 40, 70, and 100 mL·h⁻¹ to obtain the desired specific microbial growth rates ($\mu$ = 0.20, 0.35, and 0.50 h⁻¹, respectively). A portion of the tubing was submerged in a heated water bath (85 °C) to prevent microbial "back growth" and contamination of the feed reservoir.[39] Two UV lamps (Spectroline, Spectronics Co.) were also used to maintain reservoir sterility. During the experiments involving the effect of antibiotic concentration on plasmid maintenance, TC was delivered separately to the reactors via a Harvard Apparatus 22 syringe pump (Harvard Bioscience, Inc.) equipped with 100-mL gastight syringes (SGE International, Pty. Ltd.) filled with TC at levels corresponding to final reactor TC concentrations of 1 and 50 mg·L⁻¹. These concentrations are higher than typical TC levels in the environment (~1 μg/L),[40] but are representative of high concentrations in some animal farms.[41,42] The third reactor was not amended with antibiotic (TC = 0 mg·L⁻¹) to determine how the presence and concentration of TC affected plasmid stability. The dilution rate (and thus $\mu$) was set at 0.2 h⁻¹ for each reactor.

Before each run, the entire chemostat apparatus was sterilized by 3 cycles of heat sterilization (autoclaving) and subsequent 24-h purging with 20% nitric acid solution delivered from the feed reservoir. At the initiation of each experiment the reactors were seeded with an overnight-grown Tet^R culture (*E. coli* or *P. aeruginosa*) to an approximate concentration of 10⁷ cells·mL⁻¹. During the experiment, reactors were sampled via an outlet port using sterile 2-mL syringes. Samples were then stored at −80 °C until DNA extraction and qPCR analysis.

**qPCR Analysis.** Prior to DNA extraction, the $OD_{600}$ of all microbial samples was adjusted by dilution with DI water to approximately 0.2 units. DNA was extracted with an UltraClean Microbial DNA Kit (MoBio Laboratories) according to manufacturer's protocol. The concentrations of the total purified DNA in each sample were determined spectrophotometrically (Ultrospec 2100 pro spectrophotometer, Amersham Biosciences) prior to qPCR analysis which allows for detection levels of $1.4 \times 10^{-15}$ g of DNA per reaction.[43] The Tet^R strains were screened for 16S rRNA, *tet*(C) (for *E. coli*), and *tet*(A) (for *P. aeruginosa*) gene concentrations by qPCR with the 7500 Real Time PCR System (Applied Biosystems). PCR was conducted in 25-μL reaction mixtures on 96-well plates containing 1X SYBR Green Master Mix (Applied Biosystems), 2-μL aliquots of the DNA samples, forward and reverse primers at 500 nM final reaction concentration. The cycling conditions

were optimized for the three primer sets following Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) standards[44] and ran for: 1 min at 50 °C, 10 min at 95 °C, 40 cycles of denaturation for 15 s at 95 °C, annealing for 1 min at 60 °C, followed by the dissociation stage cycle (15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C).[41] Three sets of forward/reverse (For/Rev) primers were used. All of the following sequences are given in the direction of transcription, from 5′ to 3′. The first set targeted the 16S rRNA gene (For - GTGSTGCAYGGYTGTCGTCA, Rev - ACGTCRTCCM-CACCTTCCTC),[45] the second targeted the *tet*(C) gene (For - GCCTATATCGCCGACATCAC, Rev - GTAGGTT-GAGGCCGTTGAGC),[46] and the third targeted the *tet*(A) gene (For - CCTGCGCGATCTGGTTCACT, Rev - GCCAGCGAGACGAGCAAGA).[46]

Standard curves were constructed using Ct values and known DNA concentrations from PCR products of the targeted 16S, *tet*(C), and *tet*(A) genes. The standard curves were then used to estimate the concentrations of the target genes prior to normalizing the concentrations of Tet^R relative to the chromosome concentration. Slopes from the standard curves were used to calculate primer efficiencies for *tet*(C) (108%), 16S *E. coli* (77%), *tet*(A) (94%), and 16S *P. aeruginosa* (92%).

The specificity of the *tet*-gene primers was confirmed by the absence of detectable products from PCR and qPCR amplifications performed with the *tet*(A) and *tet*(C) primer sets with template DNA obtained from a nonresistant strain (*Rhodococcus jostii* RHA1). Tet^R gene concentrations were normalized to the total bacterial concentrations, determined by phylogenetic gene biomarkers, and were reported as the ratios of Tet^R to 16S rRNA genes. All samples were analyzed in triplicate to assess variability and permit evaluation of the statistical significance (if any) of differences between experimental treatments, using Student's *t* test.

## ■ RESULTS AND DISCUSSION

**Effect of Antibiotic Treatment.** The antibiotic resistance plasmids under consideration have a similar copy number within their bacterial hosts, and both harbor a stabilizing par locus, which facilitates comparison of their maintenance dynamics. In the presence of TC (50 mg/L), the resistance plasmids were maintained by both *E. coli* (Figure 1) and *P. aeruginosa* (Figure 2) as shown by the relatively high [Tet^R]/[16S] gene concentration ratios, despite moderate fluctuation likely caused primarily by experimental variation. Some fluctuations could have also been due to physiological changes (including cell division) influenced by circadian rhythm[47] or other environmental factors. In the absence of selective pressure exerted by TC (TC = 0 mg·L⁻¹), *P. aeruginosa* lost its resistance plasmid after approximately 120 generations, as shown by a decrease in the [*tet*(A)]/[16S] gene ratio from 0.42 ± 0.05 to zero (Figure 2). Complete plasmid loss was not observed for *E. coli*, although it did experience a decrease in the [*tet*(C)]/[16S] gene ratio from about 1.68 ± 0.23 to 0.63 ± 0.12 after approximately 500 generations (Figure 1). Plasmid loss without complete plasmid curing has been previously reported for *E. coli* harboring the catabolic plasmid pJEL144.[48]

To investigate the possibility of chromosomal integration of the *tet*(C) gene as the reason for resistance maintenance in the absence of TC, *E. coli* DNA (at 250 generations) was extracted separately using both a total microbial DNA and a plasmid extraction procedure. Subsequent qPCR amplification confirmed that *tet*(C) copy numbers after extraction with a total
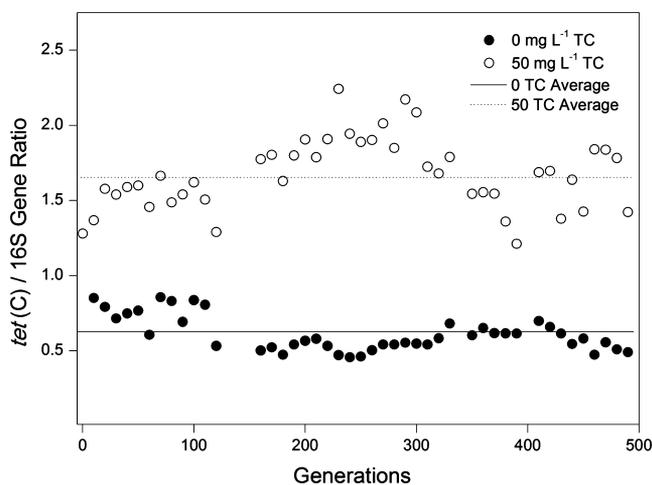
**Figure 1.** Effect of tetracycline concentration on $tet$(C)-carrying plasmid maintenance in *E. coli* (batch growth on LB medium). The absence of tetracycline resulted in a significant ($p < 0.05$) decrease in the average $[tet(C)]/[16S]$ gene ratios from $1.68 \pm 0.23$ (top, TC = 50 mg·L$^{-1}$) to $0.63 \pm 0.12$ (bottom, without TC).
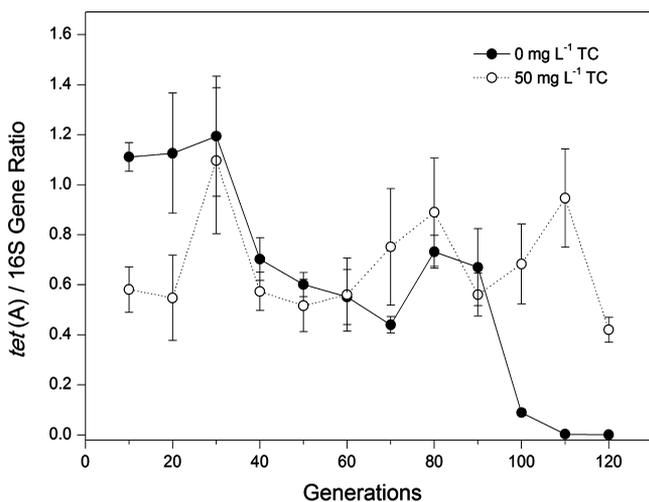


**Figure 2.** Effect of tetracycline on $tet$(A)-carrying plasmid maintenance in *P. aeuriginosa* (batch growth on LB medium). The presence of TC (50 mg·L$^{-1}$) enhanced the retention of $tet$(A). Error bars represent standard error ($n = 3$). In the absence of TC, the $tet$(A) gene was not detected after 110 generations, indicating complete plasmid curing.

DNA kit (which would extract plasmid plus chromosomal DNA) was statistically indiscernible from those obtained by extraction from the plasmid kit, suggesting no significant integration into the chromosome and indicating the continued presence of resistance genes in plasmids.

The effect of an intermediate antibiotic concentration (TC = 1 mg·L$^{-1}$), which is a relatively high concentration for the environment but representative of waste lagoons surrounding agricultural operations,[49] was also tested for *E. coli* grown on LB medium in a steady-state continuous culture ($D = 0.2$ h$^{-1}$). This resulted in an intermediate $[tet(C)]/[16S]$ ratio of $1.05 \pm 0.25$, (compared to $1.44 \pm 0.27$ for 50 mg·L$^{-1}$ TC and $0.57 \pm 0.10$ for 0 mg·L$^{-1}$ TC (Figure S1, Supporting Information). These differences are statistically significant at the 95% confidence level ($p < 0.05$). Incomplete curing in continuous culture experiments without TC corroborates the batch results,

which yielded a similar residual $[tet(C)]/[16S]$ ratio of $0.63 \pm 0.12$ (versus $0.57 \pm 0.10$).

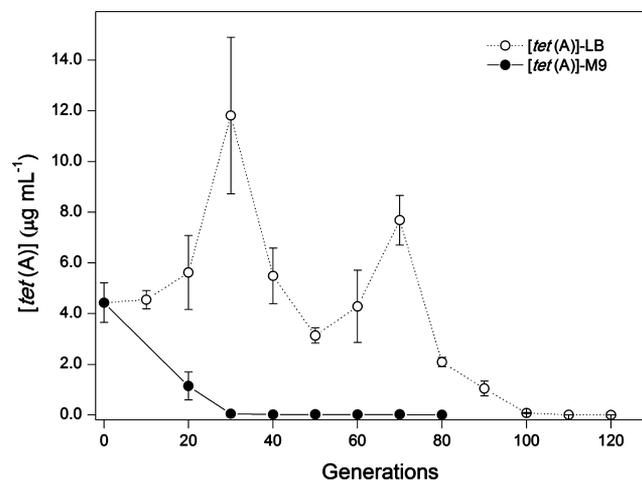**Effect of Microbial Growth Rate and Medium Richness.** Figure 3 shows $tet$(A) concentrations in *P. aeruginosa*



**Figure 3.** Gene concentration profile for *P. aeruginosa* grown in batch mode on LB versus M9 media (TC = 0 mg·L$^{-1}$). Growth on minimal M9 medium resulted in significantly ($p < 0.05$ for generations 10−80) quicker plasmid loss. Error bars represent standard deviation ($n = 3$).

during batch growth on nutrient-rich LB versus minimal M9 media. Complete plasmid loss was observed for both conditions. However, plasmid loss occurred in fewer microbial reproductions for the minimal medium ($n = 30$ for M9 versus 110 for LB). Since the maintenance of resistance plasmids can represent a metabolic burden,[50] the availability (or scarcity) of energy sources (i.e., substrates) should influence plasmid generational stability. Accordingly, the minimum M9 medium is conducive to faster plasmid loss. The relatively large size of the RP1 plasmid (56 kb),[51] which is likely to require more energy for reproduction and maintenance, may have contributed to the metabolic burden and resulting complete plasmid curing.[52] This interpretation is supported by previous observations that (i) plasmids may be stabilized during microbial growth in nutrient rich media,[22,53,54] and (ii) cells grown in minimal medium exhibit lower DNA content.[55] Thus, the gross energetic content of the growth medium affects the bacteria's ability to meet the metabolic burden of plasmid maintenance and reproduction.

The specific growth rate ($\mu$) of *E. coli* (which was controlled by the continuous culture dilution rate) in the absence of TC did not significantly affect the stability of the resistance plasmid (Figure 4). The relatively small size of the pSC101 plasmid (9263 bp) is conducive to a relatively low energy cost of replication, which contributes to stability, notwithstanding the potential confounding effect that smaller plasmids often have a higher copy number,[56] which would increase the energy demand for replication. Apparently, the rich growth medium and specific stabilizing genetic sequences (i.e., pSC101 harbors a 28-bp sequence ($psi$-site), which has been implicated in increasing its stability in microbial hosts[29]), contributed to the incomplete plasmid loss from *E. coli*. Incomplete plasmid loss was also previously reported for long-term growth studies,[57,58] and was attributed to genetic changes such as beneficial and compensatory mutations in both the plasmid and the host bacteria (including plasmid copy number reduction[59]) that
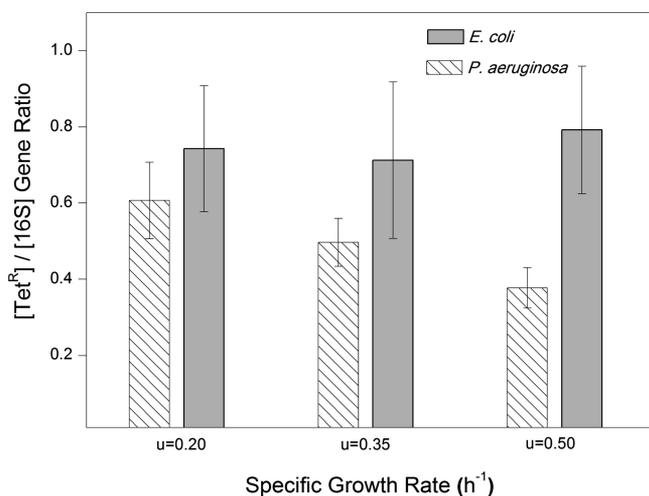
**Figure 4.** Relative ARG abundance for *P. aeruginosa* ([*tet*(A)]/[16S]) and *E. coli* ([*tet*(C)]/[16S]) continuous cultures under varying growth rates in the absence of TC. Increased plasmid loss occurred at faster growth rates, with significant differences at the 95% confidence level observed for *P. aeruginosa*. Growth rates did not exert a statistically significant effect on ARG maintenance (plasmid loss) in *E. coli*. Error bars represent standard deviation ($n = 3$).



**Figure 5.** TetR maintenance in *E. coli* under aerobic versus anaerobic conditions. In the absence of TC, resistance loss occurred earlier ($p < 0.05$) under anaerobic (fermentative) conditions. Batch cultures were grown overnight in LB medium at 37 °C. The initial plasmid loss in the anaerobic batches occurred within 29 generations, when the [*tet*(C)]/[16S] gene ratio decreased from 1.0 ($\pm0.36$) to 0.36 ($\pm0.098$), whereas the aerobic batches maintained the resistance plasmid with a [*tet*(C)]/[16S] gene ratio fluctuating between 0.74 ($\pm0.052$) and 1.34 ($\pm0.34$) until approximately 302 generations had been reached, when the ratio decreased to 0.40 ($\pm0.054$). Error bars represent standard deviation ($n = 3$).

decrease the energetic cost for plasmid carriage and increases host fitness. In contrast, *P. aeruginosa* exhibited a significant ($p < 0.05$) decrease in the [TetR]/[16S] gene ratio with increasing microbial growth rates (Figure 4). Plasmid loss for this strain increased with growth rate, possibly in part due to increased segregational instability in which plasmids are randomly distributed and lost during cell divisions.[60,61] Additional factors potentially contributing to plasmid loss include the relatively large plasmid size (56 kB) and lack of specialized gene sequences capable of imparting stabilizing characteristics onto the resistance plasmid.

**Effect of Anaerobic Environment.** In the absence of TC, *E. coli* cells under less energetically favorable anaerobic (fermentative) conditions lost the resistance plasmid in fewer generations (Figure 5), possibly to alleviate the energetic burden associated with their maintenance. This suggests that intercepting drainage from CAFOs using anaerobic barriers (e.g., anaerobic lagoons, or underground mulch barriers) might help attenuate ARG propagation from the source.

**Correlation between NADH/NAD+ and tet(C)/16S Ratios.** NADH can be oxidized to produce energy within the cell,[62] and the NADH/NAD+ ratio can be used as an indicator of a cell's energetic capacity (e.g., reducing power).[63] Figure 6 shows a significant correlation ($p < 0.005$; $R^2 = 0.83$) between the NADH/NAD+ ratio and the ability of *E. coli* to maintain the resistance plasmid ([*tet*(C)]/[16S]). A decrease in the NADH/NAD+ ratio occurs when metabolism shifts from a high-internal-energy anabolic mode that favors plasmid maintenance[64] to a catabolic mode where cells are primed to degrade available substrates for energy production.[63] Accordingly, the propensity to lose the resistance plasmid might be associated with the energetic status of the cells, which suggests that environmental conditions conducive to low energy status (e.g., limited substrates and/or oxygen availability, etc.) make it difficult for the cells to meet the metabolic burden of plasmid maintenance and favor loss of antibiotic resistance.

Overall, this study shows that environmental conditions such as substrate and oxygen availability can significantly influence
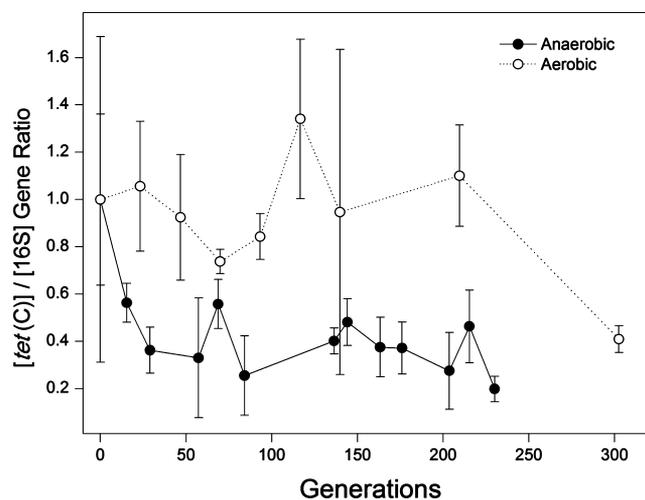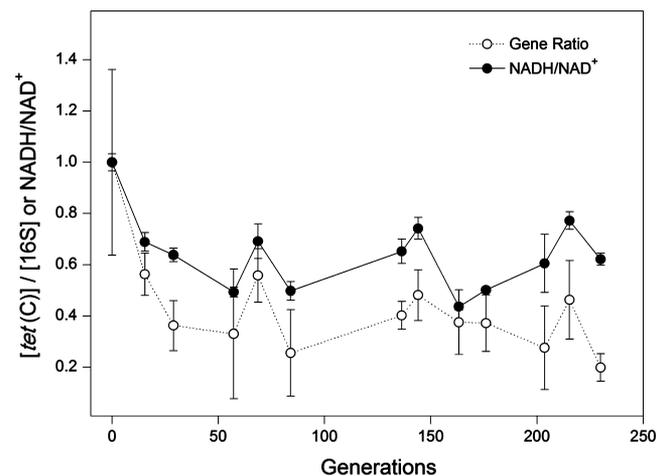


**Figure 6.** Correlation between the ratio of NADH/NAD+ and the gene ratio [*tet*(C)]/[16S] in *E. coli* grown anaerobically in batch mode without TC. The significant correlation ($R^2 = 0.83$ and $p = 0.0006$) indicates a strong connection between the cells' internal energy status (i.e., reduction potential) and maintenance of the resistance plasmid. Error bars represent standard deviation ($n = 3$).

ARG maintenance. This implies that the region of influence of antibiotic resistance vectors released from animal agriculture source zones may be influenced not only by factors known to affect bacterial and DNA transport or residual antibiotic concentrations that exert selective pressure, but also by environmental factors that influence bacterial growth and energy status, and host/plasmid specific interactions[20] that affect ARG maintenance (e.g., resistant plasmid size, copy numbers, and presence of specific stabilizing genetic sequences). Although cell regulation of antibiotic resistance genes in mobile genetic elements can alleviate their burden on

host fitness,[65] manipulation of variables that increase the metabolic burden of resistance plasmid reproduction (e.g., anaerobic barriers) should be considered to mitigate the propagation of antibiotic resistance in the environment.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Additional ARG stability data under different growth conditions. This information is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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