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A neglected and emerging antimicrobial resistance gene encodes for a serine-dependent macrolide esterase

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The discovery of unreported antimicrobial resistance genes (ARGs) remains essential. Here, we report the identification and preliminary characterization of an α/β-hydrolase that inactivates macrolides. This serine-dependent macrolide esterase co-occurs with emerging ARGs in the environment, animal microbiomes, and pathogens.

antimicrobial resistance | macrolides | hydrolase | esterase

The accumulation of antimicrobial resistance genes (ARGs) in specific environments, and eventually in pathogens, challenges the utility of antibiotics. Thousands of ARGs and resistance-conferring mutations are known and accessible in curated databases. Nevertheless, our catalog of ARGs is incomplete. Blind spots—novel and unreported ARGs—pose unknown risks to human health, animal wellness, and the sustainability of agriculture.

A novel and unreported ARG is one that cannot be identified by homology-based inference. These unknown genes are absent from the ARG databases that serve antimicrobial resistance (AMR) surveillance programs intended to inform clinical practice. Growing concerns related to antimicrobial use and efficacy prompted us to evaluate watering bowls as sentinel systems for ARG detection at a beef cattle feedlot. The identification and functional characterization of a neglected ARG that resulted form the basis of this report.

Results and Discussion

Selective cultivation of multidrug-resistant bacteria from watering bowls at a western Canadian feedlot resulted in the isolation of *Sphingobacterium faecium* WB1 (Fig. 1*A*). The *S. faecium* WB1 genome includes a 17.5-kb plasmid with a cluster of three ARGs and an unannotated α/β-hydrolase (Fig. 1*B*) A search for homologs showed that this orphaned gene occurs in animal microbiomes and pathogen genomes (Fig. 1 *C* and *D*). A retrospective analysis of relevant sequencing projects confirmed the high prevalence of the α/β-hydrolase in two known causative agents of bovine respiratory disease (BRD) (1) and bacteria infecting poultry and swine (2, 3). Moreover, this α/β-hydrolase co-occurs with *tet(X4)*, an emergent tigecycline resistance gene (4, 5). The physical location of the α/β-hydrolase near ARGs, transposases, and/or on plasmids explains its cosmopolitan distribution and presence even in the human microbiome (Fig. 1*E*). Importantly, concordance between ARGs found in an organism recovered from watering bowls and relevant pathogens highlights the pragmatism of this approach.

The α/β-hydrolases catalyze diverse reactions using a catalytic triad. The *S. faecium* enzyme has a Ser-His-Asp triad but low sequence identity to well-studied enzymes. C–O bond hydrolysis is the most frequently reported α/β-hydrolase activity, though more than a third of the superfamily members are not functionally annotated (6).

To evaluate the hypothetical role of the α/β-hydrolase in AMR, the gene and 194-bp upstream sequence were transformed into *Escherichia coli* DH5α using a high-copy plasmid. Phenotypic screening of *Escherichia coli* transformed with the α/β-hydrolase showed a ≥32- or ≥4-fold increase in the minimum inhibitory concentration (MIC) of tildipirosin or tilmicosin compared to transformants carrying a catalytically inactive S126A variant (Fig. 1*A*). Next, the α/β-hydrolase, which we named EstT, and the S126A variant were purified for a biochemical assay (Fig. 1*F*). Two forms were produced by *Escherichia coli*, and the lower molecular weight form ($EstT_{25–306}$) was characterized. Six macrolides—erythromycin, tulathromycin, gamithromycin, tylosin, tilmicosin, and tildipirosin—were tested as substrates based on variations in the size of their macrocycles from 14 to 16 atoms. The products of EstT and EstT S126A-catalyzed reactions were analyzed by high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) and mass spectrometry (MS; Fig. 1*G*). The 16-membered ring–containing macrolides tylosin, tilmicosin, and tildipirosin were EstT substrates (Fig. 1*H*). These macrolides were rapidly and completely converted to products showing mass shifts of 18 amu, consistent with the hydrolysis of the macrocyclic lactone.

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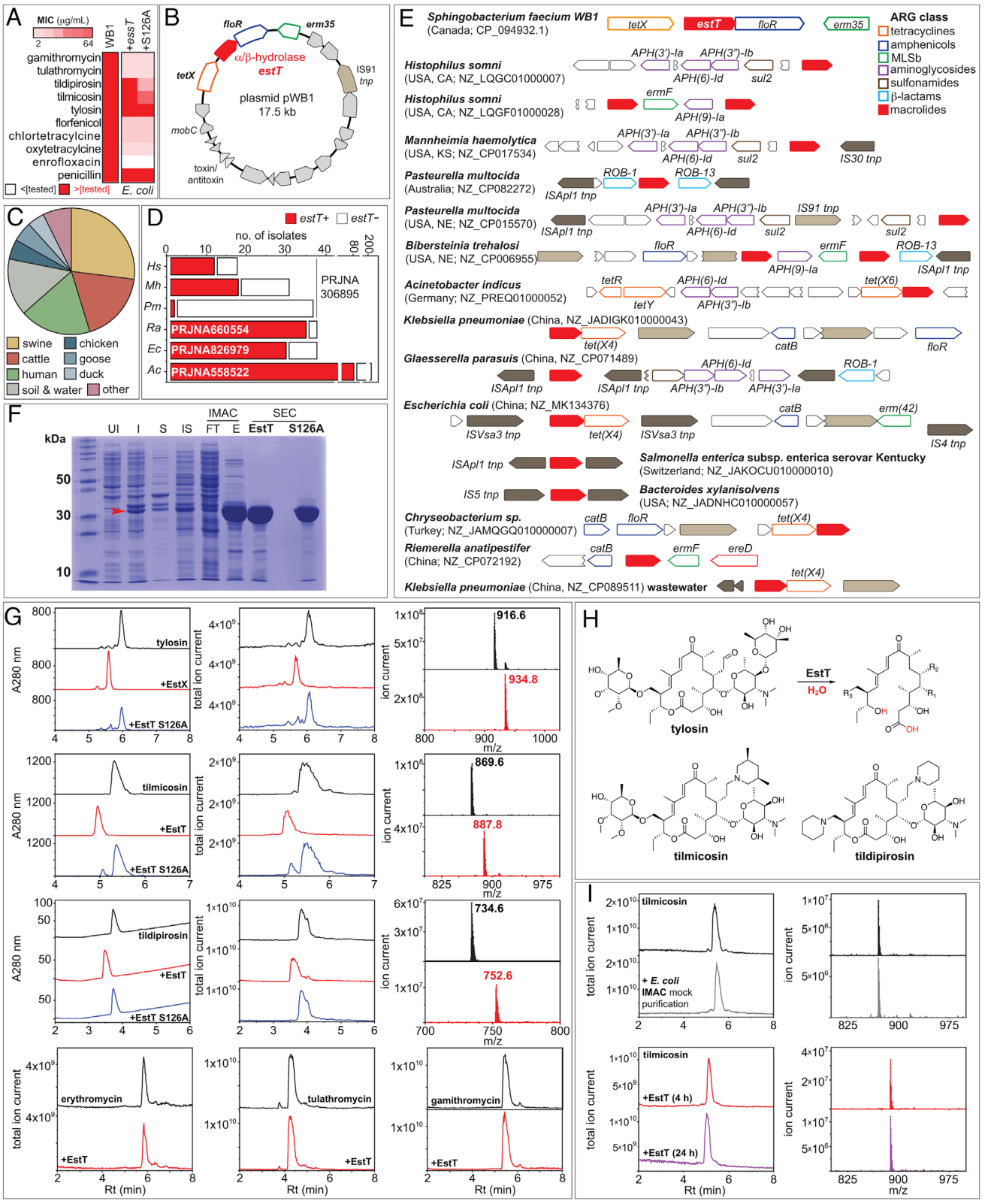

Fig. 1. (*A*) MICs of antibiotics measured for *S. faecium* WB1 and *Escherichia coli* transformants. (*B*) The *S. faecium* WB1 plasmid pWB1 map and ARG cluster showing the α/β-hydrolase (named *estT*, red). (*C*) Sample origins of bacteria with an EstT homolog (136 unique sequences >70% identity). (*D*) Prevalence of *estT* in bacterial pathogens: BRD pathogens (*Histophilus somni*, *Hs*; *Mannheimia haemolytica*, *Mh*; *Pasteurella multocida*, *Pm*), the bird pathogen *Riemerella anatipestifer* (*Ra*); *Escherichia coli* (*Ec*) found in slaughterhouses where *tet(X4)* is prevalent, and *Acinetobacter* spp. that infect swine (*Ac*). NCBI BioProject numbers are listed. (*E*) Representative *estT*-containing loci with ARGs and transposases annotated. (*F*) SDS-PAGE analysis showing the purification of EstT and purified EstT S126A. Lanes are labeled for uninduced (UI) and induced (I) cultures, soluble (S), and insoluble (IS) fractions, IMAC flow-through (FT), IMAC elution (E), and pooled ~90% pure protein after SEC. Arrows indicate two forms of EstT. The purified form (red arrow) is $EstT_{25–306}$ (N-terminal sequence: MKEKI). (*G*) HPLC/UV, HPLC/MS chromatograms, and mass spectra (MS) showing EstT and S126A reactions with six distinct macrolides: The 16-atom–containing macrolides are substrates of EstT. Parent $[M+H]^+$ ions are labeled. Representative results are shown for tylosin and tilmicosin after 30 min, whereas the other four reactions were observed after 4 h. (*H*) Structure of EstT substrates and the proposed reaction. (*I*) Chromatograms and MS showing control reactions: *Escherichia coli* IMAC impurities do not hydrolyze tilmicosin and the ring-opened product is relatively stable.

In contrast, the 14- and 15-atom–containing macrolides were not hydrolyzed by EstT. In control experiments, no hydrolysis of tilmicosin was observed using cytosolic *Escherichia coli* proteins that were adventitiously enriched by IMAC, and the hydrolytic product appeared to be relatively stable (Fig. 1*I*). The limited hydrolysis observed for S126A:macrolide reactions may have resulted from water acting as a nucleophile in the variant active site, calling for further investigation of the catalytic mechanism. Robust characterization of EstT and homologs should be completed to better define the substrate specificities of these enzymes.

The discovery of a novel macrolide esterase is remarkable. It is an example of convergent evolution to exploit lactone hydrolysis for macrolide inactivation. The erythromycin esterases (EreA-D) are the only other known macrolide esterases, and in contrast to EstT, they efficiently hydrolyze 14- and 15-atom macrolactones using a Glu–His dyad formed by a distinct protein structural fold (7–9). In the context of our agriculture-focused search, the presence of *estT* reconciles previously reported discordance between AMR genotypes and phenotypes of the bovine respiratory pathogen *M. haemolytica* (1). Incredibly, *estT* has been overlooked despite preeminent reports of it within emerging tigecycline resistance loci (4, 5). Moreover, another α/β-hydrolase of an unknown function named EstX was found alongside a known ARG in bacteria isolated from pigs, sewage, and people in the 1980s (10, 11). EstX and EstT share 44% identity, making our characterization of EstT a case study in how poorly annotated genes can go unnoticed or unstudied for decades despite a pattern of proximity to known and readily identified ARGs. Ultimately, our results speak to a need for systematic analyses to identify new ARGs based on how they are collectively disseminated.

Materials and Methods

Bacterial Isolation, Whole-Genome Sequencing, and Analysis. *S. faecium* WB1 was isolated from a pooled water sample from 10 bowls using a two-step enrichment for bacteria capable of surviving five classes of antibiotics. DNA prepared from a 100-mL culture grown in tryptic soy broth (TSB) at room temperature was subjected to long-read (PacBio) nucleotide sequencing at the University of Maryland Institute for Genome Sciences. Taxonomy was determined by using the Public databases for molecular typing and microbial genome diversity (PubMLST) (12) and ARGs identified using the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) tool (13).

Antimicrobial Susceptibility Tests. *S. faecium* or *Escherichia coli* DH5α transformants carrying *estT* or $estT_{S126A}$ and a 194-bp upstream region were grown in TSB or lysogeny broth and diluted to an optical density of 5×10^{-5} prior to inoculation in a beef feedlot-specific Sensititre antimicrobial sensitivity test (AST) plate (Thermo Fisher). Cultures' growth at room temperature (RT) or 37 °C was assessed after 18 h.

Protein Production and Purification. EstT and S126A were produced in *Escherichia coli* Rosetta (DE3) form pET29b(+) by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, followed by overnight growth at 16 °C with shaking at 200 rpm. Cells were harvested; resuspended in 20 mM tris(hydroxymethyl)aminomethane (TRIS) and 150 mM NaCl, pH 7.5; and lysed at 25,000 pounds per square inch (PSI). Lysates were clarified by centrifugation at 31,000 *rcf* (30 min, 4 °C). Purification of C-terminal His_6-tagged EstT and S126A was accomplished by immobilized metal affinity (IMAC) and size exclusion chromatography (SEC). Purity was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). N-terminal peptide sequencing was performed by the Protein Facility of the Iowa State University Office of Biotechnology. The lysate of untransformed *Escherichia coli* Rosetta (DE3) was subjected to IMAC for control reactions.

Assay of EstT Activity. A total of 140 nM EstT and 200 μM macrolide were reacted in 100 mM potassium phosphate buffer, pH 7.0, at RT. Dimethyl Sulfoxide (DMSO) was included at 1% or 3% v/v to solubilize macrolides, and IMAC-purified *Escherichia coli* proteins were used as a negative control. Reactions were monitored by HPLC/UV/MS after 30 min, 4 h, and/or 24 h using a Phenomenex Kinetex F5 column (100 Å, 5 μm, 100 × 4.6 mm) and a linear gradient from 90% solvent A (water, 0.05% formic acid) to 100% solvent B (acetonitrile, 0.05% formic acid) in 10 min, operating at 0.7 mL/min. An Advion compact single-quadrupole mass detector was connected to the HPLC through an electrospray ionization source.

Data, Materials, and Software Availability. The nucleotide sequences of the *S. feacium* WB1 genome are available through GenBank: CP094931.1 and CP094932.1. All other study data are included in the main text. Previously published data were used for this work. We analyzed publicly available data in four National Center for Biotechnology Information (NCBI) Bioprojects: PRJNA306895, PRJNA660554, PRJNA826979, and PRJNA558522. These data were obtained and made public by other researchers.

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