RESEARCH LETTER

Antibacterial enzymes from the functional screening of metagenomic libraries hosted in *Ralstonia metallidurans*

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Abstract

Phenotype-based screening of bacterial metagenomic libraries provides an avenue for the discovery of novel genes, enzymes, and metabolites that have a variety of potential clinical and industrial uses. Here, we report the identification of a functionally diverse collection of antibacterially active enzymes from the phenotypic screening of 700 000 cosmid clones prepared from Arizona soil DNA and hosted in *Ralstonia metallidurans*. Environmental DNA clones surrounded by zones of growth inhibition in a bacterial overlay assay were found, through bioinformatics and functional analyses, to encode enzymes with predicted peptidase, lipase, and glycolytic activities conferring antibiosis. The antibacterial activities observed in our *R. metallidurans*-based assay could not be replicated with the same clones in screens using *Escherichia coli* as a heterologous host, suggesting that the large-scale screening of metagenomic libraries for antibiosis using phylogenetically diverse hosts should be a productive strategy for identifying enzymes with functionally diverse antibacterial activities.

Introduction

Analyses of bacterial culture broths have traditionally been a route for the discovery of novel small molecules and enzymes (Demain & Sanchez, 2009; Trincone, 2011). While productive, these studies are limited by our inability to culture the vast majority of bacteria from the environment. The metabolomes and proteomes of a more representative sample of environmental bacteria can be accessed using functional metagenomic approaches that involve the extraction of DNA directly from environmental samples (environmental DNA, eDNA), the cloning of this DNA into model cultured bacteria, and, finally, the phenotypic screening of these clones in diverse assays (Handelsman *et al.*, 1998; Iqbal *et al.*, 2012).

To date, the majority of metagenomic library screens targeting antibiosis have relied on top agar overlay assays on *Escherichia coli*-based libraries to identify antibacterially active small molecules (Fig. 1). Large-scale functional screens using *E. coli*-based metagenomic libraries have seldom reported the discovery of heterologously expressed antibacterially active enzymes, despite the fact that cultured bacteria have been a prolific source of antimicrobial proteins (Veiga-Crespo *et al.*, 2007; Thallinger *et al.*, 2013). Phage endolysins, in particular, have recently garnered attention for their potential roles in enzyme-based antibiotic therapies (Fischetti, 2010; Thallinger *et al.*, 2013).

While most metagenomic functional screening has used *E. coli* as a host, the utility of *E. coli* as a heterologous host in functional metagenomic screens is likely to be limited due to its limited heterologous expression capacity (Gabor *et al.*, 2004). As the success of functional metagenomic screening is contingent upon the ability of the library host to heterologously express genes found on foreign eDNA, the identification of transcriptionally diverse model hosts that can express these foreign genes will likely be critical to the overall success of metagenomic screening strategies. We hypothesized that by changing the host used in phenotypic metagenomic library screens, it might be possible to begin to identify the diverse antibacterial enzymes that are undoubtedly encoded within soil microbiomes.

Here, we show that enzymes conferring antibiosis can be found in *R. metallidurans*-hosted soil DNA libraries. *R. metallidurans* is a gram-negative beta proteobacteria that we have explored as an alternative host for small molecule-based functional metagenomic studies because

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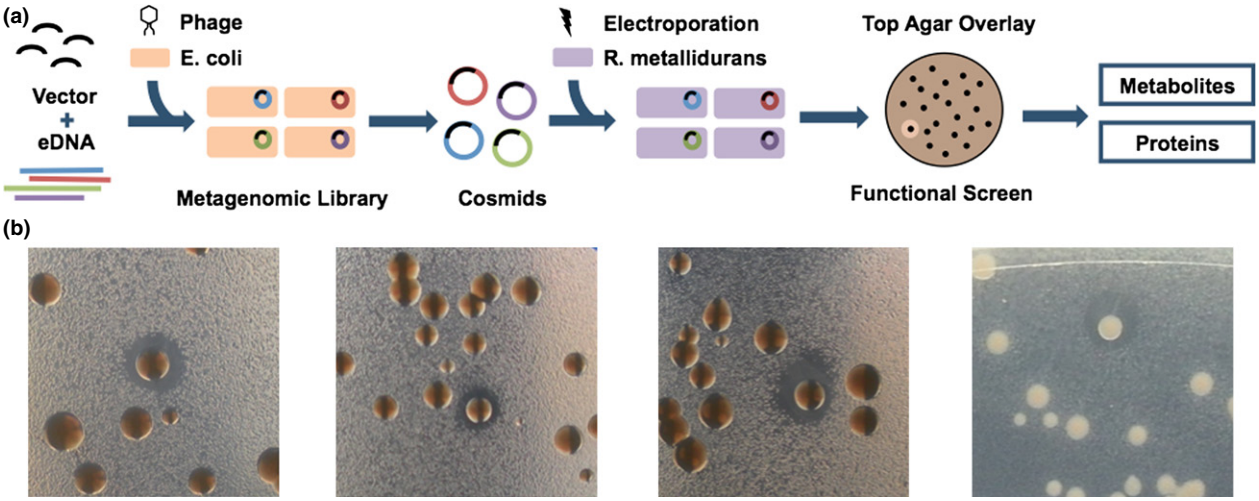


Fig. 1. (a) Overview of metagenomic library construction and screening methodology. In this study, eDNA extracted from soil samples was ligated to a shuttle cosmid vector and introduced into *Escherichia coli* by phage transduction. DNA prepared from these libraries was electroporated into *Ralstonia metallidurans* and screened for antibiosis using the top agar overlay method. (b) Representative antibacterially active hits obtained by screening soil metagenomic libraries hosted in *R. metallidurans* using the top agar overlay method. *R. metallidurans*-based libraries were arrayed onto LB-agar plates and overlaid with a layer of top agar containing a *Bacillus subtilis* assay strain; zones of growth inhibition around individual colonies indicate heterologous expression of the antibacterial phenotype.

of its previously described heterologous expression capabilities, its genetic tractability, and the ease with which it can be grown in the laboratory (Craig *et al.*, 2010). Based on recent interest in lytic enzyme-based antibiotic therapies, the large-scale screening of environmental DNA libraries hosted in *R. metallidurans* may be a productive strategy for identifying enzymes with diverse activities for potential use as novel therapeutics (Fischetti, 2010; Thallinger *et al.*, 2013).

Materials and methods

eDNA library construction

DNA extracted from soil collected in the Sonoran Desert of Arizona (USA) was used to create a 700 000-membered cosmid-based metagenomic library in *E. coli* (Brady, 2007). To obtain crude eDNA, 250 g of soil was passed through a 1/8-inch sieve to remove rocks and large debris and then incubated in lysis buffer [100 mM Tris-HCl, 100 mM NaEDTA, 1.5 M NaCl, 1% (w/v) cetyl trimethyl ammonium bromide, 2% (w/v) SDS, pH 8.0] (1 : 1 w : v) at 70 °C for 2 h (Zhou *et al.*, 1996). Heat lysed samples were centrifuged (4000 ***g***, 10 min) to remove soil particulates. Crude eDNA was precipitated from the supernatant by the addition of 0.7 volume isopropanol and collected by centrifugation (4000 ***g***, 10 min, 4 °C). The pellet was washed with 70% ethanol and the eDNA resuspended in minimum volume of TE buffer. eDNA was separated from the remaining soil material by ethidium bromide-free agarose (1%) gel electrophoresis (1 h at 100 V, 5 h at 20 V). High molecular weight eDNA (> 25 kb) was extracted from the gel by electroelution, concentrated by isopropanol precipitation, and blunt-end repaired (End-It, Epicentre Biotechnologies). Blunt-ended eDNA was ligated with either the previously reported broad-host-range cosmid vector pJWC1 or pJSS, a pJWC1 derivative with a DNA linker containing a ScaI cloning site (TGGCCTGTCATGAGCAGGATC) replacing the *sacB* gene (Craig *et al.*, 2009). Cosmids vectors were prepared for ligation by digestion with ScaI and dephosphorylation with calf-intestinal alkaline phosphatase. Ligation reactions were packaged with lambda phage packaging extracts (MaxPlax – Lambda Packaging Extracts, Epicentre) and transfected into *E. coli* EPI 300 (TransforMax, Epicentre Biotechnologies) grown to $OD_{600\ nm}$ 1.0 by shaking at 37 °C for 1.5 h. 1/1000 of the transfection reaction was plated on a LB-tetracycline plate (20 μg mL^{-1}) to estimate the size of the library. The remainder of the transfection reaction was selected overnight in LB broth containing 20 μg mL^{-1} tetracycline (37 °C with shaking). The number of colonies on the titer plate was counted to estimate the number of clones in the library. In total, 5×10^5 and 2×10^5 unique cosmid clones were constructed using vectors pJWC1 (vector size 14 kb) and pJSS (vector size 12 kb), respectively, constituting 22.5 GB eDNA. The library was stored as 15% glycerol stocks.

To transform libraries into *Ralstonia metallidurans*, cosmid DNA was miniprepped (Qiagen) from overnight cultures inoculated from library glycerol stocks and pooled together the next day in equivalent volumes. 1–2 μg of DNA was transformed by electroporation (0.8 kV per 1.0 mm cuvette) into 80-μL aliquots of electrocompetent *R. metallidurans* CH34 cells prepared as previously described (Taghavi *et al.*, 1994). Following electroporation, cells were diluted in 1 mL SOC medium and incubated for 3 h (30 °C with shaking) before plating onto LB-tetracycline plates (20 μg mL^{-1}). The resulting *R. metallidurans*-based library comprising two times coverage of the original eDNA cosmid library was scraped from the selection plates after 2 days at 30 °C and archived as glycerol stocks.

Functional screening of libraries in *R. metallidurans*

The *R. metallidurans*-based library was screened for clones with antibacterial activity in a top agar overlay assay against *Bacillus subtilis*. For this assay, the library was diluted directly from glycerol stocks and plated onto 150 mm LB-tetracycline (20 μg mL^{-1}) plates at a density of 1000–1500 clones per plate. Colonies were allowed to mature at 30 °C overnight and then incubated at room temperature for 4–5 days to allow for heterologous expression. A thin layer of 0.7% top agar (10–12 mL) was overlaid onto the plates. The top agar was inoculated with a 1 : 200 dilution of tetracycline-resistant *B. subtilis* 1E9 (*Bacillus* Genome Stock Center, Ohio) grown to $OD_{600\ nm}$ 0.5. Plates were incubated at 30 °C overnight. To cleanly obtain the naturally kanamycin-resistant *R. metallidurans* clones without residual *B. subtilis* assay strain contamination, colonies forming zones of growth inhibition in the *B. subtilis* lawn were picked from the assay plates and struck for single colonies on LB plates containing tetracycline (20 μg mL^{-1}) and kanamycin (30 μg mL^{-1}). DNA was miniprepped from overnight cultures of single colonies, retransformed into *R. metallidurans*, and patched onto LB-tetracycline (20 μg mL^{-1}) plates. These plates were re-assayed using top agar overlays; clones that showed the antibiosis phenotype were considered true hits and archived as 15% glycerol stocks in *R. metallidurans* and *E. coli*.

Sequencing and bioinformatics

High-quality cosmid DNA was obtained from antibacterially active hits by miniprepping overnight *E. coli* EPI 300 cultures induced with CopyControl induction solution (Epicentre Biotechnologies). Cosmid DNA from each clone was pooled and sequenced using 454 GS-FLX Titanium pyrosequencing technologies (MSKCC Genomics Core Laboratory) and assembled on GS *De Novo* Assembler software (Roche) (GenBank Accession numbers KF835381–KF835386 for SZR1, SZR5, WZR9, WZR11, WZR18, and WZR21, respectively). Individual cosmid clones were also end sequenced by Sanger sequencing using primers designed to recognize vector sequences flanking the eDNA cloning site. End sequencing data was used to identify contigs assembled from the 454 sequencing data. Sequences were annotated using the online tool SOFTBERRY to predict open-reading frames (ORFs), and alignments to BLAST and PFAM databases were used to predict gene function (Altschul *et al.*, 1990; Solovyev & Salamov, 2011; Punta *et al.*, 2012). For phylogenetic analysis, predicted antibacterial proteins found in clones SZR1, WZR21, and WZR9 were trimmed based on their alignment to PFAM families PF01464 (SLT Transglycosylase), PF01520 (Amidase), or PF07859 (Alpha/beta hydrolase), respectively. Phylogenetic trees were constructed from CLUSTALW alignments using the MEGA5 program with the neighbor-Joining method and 1000 bootstrap replications (Tamura *et al.*, 2011).

Subclone library construction

Cosmid DNA (2 μg) was sheared to 3 kb using Blue miniTubes in the Covaris S220 focused-ultrasonicator. Sheared DNA was blunt-end repaired, ligated into ScaI-digested dephosphorylated pJWC1, and transformed into electrocompetent *E. coli* EPI 300 cells using the same methods described in the eDNA library construction section. Subclone libraries were transformed into electrocompetent *R. metallidurans* and assayed in a top agar overlay assay for antibacterial activity. Clones displaying zones of growth inhibition were struck onto LB–tetracycline–kanamycin plates. Single colony cultures were miniprepped and transformed into electrocompetent *E. coli* EPI 300 to obtain sufficient DNA for sequencing with vector-specific primers.

Transposon mutagenesis

Escherichia coli-based random transposon mutagenesis libraries were created using the HyperMu transposon system (HyperMu <Kan-1>, Epicentre). DNA prepared from overnight cultures of the transposon mutagenesis library was transformed into electrocompetent *R. metallidurans* cells and selected on LB-tetracycline plates. The resulting *R. metallidurans-based* transposon mutant libraries were assayed using top agar overlays to identify clones that failed to display a zone of growth inhibition. DNA isolated from LB-tetracycline overnight cultures of these clones was transformed into *E. coli* EPI 300 cells,

and DNA prepped from overnight cultures of transformants was Sanger sequenced using HyperMu-specific primers.

Top agar overlays in *E. coli*

Cosmid DNA from antibacterial clones identified in our original functional screen in *R. metallidurans* was transformed into electrocompetent *E. coli* EPI 300 and assayed for the ability to confer the same antibiosis phenotype in *E. coli*. Each antibacterially active clone transformed into *E. coli*, and negative control (empty pJWC1 vector), was struck onto LB-tetracycline (20 μg mL^{-1}) plates to obtain single colonies. Plates were incubated at 30 °C overnight, followed by 3 days at room temperature, and then assayed using top agar overlays as described above.

Results and discussion

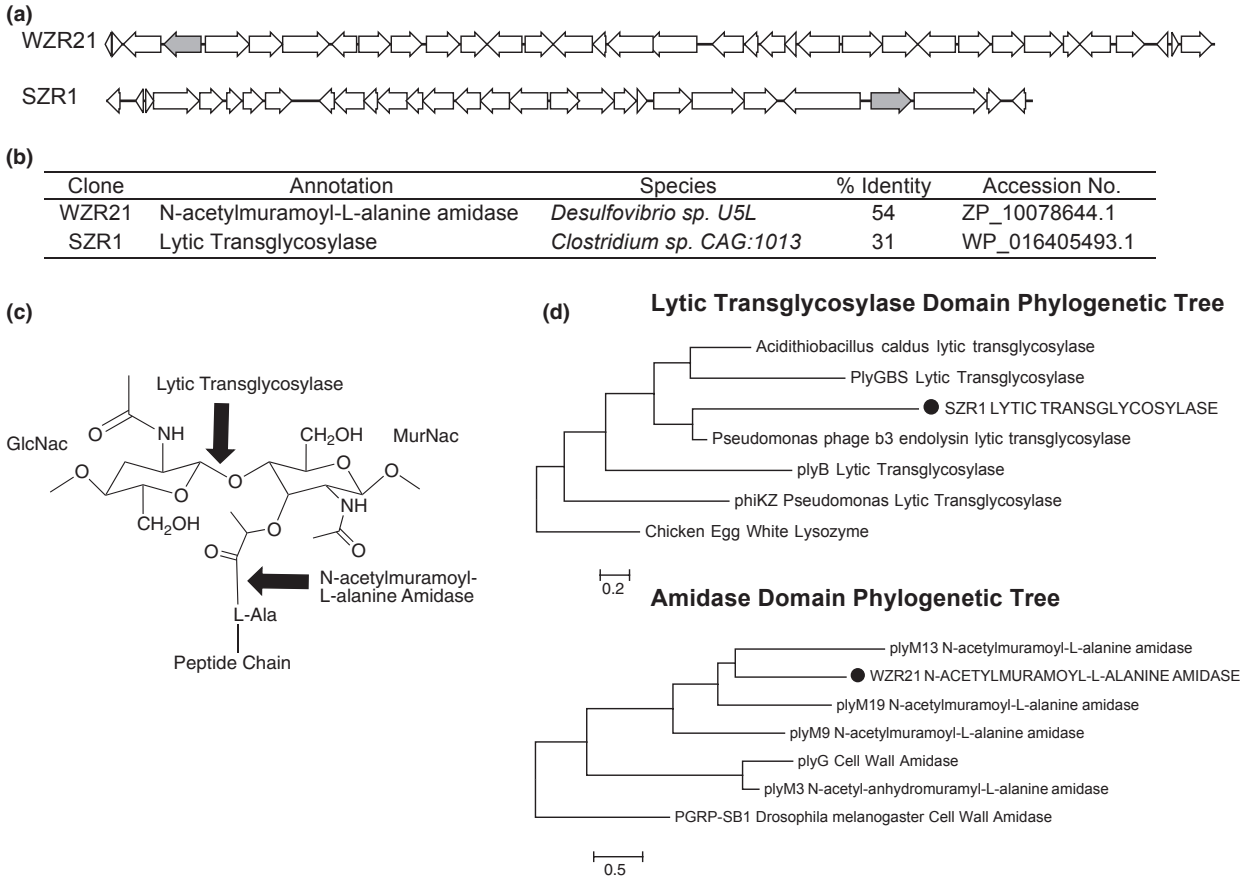
For this study, DNA extracted directly from soil collected in the Sonoran Desert (Arizona, USA) was used to construct a cosmid library in a broad-host cosmid shuttle vector. This metagenomic library comprised of 700 000 unique clones and was predicted to contain *c.* 22.5 Gb of eDNA. The library was originally constructed in *E. coli* using lambda phage packaging and transfection, and then, cosmid DNA from this *E. coli*-based parent library was electroporated into *R. metallidurans* (Fig. 1a). We selected *R. metallidurans*, a soil dwelling beta proteobacteria, as the library host for this study because it has previously shown improved heterologous expression capabilities in functional screening studies compared with *E. coli* (Craig *et al.*, 2010).

Previous *R. metallidurans*-based functional metagenomic studies have focused on the identification of antibacterially active clones producing organic extractable small molecules (Craig *et al.*, 2009, 2010). In light of recent reports highlighting the successful use of bacterial and phage enzymes as potential antibacterial therapies, we sought to explore whether antibacterially hits identified in *R. metallidurans*-based eDNA library antibiosis screens that did not produce small molecules might be a source of eDNA-encoded antibacterially active enzymes. Top agar overlays were carried out on the 700 000-membered *R. metallidurans* soil eDNA library to identify clones exhibiting antibiosis activities against a *B. subtilis* assay strain (Fig. 1b). Of the 19 clones we identified as hits in our primary antibiosis assay, cosmid DNA isolated from six clones (SZR1, SZR5, WZR9, WZR11, WZR18, and WZR21) showed the ability to confer antibacterial activity to *R. metallidurans* upon retransformation while DNA from the rest of the clones did not. In small scale fermentation studies, none of the clones with reconfirmed antibacterial activity showed the presence of clone-specific small molecule in culture broth extracts, and these clones were therefore examined both bioinformatically and functionally for the ability to encode for antibacterial active enzymes.

Cosmid DNA from each reproducible antibacterial hit was *de novo* sequenced and annotated using the online SOFTBERRY software package to identify ORFs. Putative gene functions were assigned by alignment to the BLAST and PFAM databases. Based on predicted gene functions, only two clones contained genes encoding obvious antibacterial enzymes. Clones SZR1 and WZR21 encoded enzymes with high similarity to previously well-characterized antibacterially active enzymes targeting peptidoglycan bonds (Fig. 2a and b). The cell wall lytic homolog from the first clone, WZR21, is most closely related (54% identity) to *N*-acetylmuramoyl-L-alanine amidases that cleave the amide bond connecting *N*-acetylmuramic acid to the cross-linked peptides present in the bacterial cell wall. The cell wall lytic homolog from the second clone, SZR1, is most closely related (31% identity) to lytic transglycosylases that cleave the glycosyl bond between N-acetyl glucosamine and N-acetylmuramic acid sugars in the bacterial cell wall (Fig. 2c).

PFAM-trimmed functional domains present in the predicted cell wall lytic enzymes from SZR1 and WZR21 were phylogenetically compared with soluble lytic transglycosylase and amidase domains from functionally characterized antibacterial phage endolysins (Fig. 2d). The predicted cell wall lytic domain from clone SZR1 displayed similarity to the lytic transglycosylase domain of the *Pseudomonas aeruginosa* bacteriophage B3 endolysin predicted to participate in host cell lysis (Braid *et al.*, 2004). This same analysis indicated high similarity between the predicted N-acetylmuramic-acid-L-alanine amidase domain from clone WZR21 and the antibacterially active phage amidase domain plyM13. The cell wall lytic amidase plyM13 was found in one of the only other reported metagenomic screens to identify enzymes with cell wall lytic activity, in which metagenomic libraries made from animal feces were screened using a two-step functional assay. In the two-step assay for cell wall lytic activity, phage genes were first identified by their proximity to hemolytic phage holins using a blood agar-based assay and then recombinantly expressed and tested for lytic activity against a heat-killed bacterial assay strain (Schmitz *et al.*, 2010).

For the four antibacterially active clones (WZR9, WZR11, WZR18, and WZR21) where no ORFs were found to encode enzymes commonly associated with antibacterial activity, the genetic elements required for antibacterial activity were identified through either subcloning (WZR9, WZR11, and WZR18) or transposon mutagenesis (WZR21) experiments, followed by top agar



Clone	Annotation	Species	% Identity	Accession No.
WZR21	N-acetylmuramoyl-L-alanine amidase	*Desulfovibrio sp. U5L*	54	ZP_10078644.1
SZR1	Lytic Transglycosylase	*Clostridium sp. CAG:1013*	31	WP_016405493.1

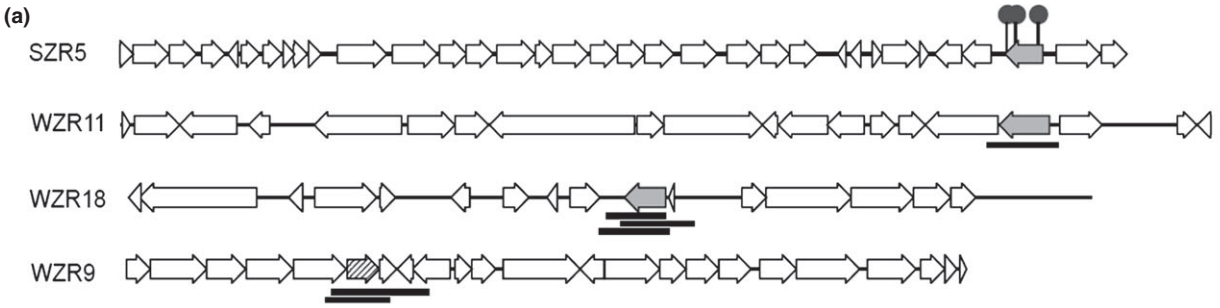
Fig. 2. (a) Sequence maps from antibacterially active clones containing putative cell wall-degrading enzymes (gray). (b) Annotation of putative cell wall-degrading enzymes associated with antibacterial activity. Species and % identity columns describe the top BLAST hit to the predicted cell wall lytic enzyme encoding gene. (c) Repeat structure of the peptidoglycan layer of the cell wall of gram-positive bacteria. Arrows indicate the points of action of the cell wall-degrading enzymes found in this study. (d) CLUSTALW-derived phylogenetic trees comparing functional domains of a representative set of cell wall lytic transglycosylases and amidases with the predicted enzymatic domains found in our phenotypic screen (highlighted in capital letters with black circles). Protein sequences were trimmed to PFAM families PF01464 (SLT Transglycosylase) or PF01520 (Amidase) domains.

overlay screening to identify either antibacterial active subclones or inactive transposon mutants.

Antibacterial activity assays run on subclone libraries made using sheared cosmid DNA from clones WZR9, WZR11, and WZR18 led to the identification of a single antibacterially active enzyme on each clone. In these studies, antibacterially active subclones were recovered and sequenced until the overlapping region on the recovered clones was reduced to the point of a single ORF (Fig. 3a). The antibacterial-associated ORFs found on clones WZR11 and WZR18 are predicted to encode for proteases, while the antibacterial ORF from WZR9 was predicted to encode for a lipase (Fig. 3a and b). Antibacterial assays conducted on transposon mutants of SZR5 yielded three unique mutants that lacked the antibacterial phenotype. The transposon insertion in all three mutants was found in an ORF that encodes for a predicted protease.

While proteases and lipases have been found in previous metagenomic screens using *E. coli* as a heterologous host, they have rarely been identified using direct screens for antibiosis. For example, previous attempts to clone proteases from environmental samples have focused on identifying industrially relevant enzymes for applications such as laundry detergent using milk agar plate assays (Kennedy *et al.*, 2011; Niehaus *et al.*, 2011). Similarly, industrially relevant lipases and esterases have been isolated in previous metagenomic studies using lipase-directed screening methods, including degradation of tributyrin or related techniques (Kennedy *et al.*, 2011; Reyes-Duarte *et al.*, 2012). There is only one case in which lipolytic enzymes conferring antibiosis were identified in an *E. coli*-based metagenomic study (Yung *et al.*, 2011).

The predicted proteases from clones SZR5, WZR11, and WZR18 were aligned to the MEROPS database – a



(b)

Clone	Enzyme	MEROPS Family	Species	% Identity	Accession No.
SZR5	Metallopeptidase	M20F	*Myxococcus xanthus*	54	YP_634146.1
WZR18	Metallopeptidase	M14B	*Leptospira santarosai*	40	WP_004466397.1
WZR11	Serine Protease	S8A	*Ferroglobus placidus*	38	YP_003434691.1
WZR9	Lipolytic	-	Uncultured bacterium	50	AAX37296.1

Fig. 3. (a) Sequence maps from clones containing antibacterially active proteases (solid gray ORFs) and lipolytic enzymes (hashed gray ORF). Antibiosis was confirmed either by the insertion of transposons (red circles) leading to loss of activity or by the expression of the antibiosis phenotype by random shotgun subclones (black lines). (b) Annotation of putative proteases and lipolytic enzymes associated with antibacterial activity. Species and % identity columns describe the closest homolog from the MEROPS database for proteases and from the BLAST database for the lipolytic enzyme.

curated collection of proteases organized into families of related enzymes by sequence similarities (Rawlings *et al.*, 2012). This analysis indicated that the SZR5 and WZR18 proteases belong to the M20F and M14B subfamilies of metallopeptidases, respectively, and the WZR11 protease belongs to the S8A subfamily of serine proteases (Fig. 3b). In a BLAST search against GenBank, the predicted lipase from clone WZR9 shows highest similarity (50% identity) to an esterase found in a soil functional metagenomic screen for ester hydrolysis activity (Kim *et al.*, 2006). The three enzymes found in the only *E. coli*-based metagenomic screen reporting antibacterial lipases show only low sequence identity (10% identity to CcAb1, 9% to CcAb2, 23% to UaAb1) to the lipolytic enzyme in clone WZR9 found in our *R. metallidurans*-based screen.

It is somewhat surprising that enzymes with antibacterial activity have not been reported more frequently in metagenomic screens given that a number of bacteria (e.g *Achromobacter lyticus* and *Myxococcus xanthus*) are known to produce bacteriolytic proteases, and genomes of some sequenced bacteria, for example predatory bacteria such as *Bdellovibrio bacteriovirus,* are rich in proteolytic enzymes (Sudo & Dworkin, 1972; Li *et al.*, 1998; Rendulic *et al.*, 2004). We hypothesized that this disparity in the finding of antibacterial enzymes in metagenomic screens may be a function of choice of heterologous host. To assess whether or not the putative enzymes identified in our *R. metallidurans*-based screens could have been discovered using the same techniques but with *E. coli* as the heterologous host, we performed the same top agar overlay assay on *E. coli* transformed with each of the six

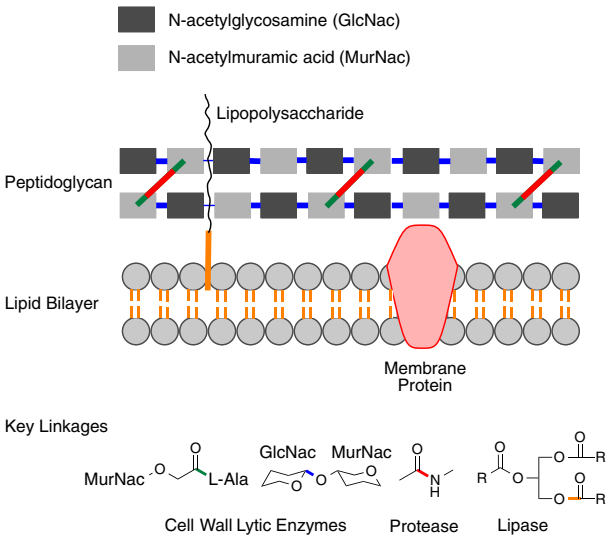


Fig. 4. The enzymes found in our metagenomic screen are predicted to target the key 'linking chemistries' (amide, ester, and glycolytic bonds) found in bacterial cell walls.

antibacterially active eDNA clones we identified. In these assays, none of the *E. coli*-based clones displayed the antibacterial phenotype observed in our *R. metallidurans*-based screens. Potential reasons for the absence of a detectable antibacterial phenotype in *E. coli* are varied. These include differences in the ability to recognize foreign promoters or ribosome-binding sites, differences in codon usage, differences in protein secretion abilities, and differences in transcription factors. This experiment

underscores the importance of diverse hosts in functional screenings of metagenomic libraries.

The six clones found in our *R. metallidurans*-based metagenomic screen for antibacterial activity contain a lipase, proteases, and cell wall lytic enzymes that are predicted to hydrolyze the three key linking chemistries (e.g. ester, amide, and glycosidic bonds) present in the bacterial cell (Fig. 4). In light of the attention that lytic enzymes have recently garnered as potential therapeutics, obtaining a diverse assortment of novel antibacterial enzymes for development as lead agents has become increasingly relevant. The diversity of antibacterial enzymes found in this study suggests that the phenotypic screening of soil metagenomes using various heterologous hosts, including *R. metallidurans*, may prove useful for identifying therapeutically relevant antibacterial enzymes in future large-scale screens of environmental samples.

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