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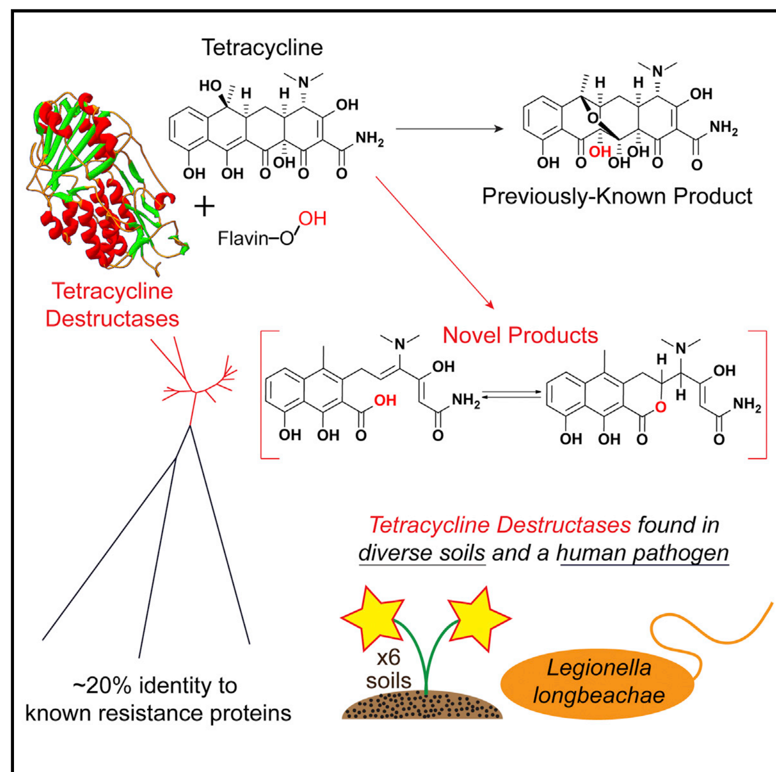
The Tetracycline Destructases: A Novel Family of Tetracycline-Inactivating Enzymes

Graphical Abstract



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In Brief

Forsberg et al. describe flavoenzymes that inactivate tetracycline antibiotics by previously known and undescribed oxidative mechanisms. These enzymes have no sequence homology to known resistance genes, inactivate tetracycline in *Escherichia coli* and when purified, and have a homolog in the human pathogen *Legionella longbeachae*.

Highlights

- Nine tetracycline-inactivating flavoenzymes are described from diverse soils
- The enzymes inactivate tetracycline when expressed in *E. coli* and when purified
- The enzymes exhibit both known and undescribed tetracycline oxidative activity
- A functional homolog exists in the human pathogen *Legionella longbeachae*

Accession Numbers

KR857681–KR857689

Forsberg et al., 2015, Chemistry & Biology *22*, 888–897
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http://dx.doi.org/10.1016/j.chembiol.2015.05.017

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The Tetracycline Destructases: A Novel Family of Tetracycline-Inactivating Enzymes

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http://dx.doi.org/10.1016/j.chembiol.2015.05.017

SUMMARY

Enzymes capable of inactivating tetracycline are paradoxically rare compared with enzymes that inactivate other natural-product antibiotics. We describe a family of flavoenzymes, previously unrecognizable as resistance genes, which are capable of degrading tetracycline antibiotics. From soil functional metagenomic selections, we discovered nine genes that confer high-level tetracycline resistance by enzymatic inactivation. We also demonstrate that a tenth enzyme, an uncharacterized homolog in the human pathogen *Legionella longbeachae*, similarly inactivates tetracycline. These enzymes catalyze the oxidation of tetracyclines in vitro both by known mechanisms and via previously undescribed activity. Tetracycline-inactivation genes were identified in diverse soil types, encompass substantial sequence diversity, and are adjacent to genes implicated in horizontal gene transfer. Because tetracycline inactivation is scarcely observed in hospitals, these enzymes may fill an empty niche in pathogenic organisms, and should therefore be monitored for their dissemination potential into the clinic.

INTRODUCTION

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 (Walsh, 2003; Thaker et al., 2010). Tetracyclines are polyketide natural products of actinomycete secondary metabolism, and have likely existed in the environment for millions of years (Baltz, 2007). Accordingly, tetracycline resistance is expected to be an ancient feature of environmental bacteria (D'Costa et al., 2011).

Intensive clinical and agricultural use over the past 65 years has selected for the expansion of tetracycline resistance in environmental microorganisms (Knapp et al., 2010), human and animal commensals (Johnson and Adams, 1992), and among bacterial pathogens (Chopra and Roberts, 2001). In the case of human pathogens, tetracycline resistance is typically acquired via horizontal gene transfer and occurs almost exclusively by ribosomal protection or antibiotic efflux (Thaker et al., 2010; Chopra and Roberts, 2001). Both of these resistance mechanisms have their evolutionary origins in the environment (Aminov and Mackie, 2007; Allen et al., 2010; Davies and Davies, 2010), but are now found widely distributed in many commensal and pathogenic bacteria (Gibson et al., 2015; Moore et al., 2013; Forsberg et al., 2012).

Ribosomal protection and drug efflux do not affect the concentration or activity of the tetracycline molecule itself, a feature that distinguishes clinical tetracycline resistance from that of the natural-product aminoglycoside, amphenicol, and β-lactam antibiotics, which are typically inactivated enzymatically (Walsh, 2003). Bacteria expressing drug-inactivating enzymes need act only once on a substrate to eliminate toxicity, rather than requiring continual activity in the presence of a drug for survival (Walsh, 2000). Despite this apparent advantage, only three genes have ever been reported to inactivate tetracycline (Nonaka and Suzuki, 2002; Diaz-Torres et al., 2003; Park and Levy, 1988; Speer and Salyers, 1988), and only one enzyme, Tet(X), has been confirmed for activity in vitro (Yang et al., 2004; Moore et al., 2005; Volkers et al., 2011). These efforts have demonstrated that Tet(X) is a flavoprotein monooxygenase that inactivates tetracycline antibiotics by monohydroxylation followed by spontaneous, non-enzymatic breakdown (Yang et al., 2004; Moore et al., 2005; Volkers et al., 2011).

The only report of human pathogens with the potential to inactivate tetracycline occurred in 2013; 11 isolates from urinary tract infections in Sierra Leone were positive for *tet*(X) (Leski et al., 2013). At present, tetracycline inactivation is rarely detected in environmental metagenomes (Thaker et al., 2010; Nesme et al., 2014), indicating that it is either a truly rare function or occurs via the activity of cryptic genes, unrecognizable as resistance-conferring based on sequence composition. Because other natural-product antibiotics are frequently inactivated enzymatically (Walsh, 2000, 2003; Davies, 1994), we hypothesize that tetracycline inactivation may also be widespread, but underestimated using standard methods for resistance gene prediction from metagenomic sources.

Flavoenzymes, which include monooxygenases such as Tet(X), are common in nature and catalyze an enormous range of chemical transformations, including multiple modifications of aromatic polyketides (Walsh and Wencewicz, 2013; Aminov, 2009). Their sequence diversity makes a priori functional

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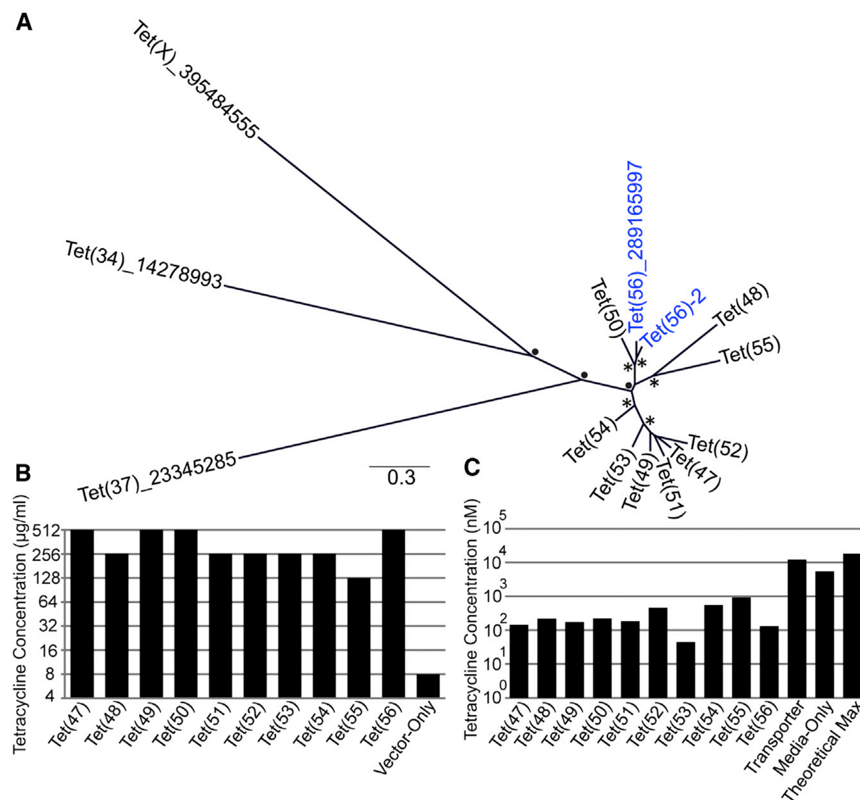

Figure 1. Tetracycline-Inactivating Proteins
(A) Ten proteins derived from soil metagenomes and four tetracycline-inactivating proteins from NCBI. Numbers following NCBI sequences indicate GenBank identifiers. Tet(56) was cloned from *Legionella longbeachae*. Asterisks denote nodes with Shimodaira-Hasegawa-like branch supports >0.95, and circles denote nodes with support >0.7. Blue labels indicate proteins with 86% amino acid identity to one another. The scale bar represents the number of substitutions per site.
(B) The minimum inhibitory concentrations of *E. coli* heterologously expressing the indicated proteins.
(C) Absolute tetracycline levels in medium conditioned by *E. coli* strains expressing the designated proteins. "Theoretical Max" indicates the initial tetracycline concentration in the medium prior to inoculation.

prediction a challenge, while their proclivity for horizontal gene transfer and gene duplication allow for facile acquisition of new function and hinder efforts to accurately measure prevalence (Walsh and Wencewicz, 2013; Aminov, 2009). Therefore, these enzymes may be undersampled relative to many other bacterial functions, and represent a potential source of undiscovered antibiotic-inactivating enzymes. Here, we describe the functional discovery and biochemical characterization of a novel family of tetracycline-inactivating flavoenzymes, previously unrecognizable as antibiotic resistance genes by primary sequence.

RESULTS

A Family of Novel Tetracycline Resistance Genes

We identified a family of putative flavin adenine dinucleotide (FAD)-dependent monooxygenases through functional metagenomic selections for tetracycline resistance from 18 grassland and agricultural soils (Forsberg et al., 2014). Of the ten full-length open reading frames (ORFs) predicted (Table S1), nine were subcloned into an *Escherichia coli* expression system (Table S2) and confirmed to provide tetracycline resistance at concentrations up to 256 μg/ml, 64-fold greater than that of an empty-vector control (Figure 1B). All ORFs conferred comparably high levels of resistance toward oxytetracycline, two conferred moderate resistance to minocycline, and none were active against tigecycline (Table 1). The novel tetracycline resistance proteins share moderate similarity with one another (average pairwise amino acid identity 65.4% ± 9.2%) but have primary sequence unlike any previously identified tetracycline-inactivation protein (Figure 1A; amino acid identity to Tet(X) is at most 24.4%). Homology modeling of these enzyme sequences predicts structural similarity to many flavin-dependent oxidoreductases, and indicates functional homology with Tet(X) (Volkers et al., 2011; Kelley and Sternberg, 2009). Therefore, we hypothesized that these genes encode tetracycline-inactivating flavoenzymes due to (1) structural similarity to Tet(X), (2) their putative FAD-binding and oxidoreductase function (Diaz-Torres et al., 2003; Yang et al., 2004), and (3) the capacity of *E. coli* transformants to darken growth medium upon tetracycline addition (Figure 2), consistent with previous observations for Tet(X) (Yang et al., 2004).

The Novel Genes Inactivate Tetracycline in *Escherichia coli*

We tested the capacity for each protein to inactivate tetracycline in *E. coli*. We grew strains of *E. coli* expressing each enzyme in tetracycline-containing medium and subsequently monitored the growth of a tetracycline-susceptible control strain in conditioned supernatants. Unconditioned medium, or medium conditioned with *E. coli* expressing a transporter that confers non-enzymatic tetracycline resistance, did not support the growth of the tetracycline-susceptible control strain. Medium conditioned by every putative flavoenzyme permitted robust growth of the susceptible strain, indicating that the expressed ORFs inactivate tetracycline (Figure S1). Confirming this observation, absolute quantification of tetracycline by liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed concentrations of the antibiotic up to 270-fold lower in medium incubated with *E. coli* expressing a putative inactivating protein compared with medium conditioned with *E. coli* expressing the transporter (Figure 1C).

Of the nine confirmed tetracycline-inactivating proteins, five were from agricultural soils and four from grassland soils (Forsberg et al., 2014), suggesting that this antibiotic resistance gene family is distributed across diverse soil types. Only two recognizable sequence homologs exist in NCBI_nr, both of which are predicted oxidoreductases and neither have been

Table 1. Activity Profiles of Various Flavoenzymes Used in this Study

		MIC Conferred to *E. coli* (μg/ml)				In Vitro Enzyme Activity			
Enzyme	Source	Versus Tetracycline	Versus Oxytetracycline	Versus Minocycline	Versus Tigecycline	Activity Versus TET/OX	Activity Versus aTC	Production of *m/z* = 387	Production of *m/z* = 461
Tet(47)	Soil S08	**512**	**>512**	8	2	ND	ND	ND	ND
Tet(48)	Soil S08	**256**	**512**	8	2	No	No	+/−	+/−
Tet(49)	Soil S11	**512**	**>512**	8	2	Yes	No	++	+/−
Tet(50)	Soil S11	**512**	**256**	8	2	Yes	No	++	+
Tet(51)	Soil S14	**256**	**512**	8	2	Yes	No	++	+++
Tet(52)	Soil S14	**512**	**256**	8	2	Yes	No	+++	+
Tet(53)	Soil S15	**512**	**256**	**32**	2	ND	ND	ND	ND
Tet(54)	Soil S19	**256**	**512**	8	2	Yes	No	+	+/−
Tet(55)	Soil S20	**128**	**256**	**16**	2	Yes	No	++	++
Tet(56)	*Legionella longbeachae*	**256**	**256**	8	2	Yes	No	++	++
Tet(X)	NA	**256**	**256**	**32**	**8**	Yes	Yes	++	+++
Empty vector	NA	8	32	8	2	NA	NA	NA	NA

Values in bold and underlined signify antibiotic resistance.
Source soils identified as in Forsberg et al. (2014).
The number of (+) symbols correspond to the amount of the indicated product detected from enzymatic tetracycline decay, as per Figure S4. (+/−) indicates that only trace amounts of product were detected.
aTC, anhydrotetracycline; MIC, minimum inhibitory concentration; NA, not applicable; ND, not determined; OX, oxytetracycline; TET, tetracycline.

implicated in tetracycline resistance. Both homologs were found in soil-dwelling bacteria, one in *Rhizobacter* sp. (GenBank: 646785269) and a second in *Legionella longbeachae* (GenBank: 289165997), a causal agent of the potentially fatal Pontiac fever and Legionaires' disease (Whiley and Bentham, 2011; Cazalet et al., 2010). The homolog from *L. longbeachae* conferred tetracycline resistance to *E. coli* at levels 64-fold higher than an empty-vector control and also functioned via drug inactivation (Figures 1, 2, and S1).

Enzymes Inactivate Tetracycline In Vitro

Each putative tetracycline-inactivating enzyme was purified as previously described (Yang et al., 2004). When assayed by UV-visible spectroscopy, seven of the eight purified flavoenzymes appeared to degrade tetracycline with a dependence on time, enzyme, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 3). In assay conditions similar to those in previous work (Yang et al., 2004; Moore et al., 2005), we show reduction in absorbance at 363 nm with reactions using (1) six of the seven purified soil-derived inactivating enzymes, (2) the homolog from *L. longbeachae*, and (3) Tet(X) as a positive control, indicating disruption of the β-diketone chromophore of tetracycline (Yang et al., 2004; Moore et al., 2005; Volkers et al., 2011). This spectral change is not observed for reactions performed with a vector-only control for co-purified native *E. coli* protein, or for those lacking enzyme (Figures 3J and 3K). An NADPH regenerating system was used in reactions. Although the absorbance spectrum of NADPH overlaps with that of tetracycline (Yang et al., 2004; Moore et al., 2005), it does not change with time (Figure 3L), and thus does not contribute to the observed reduction in absorbance at 363 nm. The decrease in absorbance at 400 nm further indicates tetracycline loss, since tetracyclines, but not NADPH, absorb at this wavelength.

Tetracycline Inactivation Proceeds via Diverse Mechanisms

The progression of each reaction was also monitored by reverse-phase high-performance liquid chromatography (HPLC) (Figures 4, S2, and S3). The tetracycline substrate disappeared with time in all but one enzyme-catalyzed experiment. In some cases tetracycline was replaced by new product peaks (e.g. Figures 4B–4D) and in others the substrate was eliminated without obvious signatures of new, stable products (e.g. Figure 4A). When these reactions were analyzed by LC-MS, the major new product gave an *m/z* value of 461 (compound **1**), equivalent to the addition of oxygen to tetracycline (*m/z* for $[M + H]^+$ equals 445 in positive ion mode). Relative proportions of ion counts for the tetracycline substrate and compound **1** were routinely mirrored by the relative heights of the corresponding HPLC peaks over time (Figures 4, S2–S4), further suggesting that the monooxygenation of tetracycline is a direct result of flavoenzyme activity. This mechanism is consistent with the reported activity of Tet(X) (Yang et al., 2004; Moore et al., 2005; Volkers et al., 2011) and with our empirical observations using the enzyme (e.g. Figures 4D and 4I). In cases where no stable product was observed by HPLC (e.g. Figure 4A), no ion with an *m/z* value of 461 was observed (e.g. Figure 4F) despite the obvious disappearance of tetracycline. This indicates that some flavoenzymes may degrade tetracycline using mechanisms different from Tet(X). When tested for activity against anhydrotetracycline (the final precursor in tetracycline biosynthesis), Tet(X), but none of the discovered flavoenzymes, could oxidize the substrate

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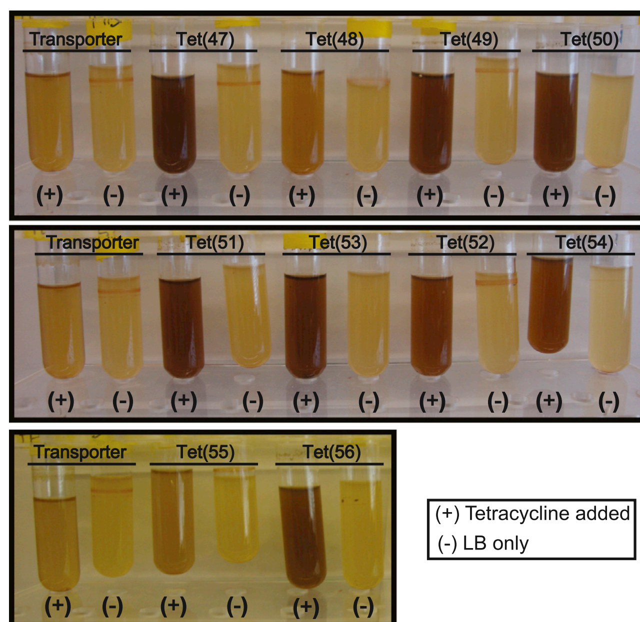

Figure 2. Expression of Tetracycline-Inactivating Genes Darkens Tetracycline-Containing Growth Media

E. coli transformants expressing either a tetracycline-resistant transporter or the indicated tetracycline-inactivating protein were grown in Luria-Bertani broth at 37°C for 4 days, protected from light. The same cultures expressing the tetracycline-resistant transporter are used across each image. Tetracycline was added at 100 μg/ml except for Tet(55); 32 μg/ml tetracycline was added to this sample due to a lower degree of tetracycline resistance conferred by this enzyme.

(Figure 5), reinforcing observations that these enzymes can act distinctly from Tet(X).

In addition to the appearance of a monooxygenated tetracycline, enzymatic reactions showed the appearance of a structure with an *m/z* value of 387 (compound **2**) as the reaction progressed (Figures 4, S4, and S5). Similarly to the monooxygenated product, this ion often replaced the tetracycline substrate over time but did not readily absorb light at the wavelengths examined. We propose two putative mechanisms whereby a product with this mass may accumulate via either a Baeyer-Villiger ring expansion or a Grob fragmentation of tetracycline, producing an unstable oxidized product that rapidly decomposes to compounds **2a** and **2b** (Figure S6). High-resolution tandem MS of tetracycline degradation reactions produce fragmentation patterns that are consistent with our proposed structures for compounds **2a**/**2b** and with the ions observed by LC-MS (Figures 6 and S5). The relative abundances of the major enzymatic products (*m/z* = 461, compound **1** and *m/z* = 387, compounds **2a**/**2b**) were dependent on enzyme, and consistent across experiments and analytical methods (Figures 4, S4, S5; Supplemental Dataset 1). Consistent enzymatic preference for production of compound **1** versus compounds **2a**/**2b** indicates that these flavoenzymes can differ in their favored mechanism of oxidizing tetracycline (Table 1).

Purified flavoenzymes were also examined for their ability to degrade the substrate oxytetracycline (Figures S2, S3, S7). Tet(X) hydroxylated oxytetracycline, as previously described (Yang et al., 2004). Similar to the results achieved using tetracycline, the flavoenzymes from soil and *L. longbeachae* appeared to degrade oxytetracycline into diverse oxidation products, some consistent with the known activity of Tet(X) and others via potentially different oxidation mechanisms. Tetracyclines ultimately degrade into a heterogeneous mixture of poorly defined (and perhaps polymeric) structures via abiotic and, in the case of Tet(X), enzymatic processes (Yang et al., 2004; Chen and Huang, 2011; Jeong et al., 2010; Palmer et al., 2010). Our results are consistent with these observations. Reverse-phase HPLC using tetracycline and oxytetracycline antibiotics showed the disappearance of characteristic substrate absorbances at 260 and 363 nm, indicating that conjugation in both the β-tricarbonyl and aryl β-diketone chromophores was disrupted (Figures S2 and S3). In addition, HPLC and MS analyses reveal a heterogeneous product mixture at late time points (Figures 4, S2, and S3), consistent with the observation of many small peaks in the chromatograms and molecular ion features with diverse *m/z* values at low to moderate abundance (Supplemental Dataset 1).

DISCUSSION

Widespread tetracycline use has fueled concerns that tetracycline residues and their decay products may persist in milk (levels measured at >2 mg/l [de Albuquerque Fernandes et al., 2014]), wastewater (Novo et al., 2013), the food supply (Silbergeld et al., 2008), and agricultural settings (Zhu et al., 2013). Tetracycline use is particularly heavy in livestock rearing (Zhu et al., 2013), with estimates that tetracyclines account for two-thirds of total therapeutic antibiotic use in animals (Ungemach et al., 2006). Tetracycline concentrations in wastewater (Novo et al., 2013) and livestock manure (Zhu et al., 2013) directly correlate with shifts in microbial community composition and increases in antibiotic resistance. The increased recognition of tetracycline as a pollutant (Chen and Huang, 2011) has sparked renewed interest in understanding its decay processes for predicting persistence in contaminated environments (Aga et al., 2005) and for use in water treatment facilities (Jeong et al., 2010). Our results highlight the diverse repertoire of tetracycline-degrading enzymes relevant to these efforts, likely stemming from long-standing evolutionary processes reflective of the intimate role of tetracycline in soil microbial ecology (Thaker et al., 2010; Baltz, 2007; D'Costa et al., 2011; Chopra and Roberts, 2001; Bassett et al., 1980).

Screening environmental isolates for bioactive secondary metabolites detects tetracycline production from approximately 1 in 1,000 actinomycetes (Baltz, 2007). Biosynthesis of tetracycline is ancient (Bassett et al., 1980), resulting in numerous enzymes capable of modifying tetracycline scaffolds (Pickens and Tang, 2009; D'Costa et al., 2006) while selecting for diverse resistance genes over long time spans (D'Costa et al., 2011) and across many habitats (Thaker et al., 2010; Chopra and Roberts, 2001; Roberts, 2012). Because antibiotics and their decay products can affect microbial communities in complex manners (e.g. tetracycline degradation selects for drug sensitivity [Palmer et al., 2010] and antibiotics can act as signaling molecules [Davies and Davies, 2010; Yim et al., 2007]), environmental selection for tetracycline degradation likely includes processes both related to, and independent of, the drug's role in microbial

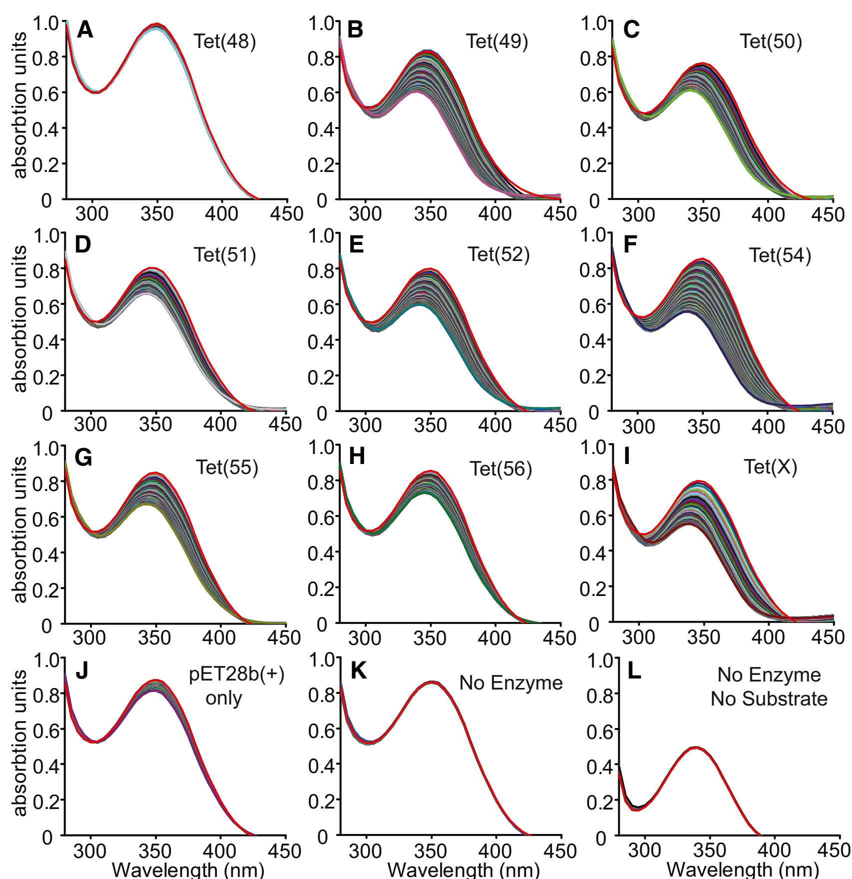

Figure 3. UV-Visible Spectrum of Enzymatic Tetracycline Degradation

(A–L) Each panel shows the degradation of tetracycline over the course of 3 hr, in a reaction containing the indicated purified enzyme (or control), tetracycline, and an NADPH regeneration system. Absorbance scans were taken at 1-min intervals. The rainbow pattern depicts a spectral change over time; absorbance at 360 or 400 nm always decreased with time. See also Figure S7.

warfare. Despite these myriad pressures for tetracycline decay, enzymes that inactivate tetracycline are rarely identified (Nonaka and Suzuki, 2002; Diaz-Torres et al., 2003; Park and Levy, 1988; Speer and Salyers, 1988). In contrast, enzymes that inactivate other natural-product antibiotics are common (Walsh, 2000, 2003). Our discovery of a novel family of tetracycline-inactivating enzymes helps to address this disparity, and suggests that enzymatic tetracycline inactivation may be a feature of natural soil habitats more widely distributed than previously recognized. Examining diverse soil metagenomes will likely uncover more tetracycline-inactivating enzymes, including additional flavin monooxygenases and, perhaps, enzymes of undescribed activity (e.g. dioxygenases, which, like monooxygenases, can oxidize polyketides and are important in the biosynthesis of tetracycline's ring structure [Zhang et al., 2008; Wang et al., 2009]).

Coincident with anthropogenic antibiotic use, resistance gene abundances have increased in environmental, commensal, and pathogenic bacteria, often disseminating through microbial populations via horizontal gene transfer (Knapp et al., 2010; Johnson and Adams, 1992; Chopra and Roberts, 2001). Relative to the staggering diversity of antibiotic resistance genes in the environment (Allen et al., 2010; Nesme et al., 2014; Forsberg et al., 2014; Allen et al., 2009; Pehrsson et al., 2013), resistance in human pathogens is typically encoded by a much smaller pool of circulating resistance genotypes (Davies and Davies, 2010; Roberts, 2012; Jacoby and Munoz-Price, 2005). The clinical resistome may be driven by the initial stochastic acquisition of resistance genes, followed by antibiotic-induced selection pressure for clonal expansion and subsequent diversification (Canton and Coque, 2006; Woodford and Ellington, 2007). This leads to a "founder-effect" scenario: once a particular resistance mechanism is widely distributed within pathogenic bacteria, a gene conferring similar function in the environment is less likely to outcompete established pathogen resistance genes for the same niche (Martinez et al., 2015). In contrast, resistance genes that can confer a fitness advantage by targeting an unfilled niche (e.g. novel activity or expanded substrate spectrum) represent larger threats for entering the circulating pathogenic resistome (Canton and Coque, 2006; Munoz-Price et al., 2013; Dortet et al., 2014).

By this reasoning, enzymes that inactivate tetracycline antibiotics may present clinical concern: drug inactivation is a preferred mechanism of pathogens to resist many antibiotics (Walsh, 2003; Davies, 1994), which is thus far scarcely observed for tetracycline. The success in clinical trials of eravacycline, a broad-spectrum fluorocycline minimally affected by tetracycline efflux or ribosomal protection resistance mechanisms (Sutcliffe et al., 2013), reinforces the importance of surveillance for tetracycline-inactivating enzymes. The canonical tetracycline-inactivation gene, *tet*(X), has been reported in pathogens only once (Leski et al., 2013), but has been identified on multiple transposable elements (Park and Levy, 1988; Speer and Salyers, 1988) and in multiple bacterial phyla (Yang et al., 2004; Leski et al., 2013; Ghosh et al., 2009). Directed evolution of Tet(X) suggests it may rapidly acquire strong resistance-conferring phenotypes (Walkiewicz et al., 2010), and its dissemination potential via horizontal gene transfer is consistent with the promiscuity of flavoprotein monooxygenases (Walsh and Wencewicz, 2013; Aminov, 2009).

Our results indicate that Tet(X) is not alone. From six soil metagenomes, we identified nine predicted flavoenzymes that inactivate tetracycline when expressed heterologously in *E. coli*, five from agricultural soils and four from grassland soils. These soils have no history of anthropogenic tetracycline addition (Fierer et al., 2012; Ramirez et al., 2010), suggesting that these enzymes represent native functional capacities of soil bacteria. The newly discovered flavoenzymes confer resistance against multiple tetracycline antibiotics and have a homolog in the soil-dwelling

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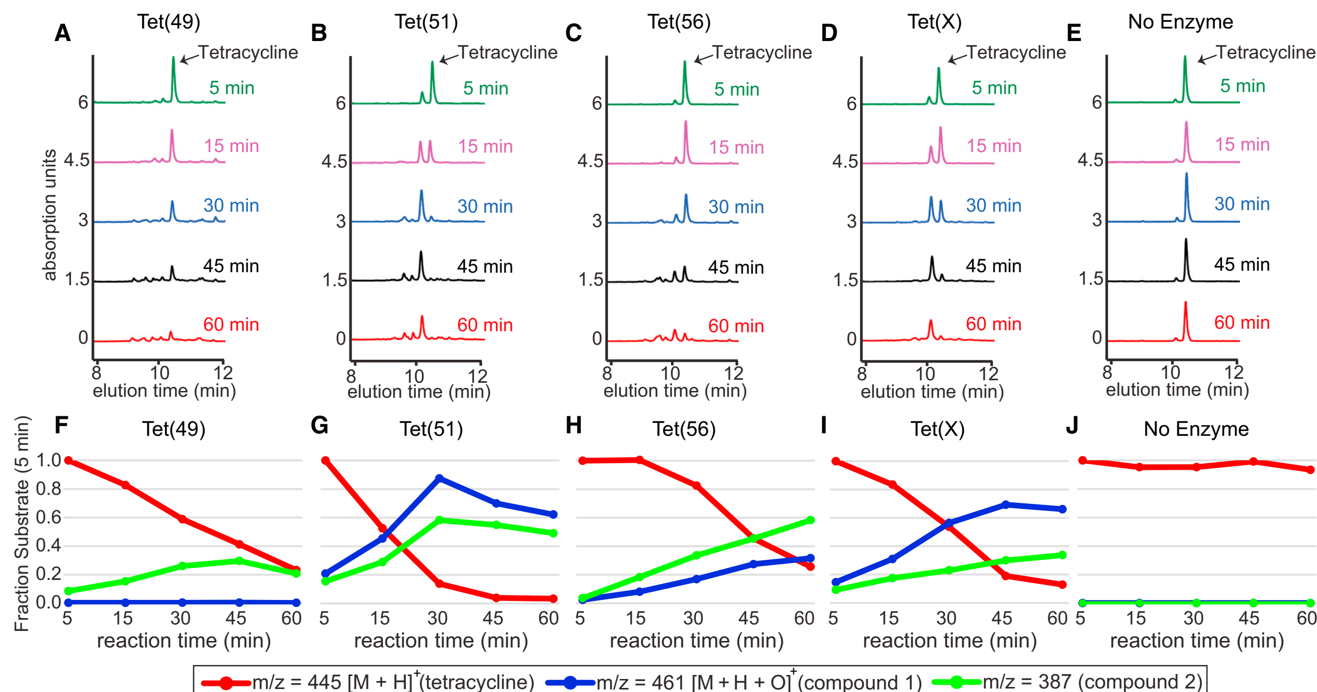

Figure 4. Tetracycline Degradation Is Catalyzed by Diverse Flavoenzymes

(A–E) Reverse-phase HPLC separation of tetracycline and enzymatically catalyzed degradation products; absorbance at 260 nm is shown.

(F–J) The relative ion counts attributable to tetracycline (*m/z* for $[M + H]^+$ equals 445 in positive ion mode) and products with *m/z* values of 461 and 387; data generated from the same reactions depicted in (A–E). The replacement of tetracycline with a product of +16 Da is consistent with monooxidation of the antibiotic and the mechanism through which Tet(X) catalyzes its degradation (Yang et al., 2004). A putative structure for the product with *m/z* = 387 is proposed in Figures 6 and S6. Flavoenzymes from soil catalyze tetracycline degradation in manners both consistent with (e.g. B, G) and alternative to (e.g. A, F) Tet(X)-mediated catalysis (D, I). Data from experiments using all purified enzymes, oxytetracycline as substrate, and measurements of absorbance at 363 nm are shown in Figures S2–S4, and S7.

human pathogen *L. longbeachae*, which was also confirmed to inactivate tetracycline when expressed in *E. coli*. Seven of these enzymes were successfully purified and shown to catalytically inactivate tetracycline via UV-visible spectroscopy. When examined by reverse-phase HPLC, LC-MS, and high-resolution tandem MS, reactions using different enzymes yielded distinct product profiles, only some of which resembled that of a reaction using Tet(X). This suggests that the soil-derived enzymes degrade tetracycline via diverse oxidative mechanisms, including strategies distinct from the established hydroxylation-mediated decay used by Tet(X). Because these enzymes function to confer tetracycline resistance in *E. coli*, their degradation products are not likely to be antibacterial at levels comparable with those of tetracycline itself. Whether these tetracycline-derived metabolites select for drug sensitivity like some abiotic tetracycline decay products (Palmer et al., 2010), can act as signaling molecules (Davies and Davies, 2010; Yim et al., 2007), or otherwise affect the structure or dynamics of soil microbial communities warrants additional work.

Tetracycline-inactivating flavoenzymes are not only distributed across diverse soils, but may also traverse diverse phylogenetic lineages. In two of ten metagenomic contigs (Table S1), a tetracycline-inactivating gene was immediately downstream of a gene predicted to encode an aminoglycoside-resistant kinase and upstream of a predicted virulence factor in one contig and a predicted resolvase in the other (Forsberg et al., 2014). These annotations are consistent with functions often present in multidrug-resistant mobile genetic elements (Sengupta and Austin, 2011; Cordero and Polz, 2014) and highlight the dissemination potential of these flavoenzymes (Aminov, 2009). A tetracycline-resistant flavoenzyme was found encoded in the human pathogen *L. longbeachae* but not its close relative *Legionella pneumophila*, indicating that tetracycline inactivation may be a flexible, mobile component of this pathogen's genome. Like other pathogens implicated in the rapid acquisition of multidrug resistance (e.g. *Acinetobacter, Pseudomonas*), *Legionella* thrives in both environmental and infectious settings (Whiley and Bentham, 2011; Cazalet et al., 2010) and is a member of a larger group of generalist Proteobacteria implicated in the exchange of antibiotic resistance between soil and clinic (Forsberg et al., 2012, 2014). Although the flavoenzymes described herein likely evolved in response to environmental selection pressures (e.g. tetracycline production by soil microbes), they should be carefully monitored due to their association with many of the risk factors predicted to promote acquisition by human pathogens.

SIGNIFICANCE

Enzymes that inactivate most naturally derived antibiotics, but not tetracycline, are commonly found in environmental

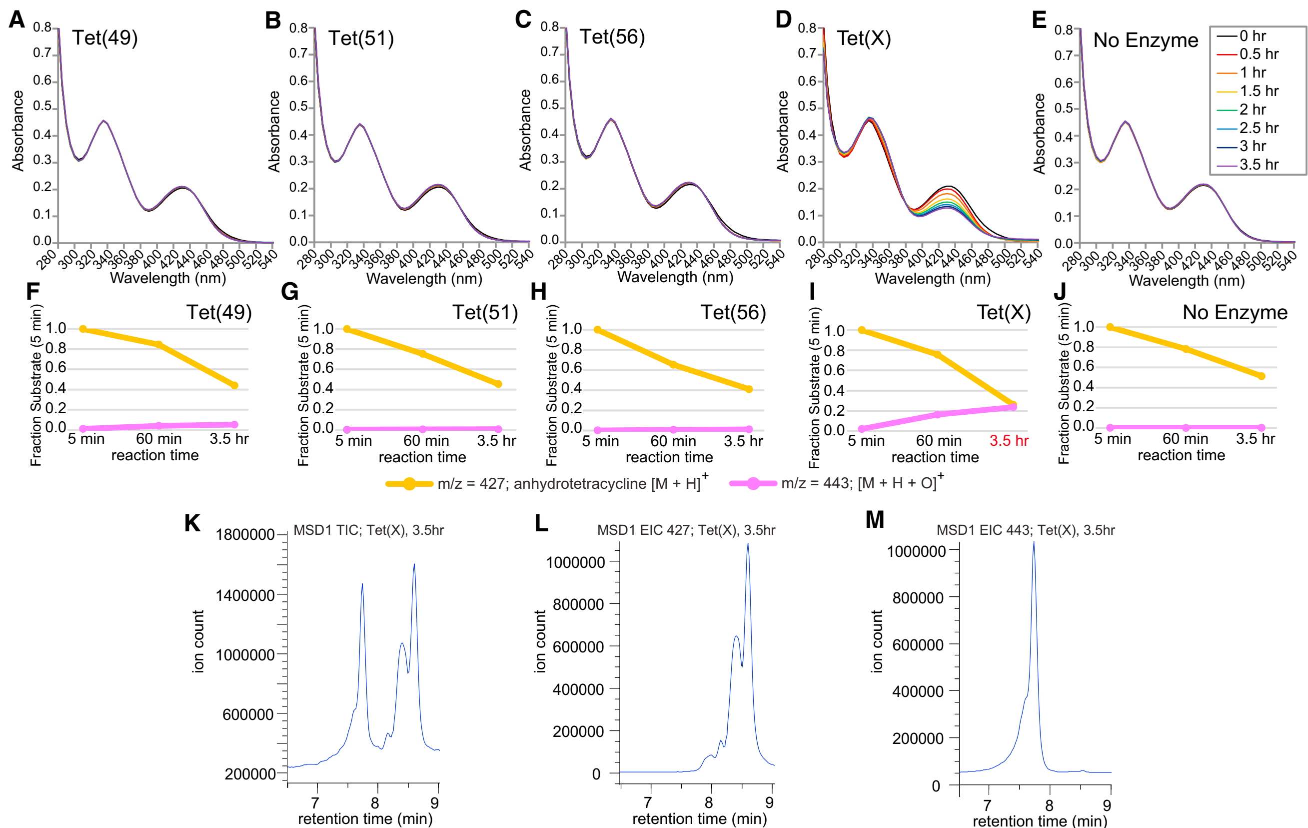

Figure 5. Tet(X), but No Other Flavoenzyme, Oxidizes Anhydrotetracycline

(A–E) Representative UV-visible spectra; each panel shows absorbance spectra taken every 30 min throughout a 3.5-hr reaction with anhydrotetracycline and the indicated enzyme. The legend in (E) applies to (A–E); Tet(X) was the only flavoenzyme to show activity toward anhydrotetracycline.

(F–J) The relative ion counts attributable to anhydrotetracycline (*m/z* for $[M + H]^+$ equals 427 in positive ion mode) and a product with +16 Da, consistent with monooxidation of the substrate.

(K–M) Representative LC-MS spectra of the indicated ions in from (I), measured at 3.5 hr as indicated in red. TIC, total ion count; EIC, extracted ion count.

and clinical settings. We reconcile tetracycline's apparent outlier status with the discovery of ten tetracycline-inactivating enzymes, previously unrecognizable as resistance genes on the basis of primary sequence. This family of flavoenzymes was confirmed to inactivate tetracycline in vitro and includes members that degrade the antibiotic via known, as well as previously uncharacterized, oxidative mechanisms. Enzymes were identified from multiple soil types, across varied geography, and from diverse phylogenetic origin, including from a causal agent of Pontiac fever and Legionnaires' disease, *Legionella longbeachae*. Because some of these enzymes show genetic signatures indicative of horizontal gene transfer, their potential for movement into hospital settings should be carefully monitored.

EXPERIMENTAL PROCEDURES

A full description of experimental procedures is provided in Supplemental Experimental Procedures.

Determination of Tetracycline Inactivation in *E. coli*

Each ORF encoding a putative tetracycline-inactivating enzyme was subcloned from its metagenomic (or genomic) source into the pZE21 expression vector (Lutz and Bujard, 1997) and transformed into *E. coli* MegaX cells (Invitrogen). Minimum inhibitory concentrations were determined using Mueller-Hinton broth and profiled via absorbance measurements at 600 nm (OD_{600}) using the Synergy H1 microplate reader (Biotek Instruments) for a minimum of 48 hr at 37°C. Luria-Bertani broth was used for darkened medium and Mueller-Hinton broth for conditioned medium experiments. Initial inocula were normalized across samples, and OD_{600} measurements used to profile tetracycline susceptibility in conditioned medium experiments using the Synergy H1 microplate reader. Tetracycline levels in supernatants of liquid growth assays were quantified via LC-MS/MS by Dr. Sophie Alvarez of the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO, USA). Tetracycline quantification assays were performed using M9 minimal salts medium and PIPES (Sigma #P6757) as an internal standard.

Enzyme Purification

All genes encoding putative tetracycline-inactivating enzymes were cloned into the pET28b(+) vector (Novagen) at BamHI and NdeI restriction sites. Constructs were then transformed into BL21-Star(DE3) *E. coli* cells (Life Technologies) and expression induced with isopropyl β-D-1-thiogalactopyranoside. Enzymes were expressed with an N-terminal His_6-tag and purified via Ni-NTA (nitrilotriacetic acid) resin, concentrated by centrifugal filtration using a 10-kDa molecular weight cutoff size-based concentration column (Millipore cat #ACK5010PG), analyzed by SDS-PAGE for purity, quantified by A_{280} measurement, flash-frozen in liquid nitrogen as 50-μl aliquots, and stored at −80°C.

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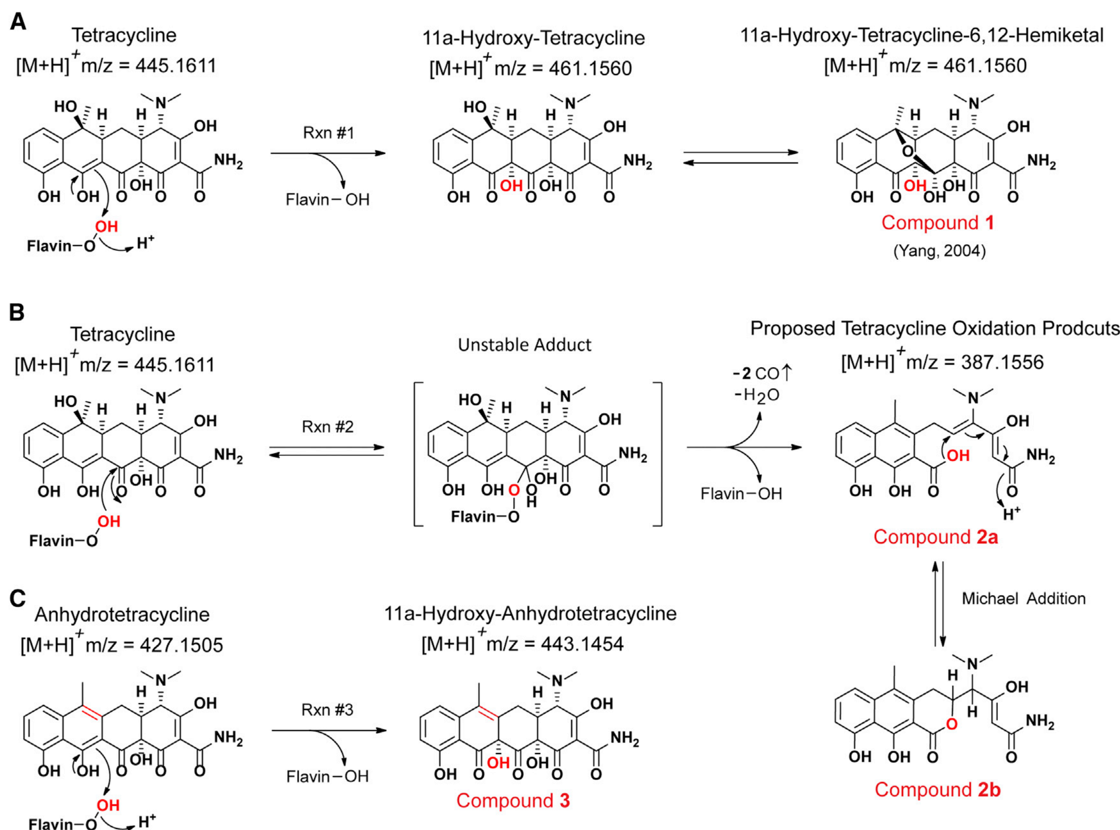

Figure 6. Enzymatic Conversions Discussed in this Article

(A) Monooxygenation of tetracycline to compound **1**, as described by Yang et al. (2004).

(B) The proposed tetracycline oxidation products, compounds **2a** and **2b**, with an *m/z* value of 387.

(C) Monooxygenation of anhydrotetracycline, depicted as is described for Tet(X)-catalyzed oxidation of tetracyclines in Yang et al. (2004).

In Vitro Determination of Tetracycline Inactivation

All reactions were performed using 100 mM TAPS buffer (pH 8.5) unless otherwise noted. Inactivation reactions contained 1.4 mM antibiotic, 350 μg enzyme, and an NADPH regenerating system in a total volume of 564 μl. For reactions using anhydrotetracycline, 1 mM substrate was used with 200 μg enzyme in a 475-μl reaction. The NADPH regenerating system consisted of the following components (final concentrations): glucose-6-phosphate (40 mM), $NADP^+$ (4 mM), $MgCl_2$ (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. Immediately upon reaction initiation, aliquots were diluted 40-fold (50-fold with anhydrotetracycline) and scanning UV-visible spectroscopy measurements taken from 280- to 550-nm wavelength light with a Cary 60 UV/Vis system (Agilent) for at least 3 hr. Reactions were subsequently sampled at indicated time points by transferring a 50-μl volume into a 200-μl quencher solution comprising equal parts of acetonitrile and 0.25 M aqueous HCl. Reactions were quenched under acidic conditions because prior work with Tet(X) demonstrated that enzymatic degradation products of tetracyclines are unstable at neutral pH but stable at low pH (Yang et al., 2004; Moore et al., 2005).

Products generated from enzymatic inactivation of both tetracycline and oxytetracycline were separated by reverse-phase HPLC using a Phenomenex Luna C18 column (5 μm, 110 Å, 2 × 50 mm) and 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as mobile phase. Injections of 25 μl sample volume were eluted using a linear gradient from 25%B to 75%B over 14 min at a flow rate of 1 ml/min. Samples were analyzed by LC-MS using an Agilent single-quadrupole LC/MS 6130 fitted with an autosampler and diode array detector. Reaction products were first separated by reverse-phase HPLC using a Phenomenex Gemini C18 column (5 μm, 110 Å, 2 × 50 mm) and 0.1% formic acid in water (A) and acetonitrile (B) as mobile phase. Injections of 30 μl sample volume were eluted using a linear gradient of 0%B to 95%B over 14 min at a flow rate of 0.5 ml/min. Electrospray ionization was used for analysis of reaction products by MS and ion counts for a particular *m/z* peak determined by peak height. Panels in Figures 4, S4, and S5 depict ion counts of various analytes from each enzymatic (or control) reaction, normalized to the counts observed for the peak associated with the tetracycline substrate (*m/z* of 445) at the first time point taken (5 min after reaction initiation).

Reactions performed for high-resolution tandem MS were prepared as described above and 45-min time points used for analysis. Samples were diluted 6-fold with 50% MeOH/0.1% formic acid and run on LTQ-Orbitrap Velos by direct infusion using the Advion Triversa Nanomate. The samples were acquired using a high-resolution (60,000) mass spectrometer. The MS scan was acquired from 300 to 550 *m/z*. The 387 *m/z* compound was fragmented by MS2 and MS3-CID with collision energies of 15 and 25 eV, respectively.

ACCESSION NUMBERS

The accession numbers for the gene sequences reported in this paper are GenBank: KR857681–KR857689.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, seven tables, and one Supplemental Dataset and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.05.017.

AUTHOR CONTRIBUTIONS

K.J.F. identified the novel inactivating enzymes and conceived the *E. coli* experiments with G.D; K.J.F., G.D., and T.A.W. conceived in vitro experiments; K.J.F. and S.P. performed *E. coli* experiments; K.J.F. performed in vitro experiments with assistance from T.A.W; K.J.F. wrote the manuscript with contributions from T.A.W. and G.D.

ACKNOWLEDGMENTS

We thank J. Vogel for graciously sharing the *L. longbeachae* strain, B. Wang for contributions to Illumina library preparation that enabled discovery of these enzymes, S. Alvarez at the Proteomics & Mass Spectrometry Facility of the Danforth Plant Science Center for help quantifying tetracycline and with high-resolution tandem MS, the Genome Technology Access Center at Washington University School of Medicine for Illumina sequencing services, and members of the Wencewicz and Dantas labs for their help with protocol optimization and for thoughtful discussions on the results and analyses presented herein. Research reported in this publication was supported in part by grants to G.D. by the NIH Director's New Innovator Award (http://commonfund.nih.gov/newinnovator/), the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK: http://www.niddk.nih.gov/), and the National Institute of General Medical Sciences (NIGMS: http://www.nigms.nih.gov/), of the NIH under award numbers DP2DK098089 and R01GM099538, and the Children's Discovery Institute under award number MD-II-2011-117. T.A.W. was supported by start-up funds from Washington University in St. Louis. K.J.F. is a National Science Foundation graduate research fellow (award number DGE-1143954) and received support from training grants through the National Human Genome Research Institute (NHGRI: https://www.genome.gov/) and the NIGMS through award numbers T32HG000045 and T32GM007067. This material is based upon work supported by the National Science Foundation under Grant No. DBI-0922879 for acquisition of the LTQ-Velos Pro Orbitrap LC-MS/MS. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

Received: February 26, 2015
Revised: May 5, 2015
Accepted: May 17, 2015
Published: June 18, 2015

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