Tetracycline-inactivating enzymes from environmental, human commensal, and pathogenic bacteria cause broad-spectrum tetracycline resistance

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Tetracycline resistance by antibiotic inactivation was first identified in commensal organisms but has since been reported in environmental and pathogenic microbes. Here, we identify and characterize an expanded pool of tet(X)-like genes in environmental and human commensal metagenomes via inactivation by antibiotic selection of metagenomic libraries. These genes formed two distinct clades according to habitat of origin, and resistance phenotypes were similarly correlated. Each gene isolated from the human gut encodes resistance to all tetracyclines tested, including eravacycline and omadacycline. We report a biochemical and structural characterization of one enzyme, Tet(X7). Further, we identify Tet(X7) in a clinical Pseudomonas aeruginosa isolate and demonstrate its contribution to tetracycline resistance. Lastly, we show anhydrotetracycline and semi-synthetic analogues inhibit Tet(X7) to prevent enzymatic tetracycline degradation and increase tetracycline efficacy against strains expressing tet(X7). This work improves our understanding of resistance by tetracycline-inactivation and provides the foundation for an inhibition-based strategy for countering resistance.
environnmental microbes are ancient and diverse reservoirs of antibiotic-resistance genes (ARGs), and are the likely evolutionary progenitors of much clinical resistance1–3. Nonpathogenic bacteria from habitats as varied as soils and healthy human guts have exchanged multidrug-resistance cassettes with globally distributed human pathogens2. With widespread antibiotic use in clinical and agricultural settings, there is strong selective pressure for pathogens to acquire resistance genes with novel activities and substrate specificities from environmental resistomes6. The discovery and characterization of environmental resistance genes with novel activities before they are acquired by pathogens can help lessen their potential clinical impact and inspire proactive approaches to address emerging resistance early in antibiotic development and improve understanding of the ecology, evolution, and transmission of resistance genes across habitats4,7. Furthermore, understanding the evolutionary origins, genetic contexts, and molecular mechanisms of antibiotic resistance is critical to devising strategies to curb the spread of resistant organisms and their ARGs, and for sustainable development of new antimicrobial therapies.

While tetracycline resistance most frequently occurs via efflux or ribosomal protection8, enzymatic detoxification of tetracycline was first reported in 19889,10. This mechanism of resistance, originally detected in the commensal Bacteroides fragilis, has since been identified in broader commensals, environmental microorganisms, and pathogens. In 2015, we substantially expanded the catalog of “tetracycline destrucases” by identifying a family of tetracycline-inactivating enzymes, (Tet(47)–Tet(55)), from functional selections of environmental metagenomes11. Based on sequence homology to these soil-derived enzymes, we identified an additional enzyme, Tet(56), as a previously uncharacterized tetracycline resistance determinant in Legionella longbeachae. These enzymes are structurally and functionally homologous to Tet(X)12, the flavin-dependent monooxygenase (FMO) originally discovered in Bacteroides fragilis13. Recently, we described the structural details of two complementary inhibitory mechanisms for the soil-derived tetracycline-inactivating enzymes: competitive inhibition by blockade of substrate binding, and mechanistic inhibition by restraining FAD cofactor dynamics12. In 2019, two plasmid-encoded variants of Tet(X), named Tet(X3) and Tet(X4), were discovered in Enterobacteriaceae and Acinetobacter strains isolated from animal and human sources. The Tet(X3)- and Tet(X4)-containing plasmids were widely dispersed, transferable, and stable in human pathogens, and conferred high levels of resistance (up to 128-fold increase) against all tetracycline antibiotics, including latest-generation tigecycline, eravacycline, and omadacycline, in antibiotic susceptibility assays and murine infection models14,15. Given that tetracycline antibiotics have been used clinically for seven decades and are currently widely deployed in agricultural settings16, it is likely that anthropogenic emissions have driven spread of Tet(X)-like FMOs that now threaten the clinical efficacy of next-generation tetracycline antibiotics.

In human pathogens, tetracycline resistance was thought until recently to occur almost exclusively by ribosomal protection or antibiotic efflux8. Eravacycline and omadacycline were developed, in part, because the synthetic scaffold modifications of the D-ring overcome these traditional clinical resistance mechanisms, similar to tigecycline. It is noteworthy that tigecycline was approved by the FDA in 2005 and saw somewhat limited use until the appearance of infections caused by multidrug-resistant (MDR) Gram-negative bacteria, including carbapenem-resistant Enterobacteriaceae17 and recalcitrant ventilator-associated pneumonia18, complicated urinary tract infections, and complicated intra-abdominal infections19. Tet(X) and homologs conferring resistance to tigecycline, eravacycline, omadacycline, and all other classes of tetracycline antibiotics have now been discovered to be present in carbapenem and colistin-resistant MDR organisms harboring the blaNDM-1 and mcr-1 genes, respectively, creating a scenario of pan-antibiotic resistance emerging in Gram-negative pathogens1,20. The widespread distribution of tetr(X)-like genes capable of covalent inactivation of tetracycline scaffolds threatens the future clinical efficacy of this drug class in the same way consecutive generations of aminoglycosides, amphenicols, and beta-lactams have become vulnerable to enzymatic inactivation as a dominant resistance mechanism20.

All three types of tetracycline resistance have evolutionary origins in the environment, but are now found widely distributed in commensal and pathogenic bacteria8. Flavoenzymes, including Tet(X)-like FMOs, are an abundant and diverse enzyme family and display a proclivity for horizontal transfer and gene duplication, allowing them to spread between species and acquire novel functionality21. Thus, these genes are candidates for dissemination, potentially compromising new tetracycline antibiotics and motivating surveillance of the prevalence and abundance of this gene family across microbial habitats. The soil tetracycline-inactivating enzymes and Tet(X)-like FMOs have low overall sequence identity (20.1% mean ± 1.2% s.d. percent amino-acid identity between Tet(X) and soil-derived tetracycline-inactivating enzymes) making ARG prediction difficult and functional validation of putative ARGs necessary. Structural and biochemical characterization of the soil tetracycline-inactivating enzymes have revealed novel structural features including an extra C-terminal helix that plays a role in active site gating and substrate selectivity and mechanistically distinct oxidation patterns leading to resistance for soil tetracycline-inactivating enzymes (oxidations at C11a and CI) and Tet(X) (oxidation at C11a)12. The soil tetracycline-inactivating enzymes detoxify naturally occurring first-generation tetracyclines, such as chlorotetracycline and oxytetracycline, but fail to oxidize D-ring substituted analogs, including tigecycline12. Tet(X)-like homologs possess a constitutively open active site that accommodates D-ring substituted substrates, such as tigecycline, eravacycline, and omadacycline12,14,15, making Tet(X) homologs a clinical threat for these last-generation tetracyclines. Single amino-acid mutations in Tet(X) have been shown to provide gain-of-function under tigecycline selection through more efficient oxidative inactivation22. Thus, monitoring broadly for Tet(X) homologs, even single amino-acid point mutants, and understanding the evolutionary connection with the soil tetracycline-inactivating enzymes is critical for proactively managing this emerging resistance mechanism through optimization of next-generation tetracycline structures and the development of effective inhibitor combinations that overcome resistance by inactivation23.

To this end, we sought to characterize the tetracycline resistance across environmental and human-associated metagenomes. We used functional metagenomic selections, wherein the heterologous expression bottleneck allows only cloned fragments with a functional resistance gene to be sequenced and assembled2. Annotation of all open-reading frames in the selected fragments, using similarity to known resistance protein families (including remote homologs via hidden Markov models), enable identification of the gene likely to be responsible for the resistance phenotype in recombinant E. coli24. We previously employed this sequence- and culture-independent approach to identify the tetracycline-inactivating enzymes from 18 grassland and agricultural soil metagenomes11. Here, we expand that approach to analyze 244 additional metagenomes to ask whether novel tetracycline-resistance elements are found in other habitats, perhaps with different or expanded substrate range and specificity. We find genes encoding tetracycline-inactivating enzymes are widespread in diverse microbial communities, but cluster
by habitat of origin and resistance phenotypes also correlate with microbial habitat. We show that tetracycline-inactivating enzymes identified in the human gut metagenome confer resistance to all tetracycline antibiotics tested. Furthermore, we describe in detail Tet(X7), a tetracycline-resistance enzyme recovered from a human gut metagenome that confers resistance to tigecycline, omadacycline, and eravacycline. We characterize the phenotypic resistance profile, solve a crystal structure, and reveal the biochemical basis of Tet(X7) degradation of tetracyclines. We identify Tet(X7) in a clinical isolate of Pseudomonas aeruginosa from a cystic fibrosis patient, further supporting the clinical arrival of tetracycline resistance by inactivation. Lastly, we show that inhibition of Tet(X7) with anhydrotetracycline or analogues prevents tetracycline degradation and rescues antibiotic efficacy. Our results emphasize the need for surveillance of novel resistance determinants to antimicrobial agents in development to counter emerging antibiotic resistance.

Results
Identification of tetracycline inactivation across habitats. We sought to better understand the prevalence of tetracycline inactivation by FAD-dependent oxidoreductases as a resistance mechanism across habitats. Tetracycline resistance by inactivation is relatively uncommon as compared with efflux or ribosomal protection, so current resistance databases are biased toward these latter two mechanisms. Functional metagenomics circumvents limitations imposed by identifying resistance via sequence similarity to a database. We conducted a retrospective analysis of functional metagenomic libraries that had previously been selected on tetracycline and tigecycline. We selected for further analysis are indicated by gray dots at branch tips, and clinically implicated tet(X) variants are labeled. b Percent amino-acid identity heatmap indicates greater relative sequence diversity in the soil-derived set compared with the gut-derived set, but low identity between these two sets.

Sixty-nine tetracycline inactivators were identified from tetracycline and tigecycline selections of diverse metagenomic libraries. Genes selected for further analysis are indicated by gray dots at branch tips, and clinically implicated tet(X) variants are labeled. b Percent amino-acid identity heatmap indicates greater relative sequence diversity in the soil-derived set compared with the gut-derived set, but low identity between these two sets. The widespread resistance to tigecycline by inactivation of tetracycline inactivating enzymes in addition to the 10 tetracycline-inactivating genes from soil metagenomes that had previously been described. We found that these genes formed two distinct clades that were correlated with habitat of origin. There was greater sequence diversity within the soil-derived subset (mean ± s.d. percent amino-acid identity of 66.5% ± 9.4%) compared with the gut-derived subset (mean ± s.d. percent amino-acid identity of 91.4% ± 4.7%), but low identity between the two habitats (mean ± s.d. percent amino-acid identity of 20.8% ± 1.5%, Fig. 1b).

We selected eight genes from this set for further analysis and subcloned them from their metagenomic source into a pZEl vector and transformed into E. coli DH10B. These genes were originally selected from fecal samples (six of eight) and latrine samples (two of eight) from a peri-urban setting in Peru. We additionally include nine genes of environmental origin which were originally selected from agricultural soils in Michigan (five of nine) and grassland soils in Minnesota (four of nine) (Supplementary Data 1). Lastly, we included one homolog identified in the human pathogen L. longbeachae (tet(56)) and tet(X), which was originally discovered in the commensal B. fragilis, but has since been identified in numerous pathogens. These genes were selected to encompass the representative phylogenetic diversity in the broader set and based on the availability of metagenomic DNA for subcloning. We performed antimicrobial susceptibility testing using broth microdilution for these recombinant constructs against 11 tetracycline compounds, including anhydro-tetracycline. All computationally predicted tetracycline-inactivating enzymes tested had a bona fide resistance function when subcloned from their metagenomic source into E. coli. We found that resistance phenotypes, like genotypes, clustered according to habitat of origin (Fig. 2). Each of the eight human gut-derived genes displayed pan-tetracycline resistance. While all gut-derived tetracycline-inactivating enzymes conferred high-level resistance to tigecycline, minocycline, eravacycline, and omadacycline, soil-derived enzymes were all susceptible or intermediate to these drugs. Thus, resistance to latest-generation tetracyclines mediates phenotypic clustering between the soil-derived and gut-derived genes and discriminates between habitats of origin. Our functional identification of 69 diverse tetracycline inactivators from soil, human gut, animal gut, and latrine metagenomes indicate that tetracycline resistance by inactivation is widespread.
in diverse metagenomes14,15. This includes, in the case of genes identified from gut metagenomes, high-level resistance to latest-generation tetracyclines.

**Tet(X7) confers tetracycline resistance.** Each of the eight gut-derived tetracycline-inactivating enzymes encoded resistance to every tetracycline currently approved for clinical use, including tigecycline, eravacycline, and omadacycline. Tigecycline was approved for human use in 2005, and is an antibiotic of last resort for infections, including those caused by carbapenem-resistant *Enterobacteriaceae*17,31. Eravacycline was approved in August 2018 for treatment of complex intra-abdominal infections, and omadacycline was approved in October 2018 for treatment of community-acquired bacterial pneumonia and acute skin and skin structure infections32,33. Reports of resistance to eravacycline and omadacycline have been limited to date. Eravacycline resistance in *Klebsiella pneumoniae* has been attributed to overexpression of the OqxAB and MacAB efflux pumps34, and nonsusceptibility (MIC 4 µg/mL) has been observed in *Escherichia coli* DH10B heterologously overexpressing Tet(X). The recent reports of plasmid-encoded Tet(X3) and Tet(X4) in *Enterobacteriaceae* and *Acinetobacter* strains isolated from animal and human sources, encoding phenotypic resistance to eravacycline and omadacycline in both whole-cell assays and murine infection models, are of potential immediate clinical concern14,15.

Approval of these drugs motivate further study of this mode of resistance. To better understand the mechanistic basis of resistance to latest-generation tetracyclines by these types of emerging resistance determinants, we selected a gut-derived gene, tet(X7), indicating that the mechanism of resistance is consistent with drug inactivation.

We characterized the in vitro degradation of tigecycline, eravacycline, or omadacycline by recombinant N-His6-tagged Tet(X7), indicated by a time- and enzyme-dependent decrease in the ~400-nm absorbance band in the optical absorbance spectrum that is associated with disruption of the conserved β-diketone chromophore found in all tetracyclines11 (Supplementary Fig. 1a). To understand substrate binding and catalytic efficiency for latest-generation tetracycline inactivation by Tet(X7), we measured apparent Michaelis–Menten kinetic parameters by continuously monitoring the decrease in optical absorbance at 400 nm under steady-state conditions (Fig. 3b). For comparison, we also determined Michaelis–Menten kinetic parameters of Tet(X)-mediated inactivation of tigecycline and eravacycline (Fig. 3a). The apparent catalytic efficiency of Tet(X7) was five times greater than that of Tet(X) for degradation of eravacycline ($k_{cat}/K_M$ values of 0.07 ± 0.02 and 0.01 ± 0.002 µM$^{-1}$min$^{-1}$, respectively) and eight times greater for degradation of tigecycline ($k_{cat}/K_M$ values of 0.07 ± 0.01 and 0.01 ± 0.001 µM$^{-1}$min$^{-1}$, respectively; Fig. 3c, d). This difference in apparent catalytic efficiencies is largely mediated by increased substrate turnover by Tet(X7) compared with Tet(X), as $K_M$ values are of similar magnitude across all pairwise enzyme-substrate combinations. Indeed, the apparent rate constants ($k_{cat}$) for Tet(X7)-mediated degradation of tigecycline, eravacycline, and omadacycline are an order of magnitude greater than those observed for Tet(X) (Fig. 3c). We previously reported kinetic parameters for gut-derived Tet(X) and Tet(X7) (referred to as Tet(X)_3 in ref. 23) compared with soil-derived Tet(50) using first (tigecycline, chlorotetracycline, and demeclocycline) and second (oxytetracycline) generation tetracycline antibiotics and observed that Tet(50) and Tet(X7) displayed similar catalytic efficiencies for inactivating first and second-generation tetracyclines that were ~fivefold greater than Tet(X). However, in contrast to Tet(X) and Tet(X7), the soil-derived Tet(50) was unable to inactivate tigecycline, a third-generation tetracycline (Fig. 2)23. Here, we demonstrate that Tet(X7) encodes the improved catalytic efficiency of the soil-derived enzyme Tet(50) and additionally has an expanded substrate scope for inactivation of third-generation tetracyclines.

When enzymatic reactions using Tet(X) and Tet(X7) were analyzed by liquid chromatography-mass spectrometry, we observed the primary product of omadacycline, tigecycline, and...
eravacycline degradation is consistent with monohydroxylation (Supplementary Fig. 1b, c). For each latest-generation tetracycline substrate, there was a time- and enzyme-dependent decrease in the relative extracted ion counts of tetracycline substrate (m/z for [M + H]+ equal to 586, 557, 559 for tigecycline, eravacycline, and omadacycline, respectively), and a corresponding increase in the relative extracted ion counts of monooxygenated product (m/z for [M + H]+ equal to 602, 573, 575 for tigecycline, eravacycline, and omadacycline, respectively; Supplementary Fig. 1b). Enzyme-dependent antibiotic degradation was also confirmed by HPLC with detection by optical absorbance (Supplementary Fig. 1c). Tet(X) has previously been shown to monohydroxylate the C11a-position of tetracyclines13. We show that the monohydroxylated product of Tet(X) and Tet(X7) reaction with tigecycline coelute (Supplementary Fig. 2), indicating that the site of hydroxylation by Tet(X7) is also C11a.

Tet(X7) structurally resembles Tet(X). We solved an X-ray crystal structure of Tet(X7) at a resolution of 2.55 Å (Table 1), and observed a similar architecture to previously reported tetracycline inactivators12,35 with an FAD-binding Rossmann-type fold domain, a tetracycline-binding domain, and a C-terminal α-helix that bridges the two domains (Fig. 4a–c). Structurally, the enzyme resembles Tet(X) more so than the tetracycline-inactivating enzymes Tet(50,51,55,56) owing to the presence of a single C-terminal bridging helix, rather than the two observed in the soil-derived enzymes. We examined the structural differences between Tet(X7) and Tet(X) that could explain the enhanced phenotypic resistance of Tet(X7) against tigecycline and minocycline, despite ~86% amino-acid identity. Structure

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| Substrate | Enzyme | $K_m$ (μM) | $k_{app}$ (min⁻¹) | $k_{cat}/K_m$ (min⁻¹μM⁻¹) |
|-----------|--------|------------|-------------------|-------------------|
| Tigecycline | Tet(X) | 7 ± 0.5 | 0.08 ± 0.001 | 0.008 ± 0.001 |
|          | Tet(X7) | 4 ± 0.4 | 0.3 ± 0.01 | 0.07 ± 0.008 |
| Eravacycline | Tet(X) | 4 ± 0.4 | 0.05 ± 0.001 | 0.01 ± 0.002 |
|          | Tet(X7) | 6 ± 1 | 0.4 ± 0.003 | 0.07 ± 0.02 |
| Omadacycline | Tet(X) | 9 ± 0.7 | 0.04 ± 0.001 | 0.004 ± 0.0003 |
|          | Tet(X7) | 4 ± 0.4 | 0.2 ± 0.006 | 0.05 ± 0.007 |

**Table 1 Data collection and processing statistics for Tet(X7).**

| Data collection | Crystal 1 name |
|-----------------|----------------|
| Space group     | P 2₁2₁2₁      |
| Cell dimensions | $a$, $b$, $c$ (Å) | 57.130, 131.970, 136.970 |
|                 | $a$, $b$, $c$ (°) | 90.00, 90.00, 90.00 |
| Resolution (Å) | 19.77–2.55 (2.641–2.55) |
| Rfree           | 0.09138 (0.8967) |
|Completeness (%) | 99.50 (99.74) |
|Redundancy       | 6.9 (6.5) |
|Refinement       | 17.31 (2.26) |
|Resolution (Å)  | 19.77–2.55 |
|No. of reflections | 34,427 |
|Rwork/Rfree     | 0.2070/0.2436 |
|No. of atoms    | 5800 |
|Protein         | 106 |
|Ligand/ion      | 62 |
|Water           | 62 |
|B-factors       | 63.82 |
|Protein         | 49.63 |
|Ligand/ion      | 49.27 |
|Water           | 49.27 |
|R.m.s. deviations | 0.004 |
|Bond lengths (Å) | 0.72 |
alignment of Tet(X7) and Tet(X) (PDB ID: 2XDO, chain A) demonstrated a low overall root-mean-square deviation of 0.37 Å. The FAD in Tet(X7) retains an IN-conformation analogous to the FAD in Tet(X). This structural alignment of Tet(X7) and Tet(X) is supported by the low root-mean-square deviation values. The FAD-binding residues (Glu46, Gly57, Gly58, Pro318) and substrate-binding residues (Glu366, Pro318, Met375) are conserved between the two proteins (Fig. 4d, e). Furthermore, multiple sequence alignment revealed that these residues are conserved between all Tet(X)-like FMOs.

Fig. 4 Conserved architecture of different tetracycline-inactivating enzymes. Crystal structures of Tet(X7) (a), Tet(X) (b), and Tet(SO) (c). All three enzymes have a conserved FAD-binding Rossmann-fold (green), a substrate-binding domain (pink) and a bridge helix (purple). Tet(SO) has an additional helix (cyan). The bound FAD is represented spherically. d The Tet(X7) structure is aligned to tigecycline (TIG)-bound Tet(X) structure (PDB ID: 4A6N). Nonbonded interactions of the bound FAD (stick) are conserved between Tet(X7) (blue) and Tet(X). e The nonbonded interactions of tigecycline (TIG) are also conserved between the two proteins.

Tet(X7) is functional in a clinical P. aeruginosa isolate. While functional metagenomics is a useful method for identifying resistance genes in a sequence- and culture-unbiased manner, it is poorly equipped to associate specific resistance determinants with their native hosts. Thus, we sought to identify homologs of our set of functionally validated resistance genes in sequenced clinical isolates. We identified Pa-3, a Pseudomonas aeruginosa strain isolated from a 45-year-old male cystic fibrosis patient’s tracheal aspirate in a tertiary care hospital ICU in Pakistan in December 2016. This strain was predicted to encode an FAD-dependent oxidoreductase homolog by BLAST, which we identified as having 100% nucleotide identity to Tet(X7). We conducted antimicrobial susceptibility testing on this strain using disk diffusion for a panel of clinical antibiotics. Pa-3 was resistant to all antibiotics tested with the exception of piperacillin/tazobactam, a β-lactam/β-lactamase inhibitor combination, and colistin (Table 2). P. aeruginosa is a serious clinical threat, and acquisition of resistance to latest-generation tetracyclines further exacerbates this hazard.

In addition to antimicrobial susceptibility testing by disk diffusion, we performed broth microdilution for a subset of tetracycline antibiotics for which disks are not yet commercially available, against Pa-3 (the strain encoding Tet(X7)), Pa-8 (another clinical isolate from the same collection from Pakistan which did not encode Tet(X7)), and the P. aeruginosa type strain ATCC 27853 (also lacking Tet(X7)). Each P. aeruginosa isolate displayed nonsusceptibility to tigecycline, eravacycline, and omadacycline, even in the absence of a putative tetracycline inactivator (Table 3). This is likely due to drug efflux, as low-level OprM mediated resistance to tigecycline in P. aeruginosa PAO1 has been previously reported. Although tigecycline, eravacycline, and omadacycline breakpoints do not yet exist for P. aeruginosa, Pa-3 was four- to eightfold more non-susceptible to these drugs than Pa-8 and ATCC 27853. Our results with P. aeruginosa expand the repertoire of urgent-threat MDR clinical pathogens with demonstrated nonsusceptibility to either eravacycline or omadacycline via enzymatic inactivation. We anticipate clinical deployment of these antibiotics will lead to selection for and expansion of this mode of resistance in both hospital and community settings.

Media conditioned by Pa-3 supported the growth of susceptible E. coli, while media conditioned by 27853 or Pa-8 could not, confirming that the mechanism of resistance in Pa-3, but not Pa-8 or 27853, involves antibiotic inactivation. Clinical use of latest-generation tetracyclines may select for dissemination of this strain. Pseudomonas spp. have highly plastic genomes and undergo horizontal gene transfer at rates greater than observed
Anhydrotetracycline analogues rescue tetracycline efficacy. We have previously shown that anhydrotetracycline is an inhibitor of tetracycline-inactivating enzymes in vitro and that this inhibition is sufficient to rescue tetracycline efficacy against E. coli strains heterologously expressing tetracycline-inactivating enzyme\(^{12,23}\). We reasoned that this strategy might likewise prevent degradation of latest-generation tetracyclines by Tet(X7). To this end, we evaluated in vitro inhibitory activity of anhydrotetracycline and anhydrodemeclocycline against the Tet(X7)-mediated degradation of tigecycline, eravacycline, and omadacycline. Apparent half-maximal inhibitory concentrations (IC\(_{50}\)) showed that anhydrotetracycline potently inhibited Tet(X7) degradation of tigecycline, eravacycline, and omadacycline (IC\(_{50}\) of 1.06 ± 0.108 \(\mu\)M, 6.89 ± 0.655 \(\mu\)M, and 2.37 ± 0.510 \(\mu\)M, respectively). Moreover, anhydrodemeclocycline also potently inhibited the Tet(X7) degradation of these latest-generation substrates, with IC\(_{50}\) of 0.26 ± 0.04 \(\mu\)M, 2.75 ± 0.26 \(\mu\)M, and 0.31 ± 0.04 \(\mu\)M, respectively (Fig. 5–d). We reason, based on the structural similarity to previously published cocrystal structures of Tet(50) (PDB ID: 5TUF) with anhydrotetracycline, that the mechanism of inhibition likely occurs via mixed competitive and noncompetitive inhibition\(^{12}\).

In order to extend our in vitro studies, we sought to determine whether anhydrotetracycline or anhydrodemeclocycline could restore the activity of latest-generation tetracyclines against Pa-3, the clinical P. aeruginosa isolate that encodes Tet(X7). Anhydrotetracycline at 32 \(\mu\)g/mL caused a two- to fourfold increase in sensitivity of Pa-3 to tigecycline, eravacycline, or omadacycline (Table 3). In the same way, 8 \(\mu\)g/mL anhydrodemeclocycline was sufficient to cause a fourfold increase in sensitivity to tigecycline, and a twofold increase in sensitivity to eravacycline, eravacycline, or omadacycline (Table 3). Anhydrodemeclocycline exhibits an MIC of 16 \(\mu\)g/mL against both

for other genera\(^{11,12}\), so even chromosomally encoded genes are possible threats for transfer beyond this strain. Lastly, we observed that Tet(X7) was syntenic with putative rolling circle transposases (Pfam:PF04986), suggesting that they may be poised for horizontal transfer\(^{2,41}\).
E. coli + Tet(X7) and P. aeruginosa Pa-3 (Table 3). While anhydrodemeclocycline does exhibit antibiotic activity, we test for inhibition at concentrations below the minimum inhibitory concentration. We believe that the antibiotic activity of anhydrodemeclocycline is due to targeting of the cell membrane rather than inhibition of the 30S ribosomal subunit, based on prior investigations of anhydrotetracycline antibiotic activity. Our data indicate that the activity exhibited by anhydrotetracycline in our assay is due to inhibition of Tet(X7) rather than additive antibiotic activity. Thus, this adjuvant strategy has promise for restoring the efficacy of tetracycline antibiotics, including latest-generation tetracyclines, against bacteria that resist tetracycline through inactivation.

Discussion
Since their discovery from extracts of Streptomyces aureofaciens in 1948, the tetracyclines have become one of the most widely used classes of antibiotics in agriculture, aquaculture, and the clinic due to their broad antimicrobial spectrum, oral availability, and low cost. Extensive use over the past seven decades has selected for the expansion of tetracycline resistance in environmental microorganisms, human and animal commensals, and among bacterial pathogens. Tetracycline use is particularly prevalent in agriculture, with tetracyclines comprising 66% of total therapeutic antibiotic use in livestock. Widespread anthropogenic use has resulted in detectable ng/µL to µg/L quantities of tetracyclines in livestock manure and wastewater, with tetracycline concentration directly correlating with changes in microbial community composition and increase in antibiotic resistance. As a result of widespread anthropogenic use, tetracycline resistance is now widespread.

The rise in tetracycline resistance has been partially countered by the development of fully synthetic (tigecycline) latest-generation tetracyclines. The first-generation natural product antibiotics, including chlorotetracycline (1948), oxytetracycline (1950), and tetracycline (1953), were followed by the semisynthetic second-generation tetracyclines, including doxycycline (1967) and minocycline (1971), and latest-generation tetracyclines, such as tigecycline (2005), eravacycline (2018) and omadacycline (2018). Meeting clinical needs and overcoming established resistance mechanisms, such as efflux and ribosome protection, has motivated each new wave of tetracycline development. As a result, the tetracyclines are still widely used in agriculture and medicine, and remain viable therapies for a range of indications. The emergence of Tet(X) in clinical resistance mechanism. It is unclear if modification of the tetracycline scaffold will be sufficient to keep pace with the emergence of genes encoding tetracycline inactivation on the global scale.

Fig. 5 Anhydrotetracycline and analogues inhibit enzymatic inactivation of latest-generation tetracyclines by Tet(X7). a In vitro, anhydrotetracycline, anhydrochlortetracycline, and anhydrodemeclocycline inhibit Tet(X) modification of tigecycline. Likewise, anhydrotetracycline and anhydrodemeclocycline inhibit in vitro enzymatic modification of tigecycline (b), eravacycline (c), and omadacycline (d) by Tet(X7). Error bars represent standard deviation for three independent trials.

Until recently, Tet(X) was the only known enzyme capable of providing both tetracycline and tigecycline resistance, first identified in nonpathogenic bacteria. In 2013, tet(X) was discovered to be present and functional in human pathogens. Eleven isolates from the Enterobacteriaceae, Comamonadaceae, and Pseudomonadaceae families from clinical urine specimens in a hospital in Sierra Leone were found to encode tet(X). This resistance determinant has now been reported in four out of the six ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species), which are the leading causes of hospital-acquired infections around the world. The discovery of a gene encoding tetracycline inactivation in nosocomial pathogens foreshadowed the increasing clinical resistance to latest-generation tetracycline antibiotics, including the discovery of plasmid-encoded tet(X3) and tet(X4) in...
with tetracycline antibiotics. We provide evidence that anhydrotetracycline and analogs can inhibit broad-spectrum tetracycline-inactivating enzymes, including Tet(X) and Tet(X7) present in clinical pathogens, to rescue tetracycline activity in whole cells. Combination therapy appears to be the gold standard for managing resistance by antibiotic inactivation as supported by the steady development of β-lactam/β-lactamase inhibitor combinations. It is crucial to invest in the development of combination therapies before the isolated use of last-generation tetracyclines as stand-alone agents becomes functionally obsolete. This is of particular import given a recent report that tet(X3) compromises tigecycline efficacy in a mouse model of Acinetobacter baumanii thigh infection. Our data suggest that further exploration of the anhydrotetracycline scaffold as a source of novel inhibitors to rescue tetracycline activity in the face of widespread tetracycline resistance by inactivation is warranted.

While the environmental resistome has been shown to be extensive, unambiguous links between the environmental and clinical resistomes have remained elusive. Here, we explicitly demonstrate overlap between microbial habitats by identifying a family of genes conferring resistance to next-generation tetracycline-inactivating enzymes present in environmental, commensal, and pathogenic bacterial hosts. The results presented herein motivate continued surveillance of tetracycline resistance by inactivation in environmental and clinical settings to better understand the origins, evolution, and dissemination of resistance. This work underscores the value in proactive screening of antimicrobial agents in the pipeline for resistance determinants present in diverse microbial communities such that strategies to minimize their eventual clinical impact can be explored early in the development pipeline.

Methods
Identification of candidate tetracycline inactivators in sequenced functional selections. In order to identify additional tetracycline inactivators, we queried over 2000 sequenced functional selections of human gut, animal gut, larval, and soil metagenomes[22,25-28] (GenBank accession numbers: JX009202-JX009380, KJ691878-KJ696532, KU605810-KU608292, KF626669-KF630360, KX121504-KX128902, KU543693-KU549046). While the functional metagenomic protocol is described in detail in the papers accompanying these depositions, we briefly describe the method below:

Functional metagenomic library creation: Functional metagenomic libraries were constructed as previously described[14,38]. Approximately 5 μg of purified extracted total metagenomic DNA was used as a starting material for metagenomic library construction. To create small-insert metagenomic libraries, DNA was sheared to a target size of 3000 bp using the Covaris E210 sonicator following the manufacturer’s recommended settings. Sheared DNA was concentrated by QIAquick PCR Purification Kit (Qiagen) and eluted in 30 μl nuclease-free H2O. Then the purified DNA was size-selected on an agarose gel to a range of 1000–6000-bp DNA fragment through a premade 0.75% Pippin gel cassette. Size-selected DNA was then end-repaired using the End-It DNA End Repair kit (Epicenter). End-repaired DNA was then purified using the QIAquick PCR purification kit (Qiagen), and quantified using the Qubit fluorometer BR assay kit (Life Technologies) and ligated into the pZE21-MCS-1 vector at the HinII site. The pZE21 vector was linearized at the HINCI site using inverse PCR with PFX DNA polymerase (Life Technologies). End-repaired metagenomic DNA and linearized vector were ligated together using the Fast-Link Ligation Kit (Epicenter) at a 5:1 ratio of insert:vector. After heat inactivation, ligation reactions were dialyzed for 30 min using a 0.025-μm cellulose membrane (Millipore membrane number VSWP0925), and the full reaction volume used for transformation by electroporation into 25 μl E. coli MegaX (Invitrogen) according to the manufacturer’s recommended protocols. Cells were recovered in 1 ml recovery medium (Invitrogen) at 37 °C for 1 h. Recovered cells were inoculated into 50 ml of LB containing 50 μg/ml kanamycin, and grown overnight. The overnight culture was frozen with 15% glycerol and stored at −80 °C for subsequent screening.

Antibiotic selection of functional metagenomic libraries: Each metagenomic library was selected for resistance at a concentration of 8 μg/ml tetracycline or 2 μg/ml anhydrotetracycline plus 50 μg/ml kanamycin for plasmid library maintenance on Mueller Hinton agar. After plating (using sterile glass beads), antibiotic selections were incubated at 37 °C for 18 h to allow the growth of clones containing an antibiotic-resistance-conferring DNA insert. After overnight growth, all colonies from a single antibiotic plate (library by antibiotic selection) were collected by a single antibiotic plate (library by antibiotic selection) were collected by picking with a sterile toothpick and inoculating the antibiotic plate with 1 μl of the original metagenomic library DNA solution. After incubation at 37 °C for 18 h, individual colonies were picked and inoculated into 5 ml of M9 medium with appropriate antibiotic selection using a sterile toothpick. After 

M. D. R. Enterobacteriaceae and Acinetobacter spp[14,15], along with chromosomal tet(X7) in Pseudomonas aeruginosa (this work). It is now clear that future dependence on new tetracycline antibiotics including tigecycline, eravacycline, and omadacycline to treat infections caused by MDR Gram-negative pathogens may be compromised by widespread tetracycline-inactivating enzymes. Expanded global use of last-generation tetracyclines in clinical and agricultural settings will increase selective pressure for tetracycline-inactivating genes and promote dissemination. Prospecting for environmental and clinical tet(X) homologous genes is a promising method to proactively assess the landscape of tetracycline resistance via chemical inactivation. Our work has now revealed that tetracycline inactivation occurs widely across Gram-negative bacteria[29]. The related tetracycline-inactivating gene originally discovered in human commensal Bacteroides fragilis has been acquired by human pathogenic Gram-negative bacteria[29]. The related tetracycline-inactivating enzyme is now clear that future dependence on new tetracycline antibiotics. Since FAD-dependent oxidoreductases are ubiquitous, and there is low sequence similarity between the tetracycline-inactivating enzymes and tet(X) family of genes, functional selection is critical to properly survey the resistance landscape. Expanding the pool of functionally similar resistance determinants is the first step in deconvoluting the evolutionary link between soil tetracycline-inactivating enzymes and Tet(X) homologs. Further structural and biochemical characterization of tetracycline-inactivating enzyme sub-classes is critical for understanding the molecular basis for antibiotic inactivation and finding functional connections in sequence evolution that will feed predictive models. For now, Tet(X) homologs appear to be a more likely clinical threat than the soil tetracycline-inactivating enzymes, but ignorance to the full reservoir of tetracycline-inactivating genes blunts clinicians and drug developers to the most urgent needs in therapeutic advances. The observance of human-associated genes which confer resistance to next-generation tetracyclines likely reflects the promiscuous nature of the FMO family, which is highly adaptable to a wide range of substrates. Specific selection for tetracycline substrates in human versus environmental habitats most likely arose from natural production of these types of tetracycline scaffolds in their respective microbial communities, since at least the human-associated enzymes clearly existed before the latest-generation drugs were clinically deployed. Directed evolution can be useful to fill some of the gaps in these known unknowns, as has been demonstrated for other clinically important resistance determinants (e.g., beta-lactamases)[34]. An ideal goal is to predict phenotypic resistance by sequence alone and employ preemptive strategies for managing resistance early in development instead of waiting for an increase in clinical resistance events[35].

Better understanding the molecular recognition of tetracycline substrate recognition by tetracycline-inactivating enzymes can help design the next generation of structurally modified tetracyclines that can stealthily evade inactivation or maintain efficacy after a catalyzed oxidation event. More structural information dictating enzyme function and substrate recognition, such as the 2.55 Å resolution structure of Tet(X7) reported here, is needed to guide the rational design of next-generation tetracyclines capable of evading enzymatic inactivation. Recent advancements in the total chemical synthesis of tetracyclines, such as eravacycline[36], can play a critical role in providing synthetic access to rationally designed compounds. Structure-based methods can also play a key role in designed inhibitors for use in combination therapies

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ARTICLE
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assay kit (Life Technologies).

BioLabs). The amplification
nm (OD600) at 45-min intervals using the Synergy H1 microplate reader (Biotek)

Antimicrobial susceptibility testing for Pseudomonas aeruginosa. Susceptibility
testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Hardy Diagnostics) in accordance with CLSI standards.  

Pseudomonas aeruginosa genomic isolation. A suspension of ~10 colonies from a blood agar plate was used for genomic DNA isolation with the QIAamp Blood Kit (Qiagen). The DNA isolation was performed as per the manufacturer’s protocols. Genomic DNA was quantified using a Qubit fluorometer dsDNA BR Assay (Invitrogen) and stored at ~20 °C.

Pseudomonas aeruginosa isolate sequencing library preparation. Genomic DNA was diluted to a concentration of 0.5 ng/µL prior to sequencing library preparation. Libraries were prepared using a Nextera DNA Library Prep Kit (Illumina) following the modifications described in Baym et al.58. Libraries were purified using the Agencourt AMPure XP system (Beckman Coulter), and quantified using the Quant-IT PicoGreen dsDNA assay (Invitrogen). Samples were submitted for 2 × 150 bp paired-end sequencing on an Illumina NexisQ High-Output platform at the Center for Genome Sciences and Systems Biology at Washington University in St. Louis with a target sequencing depth of 1 million reads per sample.

Antimicrobial susceptibility testing for Pseudomonas aeruginosa. Susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Hardy Diagnostics) in accordance with CLSI standards. Pseudomonas aeruginosa ATCC 27853 was used as a quality control.

Cloning, expression and purification of tetracycline-inactivating enzymes. All genes encoding tetracycline-inactivating enzymes were cloned into the pET28(b+) vector (Novagen) at BamHI and restriction sites were introduced by digestion of the plasmid and re-ligation. The expression plasmids were transformed into BL21 Star (DE3) competent cells (Life Technologies). Cells harboring the plasmid were grown at 37 °C in LB medium containing a final concentration of 0.03 mg/mL kanamycin. Once cells reached an OD600 of 0.6, cells were cooled to 15 °C, induced with 1 mM IPTG overnight. After this period, cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C. Cell pellets were suspended in 10 mL of 50 mM Tris (pH 8.0), 100 mM NaCl, 10 mM imidazole (pH 8.0), 1 mM PMSF, and 5 mM BME per 1 liter of LB medium and stored at ~80 °C.

Cells were lysed by mechanical disruption using an Emulsiflex C5. The cell extract was obtained by centrifugation at 13,000 rpm for 30 min at 4 °C, and was applied onto nickel fast run agarose beads (GoldBio) equilibrated with wash buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 20 mM imidazole (pH 8.0), and 5 mM BME). The wash buffer was used to wash the nickel column three times with five column volumes. After washing, protein was eluted with five column volumes of elution buffer (wash buffer with 300 mM imidazole). The protein sample was further purified by gel chromatography using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) equilibrated with 10 mM Tris (pH 8.0), 150 mM NaCl, 5 mM diethylolether (DTE). The fractions containing the protein of interest were pooled and concentrated using a 30 K MWCO Amicon centrifugal filter (Millipore).

Cryocrystalization, data collection, and structure refinement. Tet(II) was
concentrated to 20 mg/mL, and crystallized by hanging drop vapor diffusion at 18 °C in 0.2 M ammonium sulfate and 20% (w/v) PEG 4000. Crystals were transferred to 20% ammonium sulfate, 20% (w/v) PEG 4000, and 20% glycerol for 15–30 s and flash-cooled in the liquid nitrogen. Diffraction data were collected at 100 K on beamline 4.2.2 (Advanced Light Source, Lawrence Berkeley National Laboratory in Berkeley, California). All data processing and structure analysis were performed using SBGrid70. Diffraction data was reduced and scaled using XDS17. Tet(II) structure was solved by molecular replacement using Phaser for the substrate-
free Tet(X) structure (PDB ID: 2XDO; percentage identity: ~86%) as a starting model. Structure refinement was performed in Phenix21 and Coot24. The final model was validated using the Molprobity server25.

In vitro tetracycline-inactivation assays. In vitro reactions were performed as previously described22. Reactions were prepared in 100 mM TAPS buffer with 28.0 mM substrate, 0.24 µM enzyme, and an NADPH regeneration system consisting of the following components (final concentrations): glucose-6-phosphate (40 mM), NADP+ (4 mM), MgCl2 (1 mM), and glucose-6-phosphate dehydrogenase (4 U/ml). The regeneration system was incubated at 37 °C for 30 min to generate NADPH before use in reactions. Reactions were sampled at various time-points over a 2-h period, and 150-µL samples were transferred from the reaction vessel and quenched in four volumes of an acidic quencher consisting of equal parts of acetonitrile and 0.25 M HCl.

Products generated from the Tet(X7) enzymatic inactivation of the last-generation tetracyclines were separated by reverse-phase HPLC using a Phenomenex Luna C18 column (5 µm, 110 Å, 2×50 mm) and 0.1% triethylamine in water (A) and acetonitrile (B) as mobile phase with optical absorbance detected at 210 nm. Reactions were prepared in triplicate. The final concentrations for assay components were 100 mM TAPS buffer, 0.4 µM enzyme, 0.4 µM NADPH, 40 µM substrate, 0.24 µM enzyme, and an NADPH regenerating system consisting of equal parts of acetonitrile and 0.25 M HCl.

In vitro characterization of tetracycline inactivation. Reactions were performed in 100 mM TAPS buffer at pH 8.5 with 40-µM substrate, 0.54 µM NADPH, 5.04 mM MgCl2, and 0.4 mM enzyme. UV-visible spectroscopy measurements were taken in duplicate at 400-nm wavelength light with a Cary 60 UV/Vis system–detected to 450 nm. After the addition of enzyme, 150-µL samples were transferred from the reaction vessel and quenched in four volumes of an acidic quencher consisting of equal parts of acetonitrile and 0.25 M HCl.

Statistics and reproducibility. Biochemical assays were performed in triplicate at a minimum. Statistical analysis for in vitro enzyme kinetics and inhibition assays were done using GraphPad Prism 6. All error bars shown depict standard error for three independent trials.

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Author contributions
A.J.G., G.D., T.A.W., and N.H.T. conceived and designed the study. A.J.G. performed bioinformatics analyses. A.J.G. and B.W. subcloned tetracycline inactivators and performed susceptibility testing by broth microdilution. J.L.M., L.F., and C.T.S. purified enzymes and performed biochemical assays. H.K. crystallized Tet(X7), collected the data, and solved structure. S.I. and S.A. collected clinical sample and cultured P. aeruginosa clinical isolate. M.W. and C.-A.D.B. performed P. aeruginosa phenotyping by disk diffusion. A.J.G. wrote the paper with input from all authors.

Competing interests
The authors declare no competing interests.

Additional information
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