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Letters

Endogenous Enzymes Enable Antimicrobial Activity

Kenton J. Hetrick, Miguel A. Aguilar Ramos, and Ronald T. Raines*

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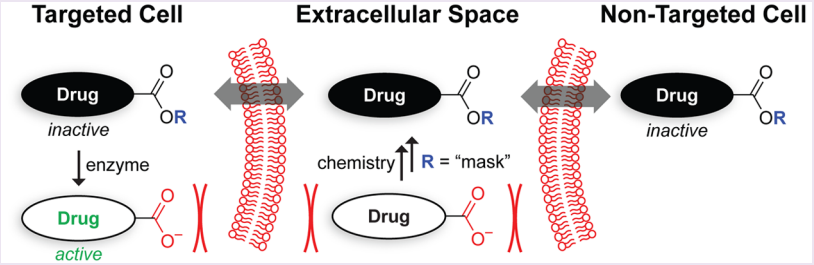

ABSTRACT: In light of the continued threat of antimicrobial-resistant bacteria, new strategies to expand the repertoire of antimicrobial compounds are necessary. Prodrugs are an underexploited strategy in this effort. Here, we report on the enhanced antimicrobial activity of a prodrug toward bacteria having an enzyme capable of its activation. A screen led us to the sulfurol ester of the antibiotic *trans*-3-(4-chlorobenzoyl)acrylic acid. An endogenous esterase makes *Mycolycibacterium smegmatis* sensitive to this prodrug. Candidate esterases were identified, and their heterologous production made *Escherichia coli* sensitive to the ester prodrug. Taken together, these data suggest a new approach to the development of antimicrobial compounds that takes advantage of endogenous enzymatic activities to target specific bacteria.

Antimicrobial resistance represents a growing threat to public health, rendering established therapies ineffective. Changes in the societal use of antibiotics, the discovery of new antibacterial compounds, and the derivatization of known antibacterial compounds all demonstrate great promise for addressing this challenge. Nonetheless, these efforts alone are unlikely to be sufficient to solve this crisis because resistance arises so quickly, even for new classes of antibiotics.[1] Rather, new strategies beyond classical broad-spectrum antibacterial compounds are called for.[2,3]

One potential strategy is the creation of narrow-spectrum antibiotics.[4−6] In contrast to broad-spectrum antibiotics, which kill virulent and avirulent bacteria, narrow-spectrum antibiotics are only lethal to particular types of bacteria, ideally, pathogens. The use of broad-spectrum antibiotics has been shown to increase the presence of resistance genes to both the specific antibiotic and other groups of antibiotics.[7] In comparison, narrow-spectrum antibiotics apply less selective pressure to the development of resistance because only a few bacterial species present in a treated patient (rather than the entire microbiome) would benefit from resistance. Such drugs could therefore have extended utility.[6] In addition, treatment with a narrow-spectrum antibiotic would allow the native fauna of the microbiome to persist, thereby reducing the likelihood of secondary infections and other complications.[8]

We reasoned that a prodrug approach to antimicrobial compound development could be a novel means of developing targeted antimicrobial compounds if activation were dependent on the activity of an enzyme found only in the target species. Most of the limited work on antimicrobial prodrugs has focused on improving solubility[9] or broad-spectrum effectiveness.[10] One report has shown, however, that ciprofloxacin can be targeted to pathogenic *Escherichia coli* when linked as an ester to the siderophore enterobactin, leveraging the receptor for the siderophore to endow specificity.[11] Another report has demonstrated the illumination of a fluorogenic probe by an enzyme endogenous in mycobacteria.[12] Here, we sought to test the hypothesis that an antibiotic could be targeted through the substrate specificity of an endogenous enzyme.

RESULTS AND DISCUSSION

Screen of Microbial Esterase Activity. We began by seeking two bacterial species that have vastly different esterase activities. A previous literature report suggested that *E. coli* contained low esterase activity,[13] and the carboxylic ester hydrolase enzymes (CASTLE) database further suggested that *E. coli* DH10B contained no known carboxylesterases.[14] Reports suggest that *Mycolycibacterium smegmatis* (basionym *Mycobacterium smegmatis*), on the other hand, has higher levels

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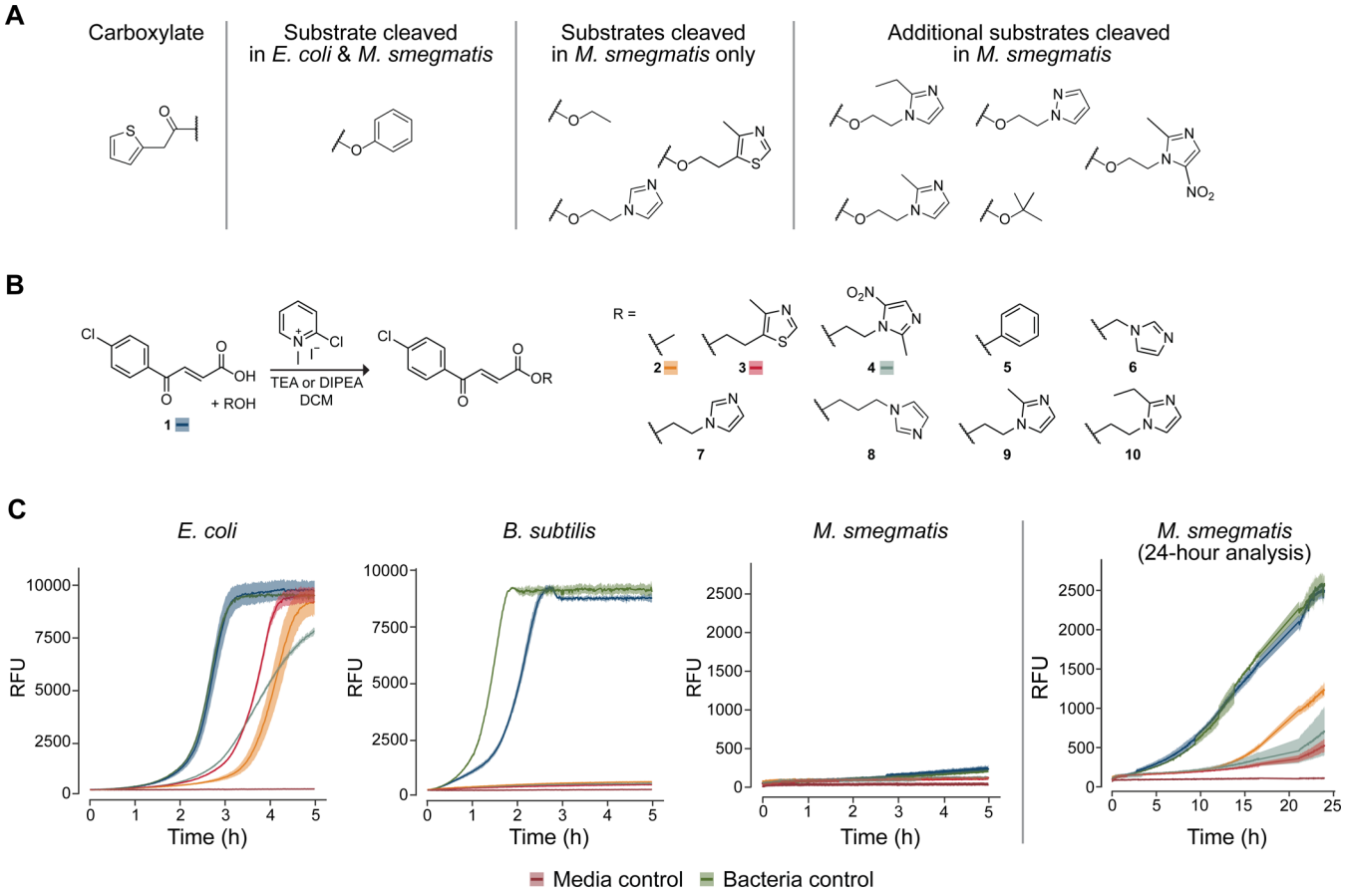

Figure 1. Microbial esterase activity assays. (A) A small panel of esters of 2-thiopheneacetic acid was assessed for cleavability in *E. coli* DH10B or *M. smegmatis* mc^{2}155 lysate. Only the phenolic ester was cleaved in *E. coli* lysate, whereas all four esters initially tested were cleaved in *M. smegmatis* lysate. Consequently, a larger panel of esters was screened in *M. smegmatis* lysate, and it was found that all esters were subject to cleavage. (B) Synthetic route to esters of *trans*-3-(4-chlorobenzoyl)acrylic acid. Note that acid **1** and esters **2**−**4** are labeled with the corresponding color for viability curves in panel (C). (C) Viability curves for bacteria incubated with acid **1** and esters **2**−**4** (100 μM), which have high antimicrobial activity. Viability was assessed through the addition to the medium of resazurin dye, which fluoresces upon reduction in the presence of live, metabolizing cells. Medium: *E. coli* and *B. subtilis*, CAMHB; *M. smegmatis*, 7H9. Lines are the average of triplicate experiments; ribbons depict one standard deviation.

of esterase activity.[15,16] To confirm these reports, we screened a small library of esters for cleavage in *E. coli* DH10B or *M. smegmatis* mc^{2}155 lysates using an esterase-activity assay established in our laboratory (Figures 1A and S1).[17] The results suggested that, in line with the literature reports, *E. coli* cells harbor little esterase activity whereas *M. smegmatis* has much higher levels. Hence, we explored the esterase activity of *M. smegmatis* further and established that all of the esters that we tested were cleaved in the presence of its lysate. Together, these literature reports and experimental evidence suggested that *E. coli* and *M. smegmatis* possess markedly divergent esterase activity and provided a basis for testing our ester-based prodrug approach.

Screen of Ester Prodrugs. Next, we established a screen to determine the antimicrobial efficacy of esters of *trans*-3-(4-chlorobenzoyl)acrylic acid (**1**) in *E. coli* versus *M. smegmatis*. Compound **1** had been reported to have moderate efficacy against *Mycobacterium tuberculosis*, and its methyl ester had been reported to have enhanced activity along with low toxicity to mammalian cells.[18,19] Still, compound **1** has not been used clinically, and is likely to target more than one species.[18,20]

We reasoned that different esters of acid **1** would differ in their ability to kill bacteria, depending on both cellular permeability and the rate of esterase-catalyzed hydrolysis. Accordingly, we synthesized an array of nine esters of acid **1** (Figure 1B). Using resazurin dye fluorescence turn-on as a proxy for cell viability, we screened these esters to assess their ability to inhibit the growth of *E. coli* (Figure S2) or *M. smegmatis* (Figure S3) at a concentration of 50 μM in Luria−Bertani broth. We found that the free acid form had low activity in both species and that the esters presented differential, species-dependent antimicrobial activity. In *E. coli*, the methyl ester was highly effective, whereas in *M. smegmatis* the ester with sulfurol[21] showed the most activity, followed closely by the metronidazole ester. Imidazole ester **7**, which showed high activity against both species, was observed to undergo rapid hydrolysis in aqueous solution, consistent with previous reports of α-(1*H*-imidazol-1-yl)alkyl (IMIDA) carboxylic acid esters,[22] and was not pursued further.

Intrigued by the differential activity of the esters in *M. smegmatis* compared to *E. coli*, we probed for differences in a Gram-positive organism. Specifically, we added *Bacillus subtilis* OI1085 to our screen because this Gram-positive bacterium, like *M. smegmatis*, is known to harbor many esterase-encoding genes.[23] We chose the three most active compounds identified in our initial screen and increased their concentration to 100

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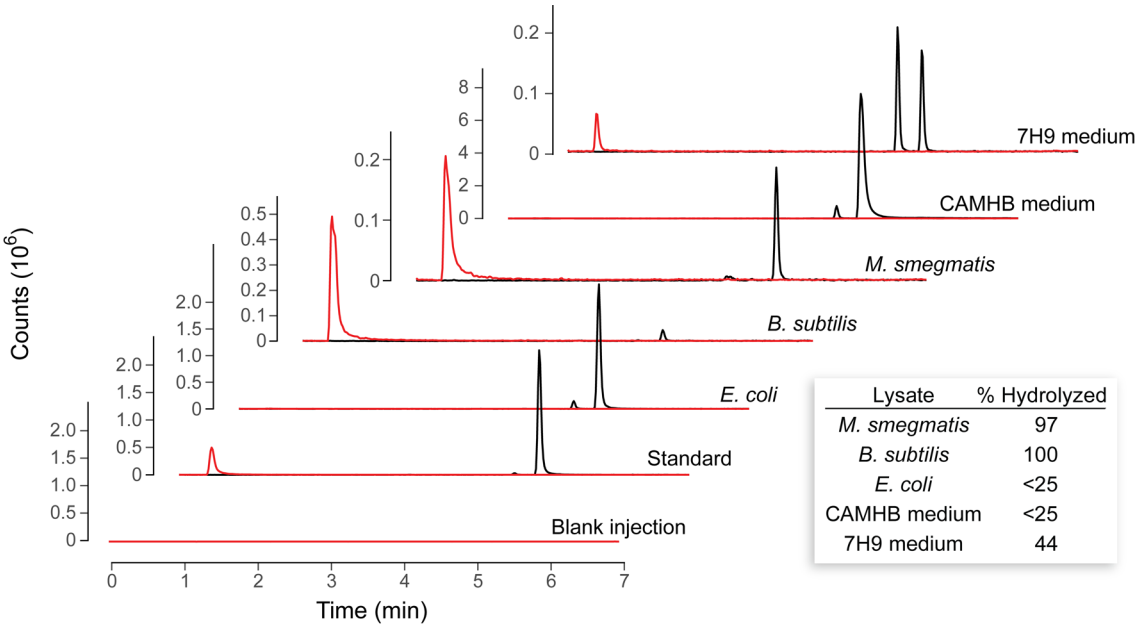

Figure 2. LC−MS extracted ion chromatograms (EICs) of sulfurol and sulfurol ester **3** after incubation with a cell lysate. Red EIC has m/z 144 (sulfurol), and black EIC has m/z 336 (sulfurol ester **3**) from the same LC−MS run. The small peak in the black trace is likely the *cis* isomer of sulfurol ester **3**. Standard traces are from a solution containing 200 μM of both sulfurol and sulfurol ester **3**. Inset: Sulfurol ester **3** % hydrolyzed was determined from calibration curves as described in the Supporting Information. Note: In addition to hydrolysis, the 7H9 medium led to isomerization.

μM. Cation-adjusted Mueller−Hinton broth was used for the screen of *E. coli* and *B. subtilis* cells, as is typical for antimicrobial tests in these species, whereas 7H9 medium was used for *M. smegmatis*. Viability curves confirmed the earlier pattern observed in *E. coli* and *M. smegmatis*. In *B. subtilis*, however, the acid form of the drug showed some activity and all ester variants inhibited bacterial growth for the duration of the viability screen (Figure 1C). We performed a similar screen in the presence of the constituent alcohol moieties alone and found that none of the alcohols influenced the growth of any of the bacteria (Figures S4−S6). MIC values correlated with the trends seen in the viability assays (see the Supporting Information). Specifically, sulfurol ester **3** exhibited a lower MIC for both *B. subtilis* and *M. smegmatis* but not for *E. coli*, consistent with our hypothesis that the presence of esterases capable of cleaving the sulfurol ester led to its enhanced toxicity.

Analysis of Microbial Esterase Activity. Next, we sought to establish whether the sulfurol ester was indeed hydrolyzed by esterases endogenous in *M. smegmatis* and *B. subtilis* but not *E. coli*. Small cultures of the three bacteria were grown overnight and then lysed via sonication. Ester **3** was then added to these lysates, which were incubated at 37 °C for 2 h, extracted with dichloromethane, and analyzed with LC−MS (Figure 2). To provide a semiquantitative assessment of the extent of hydrolysis, standard curves were constructed by spiking known amounts of authentic sulfurol ester, sulfurol, and *trans*-3-(4-chlorobenzoyl)acrylic acid into lysates of each bacterial species to mimic hydrolysis extents ranging from 0% hydrolyzed to 100% hydrolyzed. A linear relationship was discovered in the range from 25−100% hydrolyzed (Figure S7). The extracted ion chromatograms from the same LC−MS run allowed for integration of the sulfurol ester peak (m/z 336) and the sulfurol ester hydrolysis alcohol peak (m/z 144) using SpectraGryph software (version 1.2.15). Comparison of the ratio in the sample to the ratios determined from an external calibration curve allowed for semiquantitative assessment of the extent of hydrolysis. (The acid itself was excluded from the analysis because its detection was found to be too variable to allow for reliable semiquantitative analysis (Figure S8), perhaps because of its formation of covalent adducts with target enzymes as has been suggested previously.[18]) In both CAMHB medium and *E. coli* cell lysate, less hydrolysis was observed than could be determined via the semiquantitative method. In 7H9 medium, however, both partial hydrolysis and partial isomerization were apparent. In the presence of *M. smegmatis* or *B. subtilis* lysate, the ester compound was nearly completely hydrolyzed, suggesting that the sulfurol ester is a substrate for one or more esterases in these species.

Bioinformatic Analysis of Bacterial Esterases. Having demonstrated that *M. smegmatis* and *B. subtilis* but not *E. coli* are capable of hydrolyzing sulfurol ester **3**, we used bioinformatics to identify a candidate esterase. We collected the sequences of all proteins annotated as "carboxylesterases" from *E. coli* DH10B, *M. smegmatis* mc^{2}155, and *B. subtilis* subsp. *subtilis* 168 through searching of the NCBI protein database. *B. subtilis* subsp. *subtilis* 168 was used in place of *B. subtilis* OI1085 because it has a fully sequenced genome and strain OI1085 is descended from strain 168.[24−26] Using the tools from the Enzyme Function Institute,[27] we uploaded all the sequences thus identified and found 209 with UniProt ID matches, 116 of which were unique (Table S1). We then created a sequence similarity network using a minimum alignment score of 37 (Figure 3A; for determination of minimum alignment score, see the Supporting Information and Figure S9). Analysis of the network revealed a single cluster that contained esterases from both *M. smegmatis* and *B. subtilis*. Because both *M. smegmatis* and *B. subtilis* were capable of hydrolyzing ester **3**, we concluded that this cluster likely

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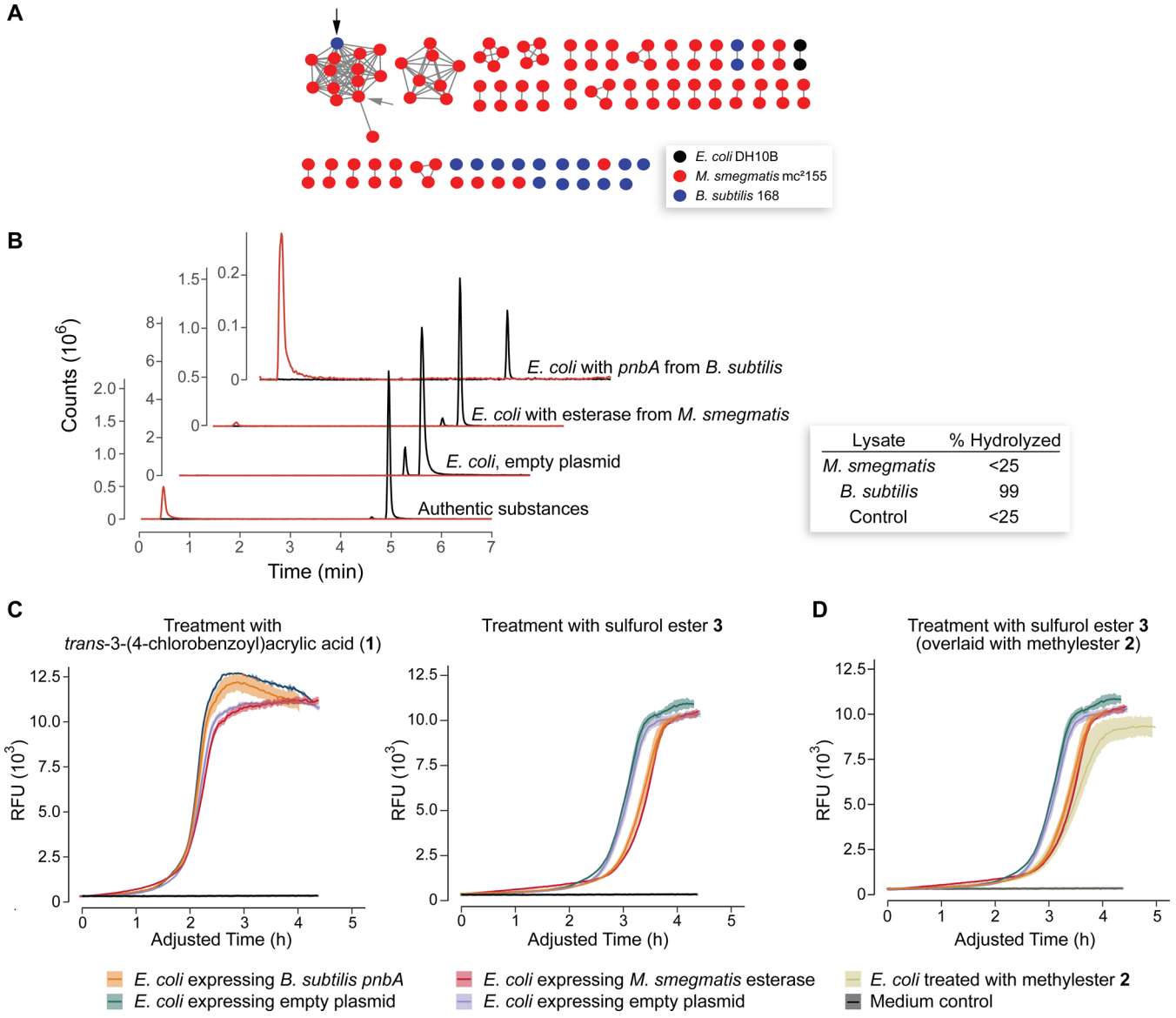

Figure 3. Identification of *pnbA* and effect of its expression in *E. coli* DH10B. (A) Sequence similarity network of annotated carboxylesterases from *B. subtilis* 168, *E. coli* DH10B, and *M. smegmatis* mc^{2}155 (Table S1). The black arrow indicates the pnbA carboxylesterase from *B. subtilis* 168. The gray arrow indicates carboxylesterase A0QYI2 from *M. smegmatis*. (B) LC−MS extracted ion chromatograms (EICs) of dichloromethane extract from cultures of *E. coli* exposed to 200 μM of sulfurol ester **3**. The "Authentic substances" traces are from a solution containing 200 μM of both sulfurol and sulfurol ester **3**. The red EIC has m/z 144 (sulfurol), and the black EIC has m/z 336 (sulfurol ester **3**) from the same LC−MS sample. Inset: The sulfurol ester **3** % hydrolyzed was determined from calibration curves as described in the Supporting Information. (C) Viability curves for *E. coli* expressing the identified carboxylesterase incubated with acid **1** and ester **3** (100 μM). (D) Viability curves for *E. coli* expressing the identified carboxylesterase incubated with esters **2** and **3** (100 μM). In panels (C) and (D), the ordinate was adjusted such that the bacteria-only growth curves from each gene expression condition have the same inflection point, allowing for a comparison between the antimicrobial compound dose curves. Lines are the average of triplicate experiments; ribbons depict one standard deviation.

represents carboxylesterases that hydrolyze ester **3** and contribute to its activity.

Heterologous Esterase Production. Two nodes from this cluster were cloned for further analysis. The first, a node from *B. subtilis* annotated as a *para*-nitrobenzylesterase A (pnbA, UniProtKB P37967) and having a known three-dimensional structure,[28] had been produced previously in *E. coli*.[29] The second, a node from *M. smegmatis* that is annotated, was annotated as a carboxylic ester hydrolase and to the best of our knowledge had not been characterized experimentally (UniProtKB A0QYI2). To confirm that proteins from this cluster were capable of hydrolyzing ester **3**, we constructed two pET22b plasmids, each harboring one of the esterase genes identified above under the control of a T7 promoter. The indicated esterase gene and a gene encoding T7 RNA polymerase on plasmid pCS6[30] were coexpressed overnight in *E. coli* DH10B cells, the culture was lysed, and the sulfurol ester was added to the lysate. As a control, an empty pET22b plasmid and plasmid pCS6 were treated in the same fashion. LC−MS analysis of the extracts following 2 h at 37 °C demonstrated that the heterologous production of the pnbA esterase from *B. subtilis* enabled *E. coli* DH10B cells to hydrolyze sulfurol ester **3** nearly to completion using our semiquantitative method, whereas the esterase from *M.*

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smegmatis hydrolyzed sufficient quantities to produce a visible peak in the hydrolysis product extracted ion chromatogram (EIC) but that was less than our method could quantify. No hydrolysis was detectable in the control culture (Figure 3B). Encouraged by this confirmatory activity, we also sought to determine whether production of the esterases in *E. coli* enhances the antimicrobial activity of the sulfurol ester. We reasoned that even a low degree of esterase activity in the lysate might be sufficient to enhance the activity of the ester prodrug in vivo. Because the control strain harboring an empty plasmid and the strain expressing the esterase experienced a different lag time in the viability assay (Figure S10), we first had to adjust the time course such that the untreated growth curves overlapped. Hence, we fitted the growth curve of the bacterial strains growing in medium alone to Richards's logistic growth curve[31,32] to determine the inflection time point, which is the point at which the growth rate has hit a maximum. We then adjusted the time of the curves such that the untreated bacteria growth curves had identical inflection points, thereby ensuring that they overlapped. Fits were conducted with R software,[33] and they are shown in the Supporting Information. This experiment was additionally complicated by the fact that hydrolytic activity was observed in the culture supernatant of *E. coli* cells expressing *pnbA* from *B. subtilis* (Figure S11); nonetheless, we observed that if *pnbA* expression was held to a low level, thereby holding extracellular pnbA to a minimum, a significant increase in sensitization could be observed in *pnbA*-expressing *E. coli* versus *E. coli* expressing an empty plasmid (Figure 3C). Additionally, a similar significant increase in sensitization was observed in the *E. coli* expressing the esterase from *M. smegmatis*. In both cases, this increase in sensitization drove the activity of *E. coli* cells for the sulfurol ester (**3**) to match that for the methyl ester (**2**), which was the most active variant of *trans*-3-(4-chlorobenzoyl)acrylic acid for parent *E. coli* cells (Figures 3D and S10).

In conclusion, we have demonstrated that a sulfurol ester prodrug of *trans*-3-(4-chlorobenzoyl)acrylic acid is more effective against *M. smegmatis* than other esters are. Importantly, the sulfurol ester is less active against *E. coli* than the methyl ester is, but this gap is bridged by the heterologous production of an esterase that hydrolyzes the sulfurol ester in *E. coli* cells. These data indicate that esterase activity profiles can be used to craft an ester prodrug of a carboxylic acid-containing antimicrobial agent. Hence, the alcohol moiety in an ester prodrug should be viewed as more than merely a mask of a negative charge; different esters can confer markedly different activity profiles.

Our findings point to a new strategy for developing prodrugs in which a unique enzymatic activity within a target species is harnessed to unmask an antimicrobial agent, thereby providing a narrow spectrum of action that can be identifiable with a bioinformatic analysis. In this work, we relied on esterase specificity to achieve this goal. We are aware, however, that bacteria are fantastic chemists. Their enzymes catalyze a wide range of unique reactions that are only beginning to be mapped effectively.[34−37] Future work could harness a range of bacterial enzymes to develop an array of narrow-spectrum antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00894.

Procedures for the synthesis of esters **2**−**10** and cognate esters with 2-thiopheneacetic acid; biological methods; annotated carboxylesterases in the sequence similarity network; esterase activity of bacterial cell lysates; screen of antimicrobial esters against *E. coli* and *M. smegmatis*; screen of alcohol compounds against *E. coli*, *B. subtilis*, and *M. smegmatis*; calibration of sulfurol ester:sulfurol peak-area ratio versus % hydrolyzed; LC−MS spectra and calibration curves of *trans*-3-(4-chlorobenzoyl)-acrylic acid; determination of the *e*-value threshold for SSN; fit of viability curves of E. coli DH10B cells; extracted ion chromatograms of sulfurol ester **3** incubated in spent medium; NMR spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Ronald T. Raines − *Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States; Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States;* orcid.org/0000-0001-7164-1719; Email: rtraines@mit.edu

Authors

Kenton J. Hetrick − *Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States; Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States;* orcid.org/0000-0003-4657-5275

Miguel A. Aguilar Ramos − *Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States;* orcid.org/0000-0002-2537-8371

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.0c00894

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CAMHB, cation-adjusted Mueller−Hinton broth; EIC, extracted ion chromatogram; LB, Luria−Bertani lysogeny broth; LC−MS, liquid chromatography−mass spectrometry; MIC, minimum inhibitory concentration; NCBI, National Center for Biotechnology Information

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