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Examining the taxonomic distribution of tetracycline resistance in a wastewater plant

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Abstract

Microbial communities serve as reservoirs of antibiotic resistance genes (ARGs) and facilitate the dissemination of these genes to bacteria that infect humans. Relatively little is known about the taxonomic distribution of bacteria harboring ARGs in these reservoirs and the avenues of transmission due to the technical hurdles associated with characterizing the contents of complex microbial populations and the assignment of genes to particular genomes. Focusing on the array of tetracycline resistance (Tc^r) genes in the primary and secondary phases of wastewater treatment, 17 of the 22 assayed Tc^r genes were detected in at least one sample. We then applied emulsion, paired isolation, and concatenation PCR (epicPCR) to link tetracycline resistance genes to specific bacterial hosts. Whereas Tc^r genes tend to vary in their distributions among bacterial taxa according to their modes of action, there were numerous instances in which a particular Tc^r gene was associated with a host that was distantly related to all other bacteria bearing the same gene, including several hosts not previously identified. Tc^r genes are far less host-restricted than previously assumed, indicating that complex microbial communities serve as settings where ARGs are spread among divergent bacterial phyla.

Sustainability statement: Analysis of the antibiotic resistance determinants present in wastewater, and the efficacy with which wastewater treatment plants eradicate of pathogenic bacteria, directly satisfy the United Nations Sustainability Goal of fostering the availability of and access to clean water and sanitation.

Keywords: wastewater treatment plant (WWTP), antibiotic resistance genes (ARGs), microbiomes, bacterial contaminants, gene transfer and acquisition, epicPCR, host-range, tetracycline (Tc^r) resistance genes

Introduction

The broad application of antibiotics for the prevention and treatment of bacterial infections and in agriculture has accelerated the spread of genes that confer resistance (Levy and Marshall 2004, Dijkshoorn et al. 2007, Nikaido 2009). In response to this threat to public health, calls for the worldwide surveillance of antibiotic resistance have been made by a number of public health organizations (WHO 2014, Berendonk et al. 2015, WWAP 2017) yielding comprehensive information about prevalence of antibiotic resistance in several settings (Danko et al. 2021). Whereas continued efforts are being devoted to the detection of specific antibiotic-resistant pathogens, attention has shifted toward the surveillance of microbial reservoirs of antibiotic resistance genes (ARGs) that can potentially be mobilized into bacteria that are pathogenic to humans (Berglund et al. 2023).

Knowledge of the incidence of antibiotic resistance determinants in complex microbial communities provides direct evidence of the prevalence and taxonomic distribution of ARGs and can lead to a better understanding about their dissemination. However, obtaining this information is problematic. Traditional culture-based approaches are labor-intensive and incapable of assaying most taxa, since the majority of bacteria in the natural world cannot be isolated in axenic cultures (Stewart 2012). More recently, molecular approaches, such as metagenomic sequencing, have circumvented the need for cultivation (Lun et al. 2009, Kristiansson et al. 2011, Hu et al. 2013), but the assignment of ARGs to specific members of a community requires the assembly of long contigs or complete genomes, which can be difficult to obtain from environmental samples.

To overcome these limitations, we employed emulsion, paired isolation, and concatenation PCR (epicPCR)(Spencer et al. 2016) that uses overlap-extension PCR to join sequences that are present in an individual cell. We apply this method to link the DNA sequences of a specific ARG with a taxonomic marker gene, thereby allowing us to determine which bacterial hosts in a diverse microbial community possess a particular resistance gene. Moreover, this approach is particularly useful for sequences, such as ARGs, that are often encoded on extrachromosomal episomes (Bennett 2008), making them difficult to assign to a host genome. Previous studies have applied epicPCR to assay the dissemination of ARGs at wastewater treatment plants (Hultman et al. 2018, Wei et al. 2021) and shown that the bacterial hosts harboring these genes can differ between the influent and effluent.

We aimed to understand the dynamics of ARGs and their distribution in hosts in wastewater. To achieve this, we focused on the taxonomic distribution of a large panel of genes, each of which confers resistance to tetracycline. Tetracyclines are broad-spectrum antibiotics used to treat Gram-positive and Gram-negative infections (Chopra and Roberts 2001) and to promote growth in livestock (Dibner and Richards 2005). Their extensive application and overuse have caused selection

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for a very diverse array genes that confer resistance, with new classes still being discovered (Thaker et al. 2010, Forsberg et al. 2015). Our survey of the diverse bacterial communities in wastewater showed that most Tc^r genes are less host-restricted than previously considered (Roberts 2005a, b; 2019). Moreover, the occurrence of identical Tc^r genes in phylogenetically divergent taxa signals repeated transfer of ARGs among distant bacterial phyla.

Materials and methods

Wastewater collection and DNA extraction

One-liter samples of wastewater were collected at sites within the City of Austin Walnut Creek Wastewater Treatment Plant (www.austintexas.gov/walnutcreekwwtp): samples were collected from the primary treatment phase (primary sludge and primary effluent) and from the secondary treatment phase (activated sludge and secondary clarifier). A 40-ml aliquot of each sample was first centrifuged at 5000 *g* for 15 min to pellet bacterial cells, washed with TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl), and total DNA was extracted from the cellular pellet by the cetrimonium bromide (CTAB) plus bead-beating method described in Powell et al. (2014), except that incubation with CTAB and Proteinase K proceeded for 2 h and RNaseA treatment was omitted.

Assaying tetracycline resistance genes

DNA purified from the wastewater effluent was assayed by PCR for the presence of 22 tetracycline resistance genes that have been detected in wastewater (Pazda et al. 2019): *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), *tet*(Y), *tet*(Z), *tet*(30), *tet*(32), *tet*(34), and *tetB*(P). PCR reactions contained 10 ng template DNA, 2.5 µl DreamTaq buffer (ThermoFisher Scientific), 0.2 µM dNTPs, 0.5 µM forward and reverse primers, and 1 U DreamTaq (ThermoFisher Scientific) in a total reaction volume of 25 µl and proceeded as follows: 94°C for 3 min for initial denaturation followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec and 68°C for 30 sec, with a final extension of 68°C for 5 min. Reactions were visualized on 2% agarose gels for the presence of amplified fragments corresponding to the 22 assayed Tc^r genes. [Primer notations and sequences of gene-specific diagnostic PCR primers (termed "dPCR") and epicPCR primers are listed in Supplementary Material Table S1.]

EpicPCR

To link an identifying segment of each ARG to a phylogenetically informative region of the 16S rDNA gene at the level of individual cells, we developed a modified epicPCR protocol based on the procedure described in (Spencer et al. 2016). Samples were filtered three times through an 11 µm nylon filter (Millipore) to eliminate large particles and debris. Bacterial cells were pelleted by centrifugation at 3000 g for 15 min at 4°C, washed twice in cold TBS, and diluted in TBS to final concentration of 1.5×10^9 cells per ml (OD_{600} ~1.5). Ten microliters of dilute (~15 million cells) were added to PCR reactions containing 1x DreamTaq buffer, 0.2 mM dNTPs, 1 µM F1 and R2 primers, 10 nM R1-F2 and F2 primers, and 20 U of DreamTaq polymerase in a total volume of 200 µl. The entire reaction volume was added to a 2-ml tube containing a rubber syringe plunger removed from 1-ml Monoject syringe (Tyco) along with 600 µl of chilled oil mix (73% Tegosoft DEC (Evonik), 7% ABIL WE 09 (Evonik), and 20% mineral oil (Sigma–Aldrich)). Emulsions were produced with a TissueLyser LT (Qiagen) at 34 Hz for 6 min. Samples were subdivided into seven emulsion PCR reactions, each performed under the following conditions: 94°C for 3 min for initial denaturation, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 68°C for 1.5 min, with a final extension of 68°C for 5 min. Emulsion PCR reactions were pooled and centrifuged at 10,000 *g* for 5 min, and then the oil phase was removed and 75 µl of ddH_2O added to the pelleted emulsion. Emulsions were broken by vortex after the addition of 750 µl of a 25:24:1 mixture of phenol: chloroform: isoamyl-alcohol pH 8.0 (Fisher Scientific). Aqueous phases were extracted and purified on a Zymo-Spin I PCR purification spin column (Zymo Research).

Purified emulsion-PCR products were used as template for a second round of PCR using nested primers (Supplementary Material Table S1). This nested-PCR reaction consisted of 1x DreamTaq buffer, 0.2 mM dNTPs, 0.5 µM F3 (Tc^r-gene specific), and 16S-specific R3 primers, 5 µl of purified emulsion-PCR product, and 2.5 U DreamTaq in a total volume of 50 µl, and proceeded as follows: 94°C for 3 min for initial denaturation followed by 35 cycles of 94°C for 30 s, 59°C for 30 s and 68°C for 30 s, with a final extension of 72°C for 5 min. Nested-PCR products were purified with a Zymo-Spin I PCR purification spin column, quantified with Picogreen (Invitrogen, Carlsbad, CA, USA), and pooled as necessary to a concentration of 70 ng/µl and cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). Sequencing was performed at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory on an Illumina MiSeq instrument using V3 chemistry, following the Earth Microbiome Project protocol (Walters et al. 2016).

Sequence processing and analysis

Sequence reads were processed in QIIME (Caporaso et al. 2010) paired-end forward and reverse reads were joined using the command *join_paired_ends.py*, and then quality-filtered to a minimum Phred score of Q19 using the *split_libraries_fastq.py* command. Chimeric sequences were identified and removed from the dataset using the command *identify_chimeric_seqs.py* with USEARCH6.1 (Edgar 2010), and remaining sequences were clustered into 97% operational taxonomic units (OTUs) using the *pick_open_reference_otus.py* command with ULCUST (Edgar 2010). OTUs with less than 10 reads were removed from the dataset, taxonomic assignment of OTUs was performed in SILVA (Pruesse et al. 2007). The phylogenetic tree of representative sequences of each OTU was created with SeaView version 4.7 (Gouy et al. 2010) by first aligning sequences using the Clustal Omega (Sievers et al. 2011) alignment option (clostalo). This alignment was then used to generate a maximum likelihood tree using PhyML v3.1 under the GTR model (Guindon et al. 2010). Branch support was assessed by the aLRT (SH-like) function (Anisimova and Gascuel 2006), and the resulting phylogenetic tree was visualized using FastTree (Price et al. 2010.). The analysis of beta diversity was performed using the command *core_diversity_analysis.py* using a sampling depth of 14 300, which represented the lowest read-depth of all samples in the

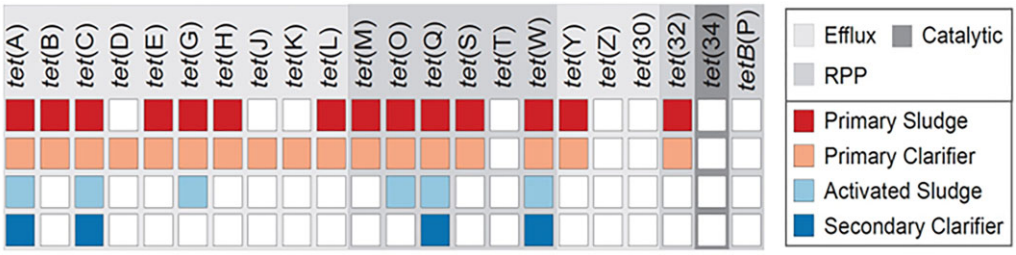

Figure 1. Survey of tetracycline resistance genes in wastewater treatment phases. DNA isolated from primary treatment phase (primary sludge and primary clarifier) and secondary treatment phase (activated sludge and secondary clarifier) were assayed for 22 Tc^r genes using PCR and electrophoresis. Detection of a given Tc^r gene is indicated by filled squares, colored according to the identity of the sample. Mechanism of tetracycline resistance are denoted by intensity of grey background shading.

group. The discovery of enriched OTUs between samples was performed with *differential_abundance.py*, which employs DEseq2 (Love et al. 2014).

Results

Wastewater treatment plant (WWTP) samples harbor a broad diversity of tetracycline resistance genes We tested samples representing four treatment phases within a WWTP for the presence of 22 tetracycline resistance (Tc^r) genes using PCR and electrophoresis: 13 tetracycline efflux pump proteins [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(K), *tet*(L), *tet*(Y), *tet*(Z), and *tet*(30)], 8 ribosome protection proteins (RPPs) [*tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), *tetB*(P), and *tet*(32)], and one enzymatic protein [*tet*(34)]. Of this set, five [*tet*(T), *tet*(Z), *tet*(30), *tet*(34), and *tetB*(P)], were observed in at least one sample. The sample from the primary clarifier contained all 17 of the Tc^r genes that were detected in this WWTP, whereas samples from secondary treatment phases have reduced subsets of these genes (Fig. 1).

To assess the bacterial diversity across treatment phases, we sequenced 16S rRNA amplicons and rarefied 97% OTUs to a common read-depth in each sample. Primary treatment samples contain many more dominant OTUs, resulting in their having lower overall OTU richness than secondary treatment samples after rarefaction (692 for primary sludge and 557 for primary clarifier vs. 1065 for activated sludge and 1169 for secondary clarifier). Samples from the primary treatment phase had greater proportions of *Epsilonproteobacteria* [*Campylobacterota*, corrig, phyl nov.)]—8.7% for primary sludge and 33.7% for primary clarifier vs. 0.7% for activated sludge and 3.8% for secondary clarifier—with most belonging to the genus *Arcobacter*, which are typically at high abundances in WWTPs (Fisher et al. 2014). Additionally, *Firmicutes* (*Bacillota* corrig, phyl nov.) are more abundant in primary treatment phase samples than in secondary treatment samples (14.2% for primary sludge and 11.3% for primary clarifier vs. 0.7% for activated sludge and 2.0% for secondary clarifier). The difference is due primarily to the reduction in the relative abundance of the *Clostridia*, a class of obligate anaerobes common in the human gut, which are inhibited by the aerobic conditions experienced during secondary treatment. The same trend was observed for two prominent families of anaerobic *Bacteriodetes* (*Prevotellaceae* and *Bacteroidaceae*), which are also abundant in the human gut.

In contrast, secondary treatment phase samples contained greater relative proportions of *Alphaproteobacteria* (1.5% for primary sludge and 0.5% for primary clarifier vs. 7.3% for activated sludge and 6.1% for secondary clarifier), *Gammaproteobacteria* (1.1% for primary sludge and 0.9% for primary clarifier vs. 10.1% for activated sludge and 6.3% for secondary clarifier), and *Saprospirae* and *Sphingobacteria*, which were virtually absent from the primary phase samples (Fig. 2). Several other taxa, such as *Nitrospirae* and *Planctomycetes*, were found exclusively in the secondary treatment samples where they are known to participate in the cycling of carbon and nitrogen that occurs in these pools (Lage et al. 2012, Daims et al. 2015).

Identification of bacterial taxa harboring Tc^r genes

We next ascertained the specific bacteria harboring each of the Tc^r genes by performing epicPCR (Spencer et al. 2016) on ~15 million single cells isolated from the primary clarifier—the sample containing the highest diversity of Tc^r genes. Cells were assayed individually for a panel of Tc^r genes that included eight efflux pump proteins and five RPPs, to capture the broad diversity of tetracycline resistance. *Arcobacter*, the most abundant genus in our sample (19%), serve as major reservoir for Tc^r genes (Fig. 3), as was also observed in an epicPCR survey of WWTPs in Finland, where abundant strains of *Arcobacter* were the predominant carriers of the four resistance-associated genes that they assayed (Hultman et al. 2018).

Distribution of Tc^r genes encoding efflux pump proteins

The distributions of the effluxpump Tc^r largely followed bacterial cell-envelop structure, with *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H) and *tet*(J) in Gram-negatives, and *tet*(K) and *tet*(L) in Gram-positives. Despite this strong partitioning, there were several instances in which a Gram-negative taxon harbored a prototypical Gram-positive Tc^r efflux pump gene and vice versa.

The Tc^r efflux pump genes in Gram-negative bacteria assorted among numerous proteobacterial subtaxa (Fig. 4). The *tet*(C), *tet*(E), and *tet*(G) genes were most commonly associated with *Aeromonas*, a widespread aquatic gammaproteobacterial genus that occurs at high frequencies in sewage and is often associated with human disease (23), with reads mapping to this genus at 39.3%, 78.2%, and 22.9%, respectively; the *tet*(D) gene was a high frequencies in enteric bacteria (85.8% of reads), and *tet*(H) occurred mainly in the order *Pseudomonadales* (56.8% of reads). The *tet*(J) gene had the narrowest distribution of any Tc^r genes assayed, with 93%

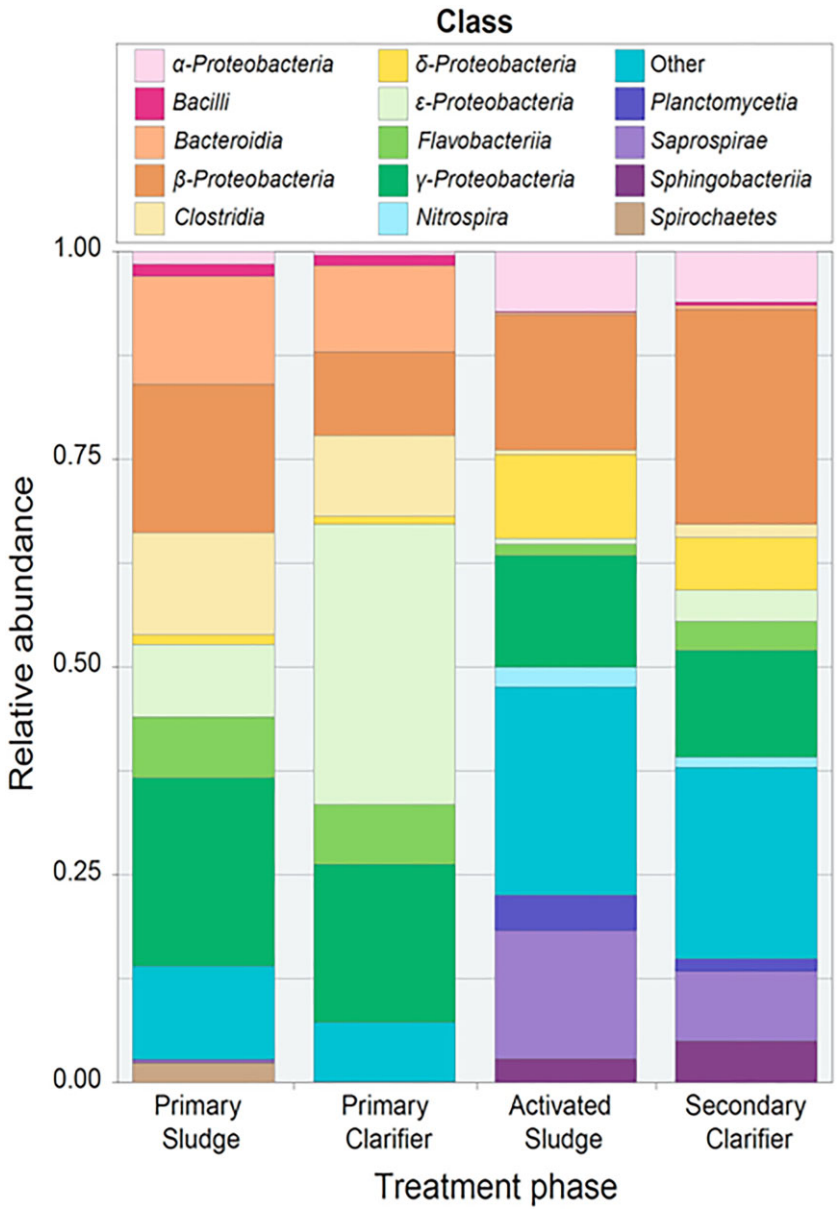

Figure 2. Class-level profiles of bacteria present in wastewater treatment phases. Included are those bacterial classes whose relative abundances based on sequencing 16S rRNA amplicons were ≥ 2% in at least one sample.

of the sequence reads assigned to the genus *Proteus*. These efflux-pump Tc^r genes were not wholly confined to *Proteobacteria*; *e.g.* *tet*(G) also occurred at fairly high frequencies in *Spirochaetae* (9.1% of reads) and *Firmicutes* (12.2% of reads) (Supplementary Material Fig. S1).

The two efflux proteins associated with Gram-positives, *tet*(K) and *tet*(L), were detected primarily in *Firmicutes* (Fig. 3). The *tet*(L) sequences were distributed among *Streptococcaceae* [*Streptococcus* (33.4%) and *Lactococcus* (20.5%)] and *Veillonellaceae* [*Veillonella* (12.2%), *Megamonas* (11.2%) and *Propionispora* (3.1%)], whereas *tet*(K) mainly occurred in *Staphylococcus* (87.5% of reads). Most of the remaining *tet*(K) reads (10.6%) were linked to a single OTU classified to the Gramnegative *Cloacibacterium* (*Bacteroidetes*), which was first isolated from a WWTP over a decade ago (Allen et al. 2006) (Fig. 3).

Distribution of Tc^r genes encoding ribosomal protection proteins (RPPs)

The Tc^r genes encoding ribosomal protection proteins (RPPs) [*tet*(M), *tet*(O), *tet*(Q), *tet*(S), and *tet*(W)] are harbored by a more diverse array of bacteria than the efflux pump Tc^r genes at almost every taxonomic level (Fig. 3, Supplementary Material Fig S1). Most genes are at high relative abundances in both Gram-positive and Gram-negative phyla, except for *tet*(Q), which predominate in the *Bacteroidetes* (66.9% of reads). As with the efflux-pump Tc^r genes, there was considerable variation in the specific bacterial families and genera associated with each of the RPP Tc^r genes, and they mostly clustered to broad phylogenetic clades (Fig. 3). Overall, *tet*(M) was the most evenly distributed, with *Campylobacteraceae*, *Lachnospiraceae*, *Marinilabiaceae*, *Porphyromonadaceae*, *Ruminococcaceae*, and *Streptococcaceae* together accounting for over 65% of the reads.

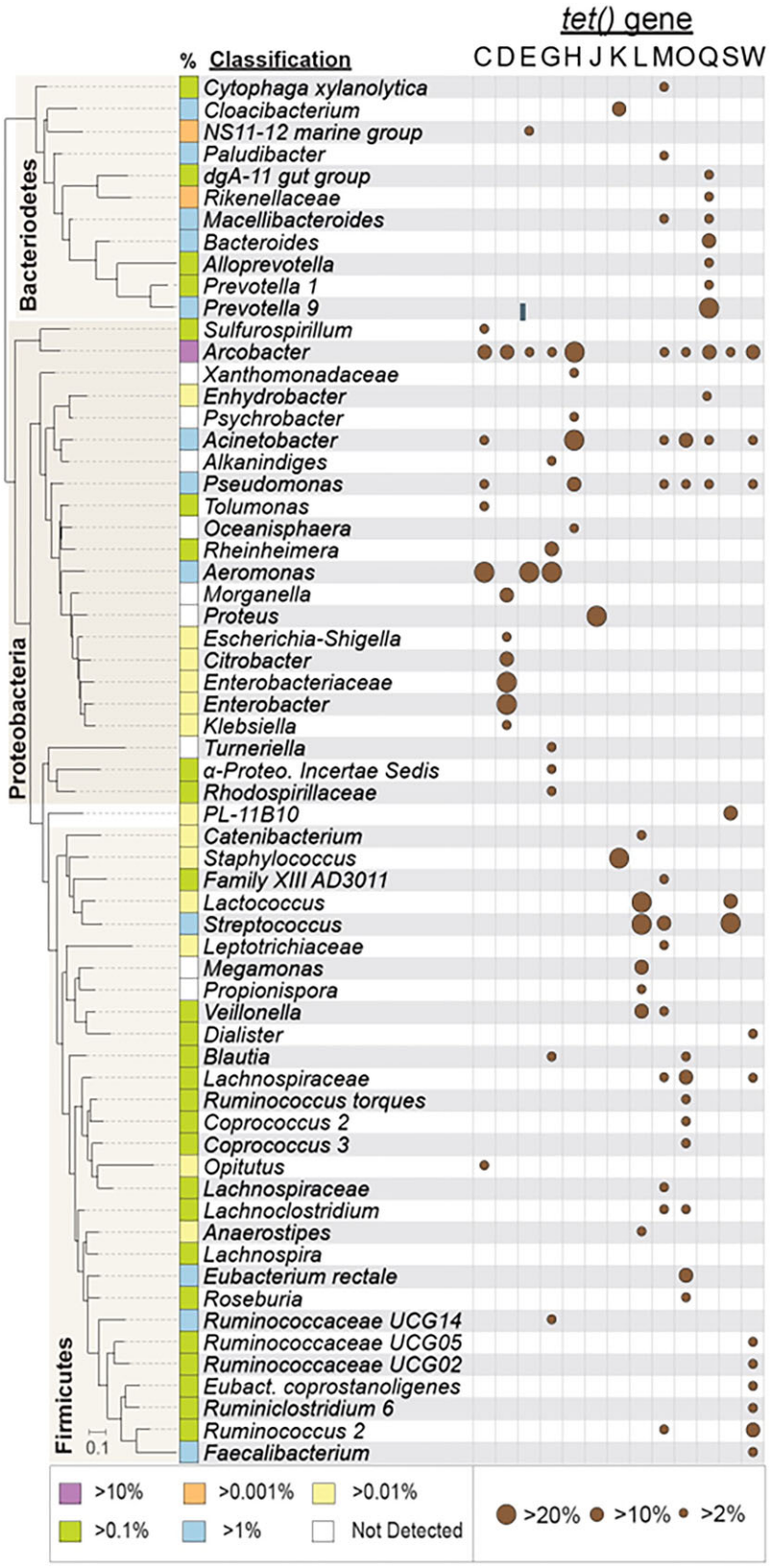

Figure 3. Phylogenetic distributions and relative abundances of tetracycline resistance genes. Phylogeny is based on representative 16S rRNA sequences of clustered 97% OTUs and includes all bacterial taxa whose relative abundances were ≥2% for at least one of the Tc^r genes assayed. Color-coded squares to the left of each classified OTU are the abundances in the WWTP population at large. Shaded circles denote the association of a particular tetracycline resistance gene with a classified OTU, with circles sized according to the relative abundance of each classified OTU within the Tc^r-selected population.

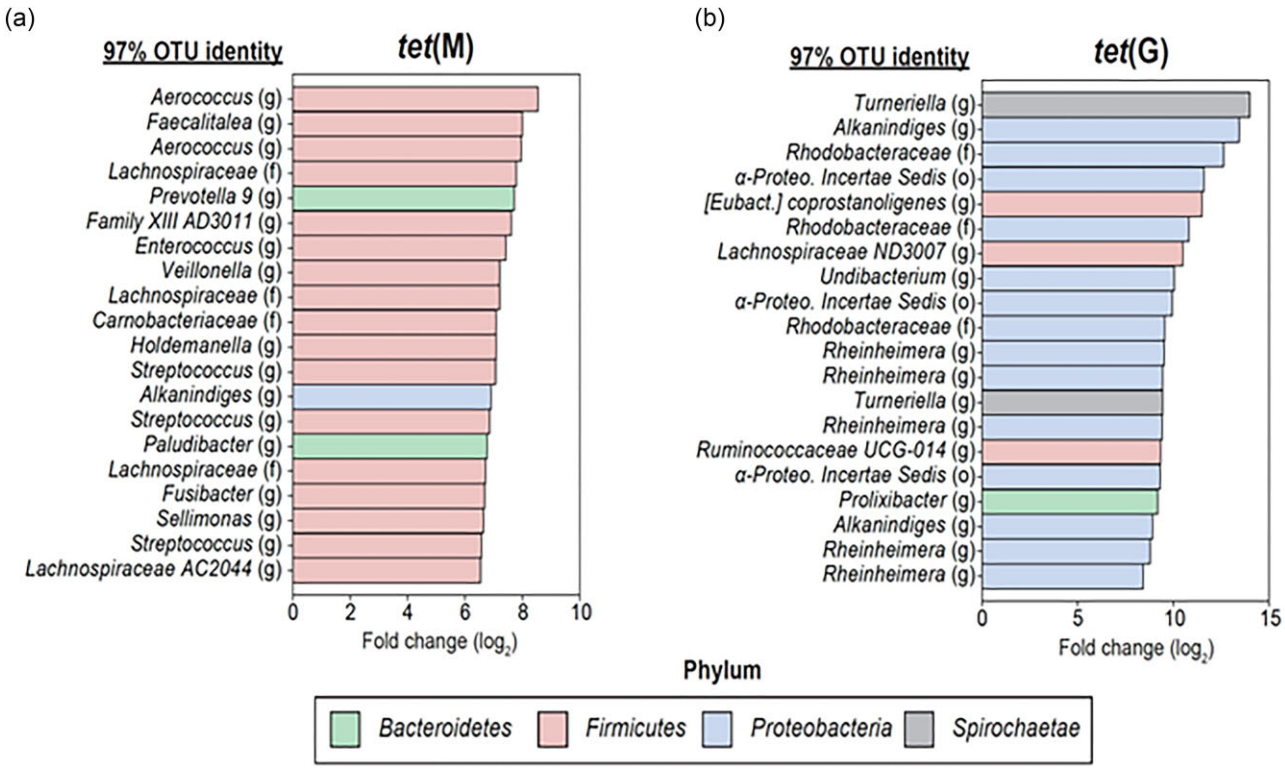

Figure 4. Enrichment of tetracycline resistance genes in rare bacterial taxa. Shown are taxonomic assignments of the 20 most highly enriched 97% OTUs associated with *tet*(M) (a) and *tet*(G) (b). Enrichment values, which denote the degree to which a given OTU changed in its prevalence between the experimental and control populations, were calculated as the $\log_2$ fold change between the normalized read counts of a given 97% OTU in the Tc^r-selected population and those in the unselected "population at large" sample. The taxonomic ranks to which each 97% OTU could be classified are as follows: (o) = order, (f) = family, (g) = genus, (s) = species.

Rare taxa harboring Tc^r genes

Whereas *Arcobacter* possess a majority (10/13) of the Tc^r genes assayed (Fig. 3), taxa that were rare (or sometimes unobserved) in our original 16S rRNA characterization of the WWTP community were often the predominant carriers of a particular Tc^r gene. For example, *Proteus* accounted for 93% of the *tet*(J) sequence reads but was not detected in the community at large, and similarly, over 80% of the *tet*(D), reads were assigned to members of the *Enterobacteriaceae* (*Enterobacter*, *Citrobacter*, *Morganella*, *Escherichia-Shigella*, *Klebsiella* and an anonymous enteric genus), each of which constituted <0.02% of the population.

To determine the full spectrum of taxa that harbor each Tc^r gene, we inventoried all 97% OTU harboring a Tc^r gene. A potential complication of this analysis is that OTUs with very low read counts could potentially arise from rare instances of mispairing between 16S- and Tc^r-gene amplicons during epicPCR. To minimize the inclusion of false positives, we limited our analyses to those OTUs that were initially rare in the population at large (and therefore unlikely to produce a false signal) but experienced a large increase in relative abundance in the *tet*-carrying population.

For each Tc^r gene, we mined the top 20 OTUs that whose relative abundances increased after epicPCR selection (Fig. 4 and Supplementary Material Fig. S2) and found that every Tc^r gene was associated with multiple 97% OTUs from the same genus. At the extreme was *tet*(K), in which 19 of the 20 most highly enriched OTUs were assigned to *Staphylococcus*. Only for *tet*(D), *tet*(K), and *tet*(L) did all of the most highly enriched taxa correspond to those at high abundances in the population (Supplementary Fig. S2).

The phylogenetic distributions of the rare carriers of Tc^r genes broadly overlapped with those of the most abundant carriers of the particular Tc^r gene. For example, *Streptococcus* is the dominant carrier of *tet*(M) based on its relative abundance and by its degree of enrichment, but three other genera—*Aerococcus*, *Enterococcus*, and an uncultured *Carnobacteria*—all in the same order as *Streptococcus* harbor the *tet*(M) gene (Fig. 4a). Likewise, for *tet*(Q), OTUs from four previously undetected genera, all within the *Bacteroidales*, were among the 20 OTUs most highly enriched for this gene (Supplementary Material Fig. S2).

Our procedures, by identifies the bacterial strains in a community that harbor a particular antibiotic resistance determinant, revealed a number of new and uncharacteristic hosts for many of the Tc^r genes. There are several examples of Gram-positive bacteria harboring efflux pump Tc^r genes that are generally associated with Gram-negative bacteria, such as the presence of *tet*(G) in *[Eubacterium] coprostanoligenes* and *Lachnospiraceae ND3007* (Fig. 4b). Most surprising was the distribution of *tet*(J), previously known only to occur in *Proteus* but now expanded to include *Coprococcus 1*, *Proteocatella*, an as-yet unidentified *Lachnospiraceae*, and two *Bacteriodetes* (*Flavobacterium* and a member of the ST-12K33 family. Our study also marks the first time that *tet*(C) has been found to be associated with members of the *Verrucomicrobia*, *Spirochaetae*, and *Fibrobacteres*, revealing that this gene is broadly distributed among Gram-negatives (Supplementary Material Fig. S2).

Discussion

The incidence of ARGs in the environment has traditionally been appraised by three approaches: (1) Plating on selective media to isolate antibiotic resistant bacteria; however, this process does not identify the specific gene or mechanism conferring resistance and is limited to the small proportion of bacteria that can be cultivated. (2) Amplicon-directed surveys, which, unlike plating, capture the diversity of cultivable and uncultivable bacteria and can assay for the presence of any ARG whose sequence is known. However, this method does not divulge which bacterial strains or species carry resistance markers, and any conclusions about the association between bacteria and ARGS are, at best, correlative (Danko et al. 2021, Javvadi and Mohan 2023). (3) Metagenomic surveys, which can reveal the complement of genes and strains in a community (Forslund et al. 2014, Crofts et al. 2017), but entail the assembly of large contigs to associate an ARG with a specific genome and are not suitable for genes residing on plasmids, which disassociate from host genomes during metagenomic library preparation. While definitively identifying an antibiotic resistance phenotype requires selective media plating approaches, linking this phenotype to a genotype is valuable for innovating solutions to resistance. However, just as important to linking a gene to a taxonomic host. We implemented epicPCR (Spencer et al. 2016), which eliminates the shortcomings inherent in other approaches and allows the direct assignment of ARGs to members of bacterial communities. The only notable inconvenience of this method is technical in that some expertise is required in the preparation of single-cell emulsion droplets.

WWTPs are densely populated with highly diverse communities of bacteria and can serve as repositories for the spread of ARGs (Szczepanowski et al. 2009, Rizzo et al. 2013, Karkman et al. 2018, Pazda et al. 2019, Wu et al. 2023). Moreover, the presence subinhibitory concentrations of antibiotics in wastewater can accelerate resistance (Andersson and Hughes 2014.) and to contribute to the increased occurrence of antibiotic-resistant pathogens (Rizzo et al. 2013, Marti et al. 2014). Tetracycline resistance genes are the most abundant family of ARGs in the human gut microbiome (Hu et al. 2013), and our survey of the Tc^r resistome in a single WWTP yielded 17 known Tc^r genes in the primary stage clarifier, with four—*tet*(A), *tet*(C), *tet*(Q) and *tet*(W)—remaining in the secondary stage clarifier. Of the Tc^r genes that we assayed, only five were not detected in wastewater samples; however, many of these [e.g. *tet*(30), *tet*(34), and *tet*(B) (P)] are considered unique to environmental bacteria (Lanza et al. 2018) and rarely encountered in samples rich in human-associated bacteria.

The host distributions of most Tc^r genes in wastewater often recapitulate what has been established from screening human-associated strains, which is not surprising considering the contributors to wastewater. For example, *tet*(J) was linked predominantly to members of the genus *Proteus*, a finding that aligns with current knowledge since this gene was initially discovered in *Proteus mirabilis* (Magalhaes et al. 1998) and its representative sequences in the NCBI database derive solely from *Proteus* isolates. *Proteus* is a common member of the human intestinal microbiome in most humans (Armbruster and Mobley 2012). However, our screening strategy discerned links between individual Tc^r genes and bacterial taxa at unprecedented depths; and for almost every gene, there were instances in which a particular Tc^r gene was associated with a bacterial taxon—sometimes even a bacterial phylum—for the first time. We detected the *Proteus*-associated *tet*(J) gene in several non-enteric bacteria, including the distantly related *Firmicutes* and *Bacteroidetes*. Therefore, by circumventing the need to cultivate bacteria to screen for resistance determinants, we were also able to analyze large taxonomic groups, such as the presence of *tet*(C) in *Spirochetes* and *Verrucomicrobia*, for which there have been few reports or surveys of tetracycline resistance.

Each of the Tc^r genes that we profiled displays a unique host distribution. Those encoding efflux proteins broadly assorted according to the cell envelope characteristics of the bacterial host (Chopra and Roberts 2001); however, there were several instances in a gene normally associated with one cell-wall type was recovered in taxa of the opposite type. For example, in our study, *tet*(K) most commonly occurred in the Gram-positive *Staphylococcus*, as shown previously (Trzcinski et al. 2000, Schmitz 2001, McDougal et al. 2010), but was also present in the Gram-negative *Cloacibacterium*, which was not previously known to carry this gene (Gay et al. 2016). Similarly, we found that *tet*(G), which is typically associated with Gram-negative taxa, displayed several unconventional hosts, including four gram-positive Clostridial taxa—*Blautia*, *Ruminococcus*, *Eubacterium coprostanoligenes* and *Lachnospiraceae ND3007*—that are regular members of the human microbiome. It is not known whether these genes confer resistance to these alternate hosts, but their sporadic occurrence in phylogenetically divergent taxa suggests that they were acquired by lateral gene transfer, and they provide additional sources for their dissemination in the population at large (Arnold et al. 2021).

The RPP class of Tc^r genes are typically distributed among a broader array of hosts than those encoding efflux proteins, and regularly include both Gram-positive and Gram-negative hosts (Roberts 2005a). Several RPP genes, including *tet*(Q), *tet*(O), and *tet*(W), are present in nearly all sampled humans (Forslund et al. 2013, Hu et al. 2013), implicating the human microbiome as a major reservoir for these genes. Within the WWTP, *tet*(Q) was most frequently associated with strains of *Bacteroides* and *Prevotella*, both of which are common constituents of the human microbiome, and the distribution of this gene has been attributed to horizontal transfer by the CTnDOT family of conjugative transposons (Shoemaker et al. 2001, Whittle et al. 2002). In our samples, *tet*(W) was the most frequently hosted by members of the *Ruminococcaceae*, and although *tet*(W) is considered the most widely distributed Tc^r gene (Roberts 2005b) and is present in many families of anaerobic bacteria in the human gut (Scott et al. 2000), there are no previous reports linking this gene to members of the *Ruminococcaceae*. Therefore, its distribution represents another case of potential gene exchange among species inhabiting the human microbiome.

Humans are currently involved in the large-scale transport and rapid global spread of bacteria, which has introduced microbes to new environments and selective pressures, such as the antibiotic challenge. On a global scale, it is estimated that <20% of wastewater is subject to any form of treatment (Sato et al. 2013, WWAP 2017), and its use for irrigation has enhanced the dissemination of antibiotic resistance determinants, which are prevalent and transferred among bacteria in both agricultural and urban settings. Under these conditions,

new ARGs may derive from genes involved in unrelated functions, as may have been the case for both *tet*(L) and *tet*(K), which are capable of transporting monovalent cations as well as tetracycline. These alternate roles help explain why these genes are present in bacterial populations even in the absence of antibiotic exposure (Krulwich et al. 2001). There is a demand for surveillance methods, such as EpicPCR, that rapidly associate ARGs to their specific host bacteria in diverse environments (Forslund et al. 2014, Berendonk et al. 2015, Pearson et al. 2016, Crofts et al. 2017), since such knowledge is needed to understand the distribution and potential spread of antibiotic resistance (Danko et al. 2021).

In summary, this study underscores the pivotal role of epicPCR in advancing our understanding of antibiotic resistance dynamics within wastewater environments. Our findings support previous studies in demonstrating that epicPCR is a powerful tool for linking ARGs to specific bacterial hosts, revealing a complex and diverse landscape of tetracycline resistance genes among various bacterial taxa, including several novel associations. This approach not only enhances our comprehension of the spread and host range of these genes but also offers crucial insights into potential pathways for the transfer of antibiotic resistance. Looking ahead, the continued application of epicPCR in wastewater surveillance promises to significantly contribute to our efforts in managing and mitigating the risks associated with antibiotic resistance, a growing concern in public health and environmental sustainability. By providing detailed insights into the taxonomic distribution of resistance genes, epicPCR stands as a key technology in shaping future strategies for monitoring and controlling the spread of antibiotic resistance in wastewater and other environmental reservoirs.

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Author contributions

Howard Ochman (Conceptualization [equal], Data curation [equal], Funding acquisition [equal], Project administration [equal], Visualization [equal], Writing – original draft [equal], Writing – review & editing [equal]), Erik M. Quandt (Data curation [lead], Formal analysis [lead], Investigation [lead], Writing – original draft [equal]), Neil Gottell (Data curation [supporting], Formal analysis [supporting], Methodology [supporting], Validation [supporting]), and Jack A. Gilbert (Funding acquisition [equal], Investigation [supporting], Project administration [supporting], Supervision [supporting], Writing – review & editing [supporting]).

Supplementary data

Supplementary data is available at *Sumbio Journal* online.

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Data availability

Sequencing reads are publicly available from the NCBI sequencing read archive under SRA accession: SRP144473.

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