TITLE: Functional metagenomic discovery of novel tetracycline and acarbose resistance genes from low biomass samples using METa assembly

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ABSTRACT

A significant challenge in the field of microbiology is the functional annotation of sequence novel genes from microbiomes. The increasing pace of sequencing technology development has made solving this challenge in a high-throughput manner even more important. Functional metagenomics offer a sequence-naïve and cultivation-independent solution. This forward genetics approach relies on the creation of functional metagenomic libraries (aka shotgun cloning) in which a microbial host such as *E. coli* is transformed with vectors containing metagenomic DNA fragments and, optimally, expresses any captured genes into a corresponding phenotype. These libraries can be screened or selected for a function of interest, such as antibiotic resistance, allowing the captured metagenomic DNA to be linked to a phenotype regardless of the sequence's novelty. Unfortunately, most methods for constructing functional metagenomic libraries require large input masses of metagenomic DNA, putting many sample types off limits to this toolset. Here, we show that our recently developed functional

metagenomic library preparation method, METa assembly, can be used to prepare useful libraries from much lower input DNA masses. Standard methods of functional metagenomic library preparation generally call for 5 µg to 60 µg of input metagenomic DNA. Here, we demonstrate that the threshold for input DNA mass can be lowered at least to 30.5 ng, a three-log decrease from prior art. These functional metagenomic libraries, prepared using between 30.5 ng and 100 ng of metagenomic DNA, nonetheless were sufficient to link three MFS efflux pumps to tetracycline resistance and capture two potential genes for degradation or resistance to the antidiabetic pharmaceutical acarbose. Our preparation of functional metagenomic libraries from aquatic samples and a model fecal swab demonstrate that METa assembly can be used to prepare functional metagenomic libraries from microbiomes that were previously incompatible with this approach. Functional metagenomic screens and selections are one of the few high-throughput methods that can link novel genes to functions and here we show that one of their significant drawbacks, a requirement for large amounts of metagenomic DNA, can now be overcome.

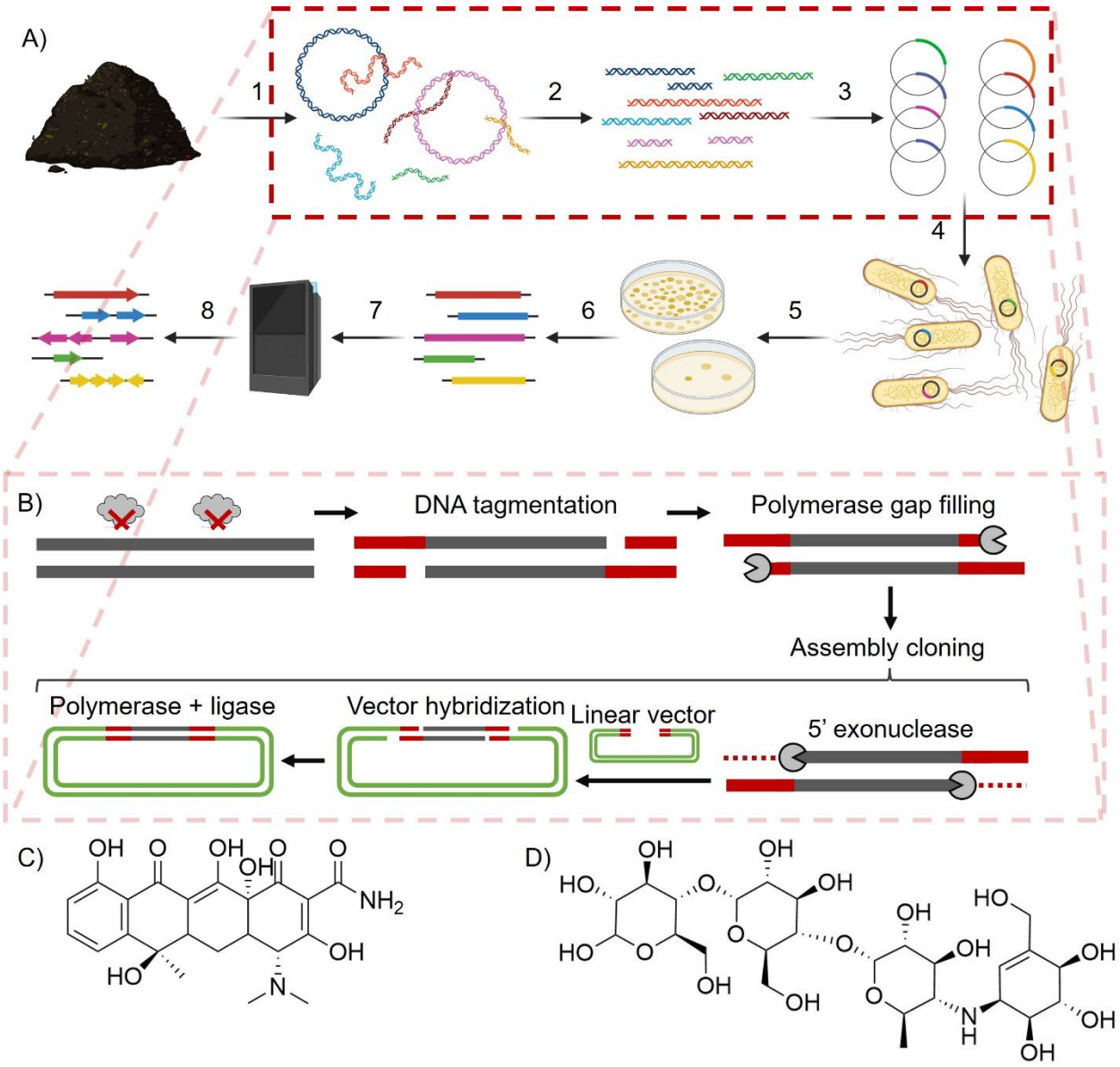

Figure 1. Functional metagenomics and METa assembly. A) General steps in the preparation and application of a functional metagenomic library: 1) Extraction of metagenomic DNA from a source microbiome. 2) Fragmentation of metagenomic DNA to preferred size range. 3) Packaging of inserts into expression vectors. 4) Transformation of host cells with vector library. 5) Screen or selection of functional metagenomic library for a phenotype of interest. 6) Collection of screened or selected metagenomic fragments. 7) Sequencing of selected inserts. 8) Open reading frame calling and annotation to identify potential genes underlying phenotypes of interest. B) Steps 2) and 3) above are modified in Mosaic ends tagmentation (METa) assembly. Fragmentation is achieved using Tn5 transposase tagmentation with mosaic end sequence oligos. Tagmented DNA is gap filled by polymerase and cloned into an expression vector with matching mosaic end sequences defining the cloning site using assembly cloning (*e.g.,* Gibson assembly). C) and D) The chemical structures of the antibiotic C) tetracycline and the diabetes pharmaceutical D) acarbose.

39 **INTRODUCTION**

Microbiomes, assemblages of bacteria, fungi, viruses and other microorganisms, are host to some of the highest levels of genetic diversity on the planet. Due to the difficulty in culturing all but a select low percentage of organisms in microbiomes (often estimated to be ~0.1% to 1%) current methods for studying microbiomes rely on DNA extracted from these communities, metagenomic DNA[1–4]. High-throughput sequencing of metagenomic DNA provides unrivaled insights into the taxonomic structure of a microbial community and can shed significant light on its functional capacity as well[3]. However, even with the sequencing revolution, the genetic diversity of microbiomes has outpaced our ability to accurately annotate novel microbial genes. Complementing homology and structure-based methods for assigning function to novel genes is the high-throughput, sequence- and cultivation-naïve method of functional metagenomics. In this method, functional metagenomic libraries, sometimes referred to as shotgun cloning libraries, are prepared by fragmenting metagenomic DNA and cloning those fragments *en masse* into an expression microorganism. Once a library is prepared, the theoretical goal is that any genes encoded within captured metagenomic DNA fragments are expressed by the host cell to give a phenotype that can be selected or screened for[4–7] (**Figure 1A**).

While functional metagenomics is a powerful method for linking novel genes to functions without requiring the growth of non-model organisms in the lab, it has its own shortcomings. These issues include the potential for gene toxicity, limited expression of foreign genes in model library hosts such as *E. coli*, limited coverage of target metagenomes, and requirements for large quantities of metagenomic DNA for preparation of the functional metagenomic library itself. We recently reported the development of a new method for preparing small insert functional metagenomic libraries (*i.e.,* 2 kb to 5 kb sized inserts) that we termed METa assembly[8]. This functional metagenomic library preparation method relies on near-random tagmentation to fragment input metagenomic DNA and an assembly-based cloning step to replace blunt ligation

(**Figure 1B**). We previously demonstrated that this method circumvents one shortcoming of functional metagenomics, limited metagenomic coverage, by showing that METa assembly libraries are more efficient at capturing DNA and can produce approximately 80-fold larger libraries per input DNA mass. We also showed that METa assembly could be used to prepare functional metagenomic libraries encoding tens of gigabase pairs (Gb) of captured DNA using sub-µg inputs of metagenomic DNA, far lower than the standard 5 µg to 20 µg input[8,9].

Here we build on our earlier results by determining if METa assembly can be used to prepare functional metagenomic libraries with significantly lower quantities of input DNA. We found that we could consistently prepare functional metagenomic libraries starting with 50 ng or less input DNA or even DNA extracted from a swab dipped into a standardized human fecal sample. These libraries were large enough that when selected for resistance to the antibiotic tetracycline and the anti-diabetic acarbose they yielded novel predicted proteins. These results suggest that many more rare or low biomass microbiomes can be studied by functional metagenomics than previously appreciated.

METHODS

Strains and materials

Routine cultivation of *E. coli* was performed at 37°C with shaking at 250 rpm for aeration in the case of liquid cultures. Cultivation for cloning and starter cultures was performed using lysogeny broth (Miller) (LB) with the addition of kanamycin at 50 µg/ml to maintain plasmids. Antimicrobial susceptibility testing was performed using Cation-Adjusted Mueller-Hinton media (Teknova, 101320-364). All *E. coli* strains used in this study are of the DH10B lineage and carry plasmids derived from pZE21[8,10,11]. *E. coli* strains were maintained at -80°C in 50% glycerol in

LB. Transposase enzymes and mosaic ends oligos were previously prepared and stored at -20°C. Standard chemicals used in this study were of high purity for molecular biology and microbiology. Kanamycin sulfate (VWR Life Science, 75856-68), acarbose (Thermo Scientific Chemical, 45908-0010), tetracycline (Aldon Corp, TT0070-5GR), ciprofloxacin hydrochloride (Corning, 61-277-RG), azithromycin dihydrate (TCI, A2076), and florfenicol (Fisher Scientific, F08115G) were stored at 4°C as powders. Working solutions were stored at -20°C as filtered aqueous solutions at 50 mg/ml concentration (kanamycin and acarbose), 5 mg/ml in ethanol (tetracycline), 10 mg/ml in alkaline water (ciprofloxacin), or 50 mg/ml in DMSO (azithromycin and florfenicol).

Preparation of 50 ng test functional metagenomic libraries

To determine if agarose extraction could be used to purify and size-select DNA following low mass tagmentation reactions, we used a DNA ladder (ThermoFisher Scientific 1 kb plus, 10787018) to mimic fragmented DNA. Mock tagmentation reactions were made with a final DNA concentration of 50 ng/µl by combining 1 µl of diluted ladder DNA with 3 µl of autoclaved milliQ water and 1 µl of 5X Taps tagmentation buffer (50 mM TAPS pH 8.5, 25 mM $MgCl_2$, 50% v/v DMF in water)[12]. We added 0.25 µl of 1% sodium dodecyl sulfate (SDS) to the 5 µl mock reactions to simulate reaction quenching followed by 1 µl of 6x DNA dye (15% Ficoll 400 w/v, 0.25% Orange G w/v in water) before loading on to a 0.7% agarose gel in TAE buffer containing SybrSafe dye (ThermoFisher Scientific, S33102). DNA bands were visualized over blue light and a clean razor was used to cut out gel portions containing bands greater than 2 kb in length. DNA was extracted from the agarose gel fragments using a gel extraction kit (New England Biolabs, T1020L) following manufacturer instructions with modification. Each agarose fragment was weighed and incubated with four volumes of gel dissolving buffer for at least 10 minutes at 55°C. The dissolved agarose was then applied to the kit's spin column, eluted, then re-applied a

second time. The standard protocol was followed until the elution step where each column was eluted twice sequentially with 6 µl of 55°C autoclaved milliQ water. DNA concentration was measured using the Quant-It DNA quantification system (Invitrogen).

To test the preparation of functional metagenomic libraries using 50 ng of input metagenomic DNA, we used a sample of previously extracted metagenomic DNA from a Canada goose fecal pellet that was stored at -20°C[8]. For library preparation, we followed our previously published METa assembly protocol[8]. Briefly, triplicate 5 µl tagmentation reactions containing 50 ng of metagenomic DNA, 1x TAPS-DMF buffer, and 25 ng of in-house transposase[12] loaded with mosaic end sequence oligos were incubated at 55°C for 7 minutes followed by quenching by addition of SDS to reach 0.05% final concentration and incubation for 5 more minutes at 55°C. To each reaction, 2 µl of 6x loading dye was added and fragmented metagenomic DNA in the range of 2 kb to 9 kb was extracted by gel excision as described above. The 12 µl of size-selected metagenomic DNA fragments were gap-filled by the addition of 12 µl of 2x Q5 DNA polymerase (New England Biolabs, M0492S) (pre-incubated at 98°C for 30 seconds) and held at 72°C for 15 minutes before purification by PCR and DNA cleanup kit (New England Biolabs, T1030S) according to manufacturer instructions with the following modifications: DNA dissolved in two volumes of binding buffer was applied to the spin column and the flow-through was applied a second time. At elution, we used two sequential applications of 6 µl of 55°C autoclaved milliQ water. Metagenomic DNA inserts were cloned into our plasmid pZE21-ME using 2x NEBuilder HiFi assembly mix (New England Biolabs, E2621L) according to manufacturer instructions (assuming an insert DNA mass of 15 ng and an average fragment size of 2 kb). The triplicate libraries were purified by PCR and DNA cleanup kit and the full 12 µl of purified library DNA was introduced into 25 µl of *E. coli* DH10B electrocompetent cells (New England Biolabs, C3020K) by electroporation at 1.8 kV in 0.1 mm cuvettes. Actual voltage reached and the electroporation time constant were recorded for all electroporation reactions to

monitor for failed or low efficiency transformations. All three libraries were rescued post-transformation by inoculation into 1 ml of SOC outgrowth medium (New England Biolabs, B9020S) pre-warmed to 37°C and incubated with aeration at 37°C for 1 hour.

Following recovery, 100 µl of 100-fold, 10,000-fold, and 1,000,000-fold diluted cultures were plated on to LB agar with kanamycin and incubated overnight at 37°C to estimate the number of unique clones resulting from each transformation. The remaining cells (~1 ml) were inoculated into 50 ml of LB with kanamycin and incubated at room temperature overnight with aeration to amplify the functional metagenomic library. Once cultures reached a 1 cm pathlength optical density at 600 nm (OD_{600}) of between 0.6 and 1.0, cells were collected by centrifugation at 4,000 rcf for 7 minutes at 4°C. Pellets were resuspended in 10 ml of LB containing kanamycin and 15% glycerol and stored at -80°C as 1 ml aliquots. The triplicate functional metagenomic library sizes were determined using the Dantas lab calculator (http://dantaslab.wustl.edu/LibSizeCalc/) considering unique cell count, determined above, average metagenomic insert size, and the proportion of clones containing an empty plasmid. This proportion and the average insert size were determined by performing colony PCR as previously described on 9 clones from each replicate library[8].

Preparation of an aquatic functional metagenomic library from a Shedd Aquarium microbiome

Preparation of a functional metagenomic library from aquatic metagenomic DNA began with a previously extracted metagenomic DNA sample. The initial sample was collected from the Shedd Aquarium in Chicago in the Underwater Beauty exhibit in May, 2019 and metagenomic DNA was extracted[13]. A functional metagenomic library was prepared from 50 ng of DNA as described above and evaluated by colony counting and colony PCR (n=14 colonies) to

163 determine library size. After preparation of the functional metagenomic library, quantification of
164 the input DNA by Quant-It revealed that only 30.5 ng of metagenomic DNA had been used as
165 input for this library and this corrected DNA mass was used in determining library preparation
166 efficiency.

167

168 **Functional metagenomic selection for antibiotic resistance**

169 The aquatic functional metagenomic library was selected for resistance to the antibiotics
170 tetracycline, ciprofloxacin, and florfenicol. Cation-Adjusted Mueller-Hinton agar plates containing
171 50 µg/ml kanamycin were prepared to contain each antibiotic at the following concentrations
172 previously established to be inhibitory to *E. coli*[14]: 8 µg/ml tetracycline, 0.5 µg/ml ciprofloxacin,
173 and 8 µg/ml florfenicol. An aliquot of the functional metagenomic library was thawed from -80°C
174 storage and a volume of suspension calculated to contain approximately 10-fold more total cells
175 than the unique cell count was plated on each antibiotic and incubated overnight at 37°C. A
176 parallel series of agar plates were plated with a similar cell count of *E. coli* cells containing only
177 empty vectors as a control.

178 Following incubation, 15 colonies each from the tetracycline and florfenicol selections
179 were collected and re-streaked onto LB agar plates containing kanamycin and either
180 tetracycline or florfenicol to obtain pure cultures of resistant strains. Plasmids were extracted
181 from each strain by miniprep kit (New England Biolabs, T1010L) and whole plasmid sequencing
182 was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and
183 annotation. The metagenomic inserts were identified by the presence of flanking mosaic end
184 sequences (5'- agatgtgtataagagacag-3') and clustered on the Clustal Omega webserver[15] to de-
185 replicate metagenomic fragments that were captured in the selection more than once. The
186 metagenomic inserts targeted for further study (TET1, TET3, TET13) were analyzed by the

MetaGeneMark program[16] to identify potential open reading frames which were subjected to analysis by Basic Local Alignment Search Tool (BLAST)[17,18] against the NCBI non-redundant database and The Comprehensive Antibiotic Resistance Database (CARD)[19,20]. The annotated metagenomic DNA fragments were made into schematics using ApE plasmid editor[21] and Microsoft PowerPoint.

Preparation of a functional metagenomic library from a simulated fecal swab

An aliquot of the ZymoBIOMICS fecal reference standard (Zymo Research, D6323) was used as a model source for a fecal microbiome swab functional metagenomic library. An aliquot of fecal standard was thawed from -80°C storage and a sterile swab was dipped into the tube and gently wrung out against the tube's side as it was removed. Metagenomic DNA was extracted from the swab using a PowerSoil Pro kit (Qiagen, 47014). The swab was agitated in 800 µl of C1 solution to release material and DNA extraction was performed following manufacturer instructions with the eluted DNA reduced in volume using a vacuum concentrator. A functional metagenomic library was prepared by METa assembly as above, with the tagmentation reaction scaled to match the 100 ng of metagenomic DNA available from the extraction. Library size was evaluated by colony count and colony PCR as above (n=14 colonies).

Functional metagenomic selection for acarbose modifying genes

To determine conditions appropriate for a functional metagenomic selection for acarbose modifying genes, we performed an agar dilution assay on minimal media plates containing maltose as the sole carbon and energy source with acarbose concentrations ranging from 0.5 µg/ml to 256 µg/ml in two-fold jumps[22]. The agar contained 0.4% w/v maltose in a modified

MDG minimal medium[23] consisting of 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.2x trace metals solution (Teknova, T1001) and was supplemented with 50 µg/ml leucine *E. coli* DH10B strains are auxotrophic for leucine. Each agar plate was inoculated with 100 µl of a 0.5 McFarland unit suspension of *E. coli* DH10B harboring an empty pZE21-ME plasmid. Plates were incubated at 37°C for roughly 48 hours then photographed to record growth.

A functional metagenomic selection for acarbose modifying genes was carried out as above with the Zymo fecal library plated to carry an estimated ten-fold excess of cells over unique clones. The functional metagenomic library was washed once in modified MDG media with no carbon added by pelleting at 8,000 rcf for 5 minutes, discarding the supernatant, and resuspending in no carbon media. The washed library was then plated on a minimal media agar plate containing 0.4% maltose as the sole carbon source supplemented with 50 µg/ml kanamycin and 256 µg/ml acarbose. In parallel, a suspension of a control *E. coli* strain with empty vector was washed as above and plated on maltose minimal media agar with acarbose at a similar predicted titer. The plates were incubated at 37°C overnight. A weather-related power outage and campus closure resulted in the plates incubating at room temperature for multiple days. Eight colonies from the Zymo fecal library plate that were judged to be substantially larger than background colonies on the empty plasmid control plate were picked, re-streaked to obtain pure cultures, and processed by miniprep kit to obtain their plasmids for sequencing as above. The plasmid sequences were analyzed as above and the most prevalent sequence (ACA1, the S-layer homology domain-containing protein) and a sequence with homology to α-amylase family glycosyl hydrolase (ACA2) were chosen for further study. The predicted amino acid sequence of ACA1 was further submitted to the Contrastive Learning-Enabled Enzyme Annotation (CLEAN) webserver for potential annotation[24].

Phylogenetic analyses

For phylogenetic analysis of the putative tetracycline efflux pumps (TET1, TET3, and TET13), we downloaded CARD amino acid sequences[19,20] for the ontology term "major facilitator superfamily (MFS) antibiotic efflux pump" and added the functionally-selected efflux pump amino acid sequences to the FASTA file. For analysis of the predicted acarbose resistance proteins (ACA1 and ACA7), we compared them to sequences for the *Klebsiella grimontii* Apg enzyme and a *Raoultella ornithinolytica* α-glycosidase from the same manuscript[25], additional reported acarbose degradation enzymes (maltogenic amylases from *Thermus* sp. IM6501, *Geobacillus strearothermophilus*, and a glycoside hydrolase from *Lactiplantibacillus plantarum*)[26–29], as well as acarbose phosphotransferases AcbK (*Actinoplanes* sp. SE50/110), GacK (*Streptomyces glaucescens*), CatK (*Streptomyces coelicoflavus* ZG0656), and Mak1 (uncultured bacterium)[30]. Phylogenetic trees were prepared by aligning the protein sequences using the Clustal Omega web server with default settings[15] and preparing a maximum likelihood phylogenetic tree from the alignment using IQ-TREE[31,32] with the "Find best and apply" substitution model, standard 100 replicates bootstrap and otherwise default settings. The resulting trees were visualized using the Interactive Tree of Life display and annotation tool[33].

Microbroth dilution antimicrobial susceptibility testing

The antimicrobial resistance of *E. coli* strains expressing metagenomic fragments encoding TET1, TET3, and TET13 were quantified by microbroth dilution assay[22,34]. Briefly, the corresponding strains and control *E. coli* carrying an empty plasmid were streaked for single colonies on LB agar with kanamycin. Approximately 10 colonies for each strain were suspended in 2 ml of MH media with kanamycin and brought to an OD_{600} of between 0.1 and 0.15 to

correspond to a 0.5 McFarland standard density, then diluted 100-fold in MH broth with kanamycin. We prepared a 96-well plate (Costar, 3370) to contain 50 µl of MH broth with 50 µg/ml kanamycin and 2-fold increasing gradients of tetracycline, chloramphenicol, or azithromycin at twice the final target concentration and added 50 µl of the diluted bacterial suspensions with each strain assayed in quadruplicate. The 96-well plates were sealed with a Breathe-Easy membrane (MilliporeSigma, Z380059) and incubated at 37°C with shaking for 20 to 24 hours. Culture density was recorded as OD_{600} with a 1 cm pathlength correction using an Epoch 2 plate reader (BioTek) and concentration-response curves were fitted to four parameter Hill equations in GraphPad Prism 10.2.3 (GraphPad Software, La Jolla, CA, USA) to determine 50% inhibitory concentration (IC_{50}) values. Differences between IC_{50} values for control *E. coli* and the tested strains (TET1, TET3, TET13) were evaluated for significance in GraphPad Prism using Brown-Forsythe ANOVA tests with multiple comparison correction using the Dunnett T3 method.

Acarbose growth inhibition

The growth of a control *E. coli* strain and strains carrying the ACA1- or ACA7-containing metagenomic DNA fragments were recorded as OD_{600} culture densities following 24 hours of aerobic growth at 37°C using a BioTek Epoch 2 plate reader. The three strains to be tested were prepared as above to give a 100-fold dilution of 0.5 McFarland suspension in modified MDG minimal media without a carbon source added. We prepared a 96-well plate where each row contained 50 µl of a different media condition (all wells containing kanamycin, acarbose concentrations are twice the final concentration): MH ± 512 µg/ml acarbose, modified MDG with 0.4% glucose ± 512 µg/ml acarbose, modified MDG with 0.4% maltose ± 512 µg/ml acarbose, and modified MDG with no carbon added. The cultures were inoculated in quadruplicate for each condition by adding 50 µl of bacterial suspension in modified MDG no carbon to the above

media. The plate was sealed with a Breathe-Easy membrane and incubated with shaking at 37°C for 24 hours before recording growth. For each media condition, final OD_{600} values were compared pairwise for each strain with or without acarbose in each media, and significance was determined in GraphPad Prism as above.

Sequence availability

Sequence files for TET1, TET3, TET13, ACA1, and ACA7 can be found as supplemental files 1 through 5.

RESULTS

METa assembly can reproducibly prepare Gb sized libraries with 50 ng of input metagenomic DNA

In our manuscript describing METa assembly, we successfully prepared functional metagenomic libraries using metagenomic DNA input quantities of 200 ng to 300 ng, approximately 25- to 67-fold less than is commonly used to prepare small insert libraries[8,9]. An advantage of our tagmentation-based fragmentation approach over physical fragmentation (*e.g., via* sonication) is its ready scalability to lower volumes and therefore lower input DNA masses. However, we suspected that DNA losses during purification and fragment size-selection by gel excision would make sub-200 ng libraries impractical. Our published METa assembly protocol tagments metagenomic DNA at a concentration of 10 ng/µl, meaning a 50 ng DNA tagmentation reaction would have a 5 µl volume where accurate pipetting starts to become difficult. To test the practicality of extracting useful DNA fragments from this size reaction, we diluted 50 ng of a

Table 1. Summary statistics of functional metagenomic libraries

Library	Input DNA	DNA mass (ng)	Ave insert size (kb)	Proportion	Library size (Gb)	Efficiency (Gb/µg)	Selections[1]
50 ng goose 1	Goose gut microbiome	50	2.48	1.00	0.89	17.8	n/a
50 ng goose 2[2]	Goose gut microbiome	50	2.62	1.00	0.10	2.09	n/a
50 ng goose 3	Goose gut microbiome	50	3.13	1.00	2.46	49.3	n/a
Aquatic	Shedd aquarium	30.5	2.77	0.88	0.72	23.6	TET, CIP, FFC
Fecal	Human fecal reference	100.8	2.70	0.90	1.48	1.48	ACA

[1]no selection (n/a), tetracycline (TET), ciprofloxacin (CIP), florfenicol (FFC), acarbose (ACA)

[2]Replicate sparked during electroporation

DNA ladder in tagmentation buffer in triplicate to a concentration of 10 ng/µl to make mock 5 µl tagmentation reactions. The triplicate mock fragmented samples were loaded on to an agarose gel for excision of bands ranging from approximately 2 kb to 10 kb (**Supplemental figure 1A**). Surprisingly to us, this resulted in 'fragmented DNA' quantities averaging 12.7 ng (± 2.7 standard deviation). Given that the total mass of the DNA bands 2 kb and higher was 16 ng, this represented a 79% yield and suggested that size-selection of fragmented DNA from a 50 ng tagmentation reaction could be feasible (**Supplemental figure 1B**).

Next, we tested this explicitly by preparing a 50 ng metagenomic DNA functional metagenomic library in triplicate. Using previously extracted goose fecal metagenomic DNA as input[8], we performed three tagmentation reactions and used gel excision to size-select for DNA fragments between 2 kb and 6.5 kb (**Supplemental figure 2A**). These fragments were used to prepare three functional metagenomic libraries by METa assembly. During the electroporation step, replicates 1 and 3 reached full voltage (1.8 kV) and had time constants of 4.6 and 4.8 seconds respectively. Replicate 2 reported a voltage of 1.65 kV and a 1.0 second time constant, consistent with the sample 'sparking'. Across the three libraries we found an average insert size of 2.75 kb with no empty vectors (**Supplemental figure 2B**), an average library size of 1.15 Gb, and a library preparation efficiency of 23 Gb/µg of input DNA. Notably, these averages include replicate 2 with a dramatically smaller library size (100 Mb compared to 890 Mb and 2.5 Gb) (**Table 1**). If replicate 2 is excluded, the average library size for the 50 ng functional metagenomic libraries increases to 1.68 Gb with an efficiency of 33.6 Gb/µg.

METa assembly preparation of a functional metagenomic library from a limited, low biomass microbiome

Two areas where input mass is limiting for classic functional metagenomic library preparation are 1) when the microbiome of interest is lower in biomass (*e.g.,* aquatic sources as opposed to soil or fecal samples) and 2) when the microbiome of interest is not readily available for re-sampling. We decided to test low-input mass METa assembly on a microbiome that fulfills both limitations. In a previous project, metagenomic DNA was extracted from the Underwater Beauty exhibit at the Shedd Aquarium in Chicago, Illinois following filtration capture of planktonic cells[13]. We used a sample of previously extracted metagenomic DNA to prepare a functional metagenomic library. Tagmentation was performed on 30.5 ng of metagenomic DNA and fragments from 2 kb to 10 kb were size selected by gel excision. Following transformation, random colonies from the functional metagenomic library were used as template for PCR to amplify captured metagenomic inserts to determine library statistics. We found that this aquatic functional metagenomic library captured 720 Mb of metagenomic DNA with an average insert size of 2.77 kb and with 12% of plasmids not containing an insert. The library preparation efficiency was calculated to be 23.6 Gb/µg (**Table 1**).

To evaluate the usefulness of this functional metagenomic library, we used it to investigate antimicrobial resistance against three antibiotics that are commonly used in aquaculture: tetracycline (**Figure 1C**), florfenicol, and ciprofloxacin[35,36]. The selection on ciprofloxacin did not yield any resistant colonies but colonies were recoverable from both the tetracycline and florfenicol plates. As an initial investigation, fifteen colonies from the tetracycline selection were collected and submitted for whole plasmid sequencing. We dereplicated repeated inserts, which resulted from plating a ten-fold excess of total cells over unique cells, resulting in three *E. coli* strains, TET1, TET3, and TET13. The metagenomic DNA captured in these strains had top nucleotide BLAST hits to *Lentibacter algarum* strain SH36 (95% coverage,

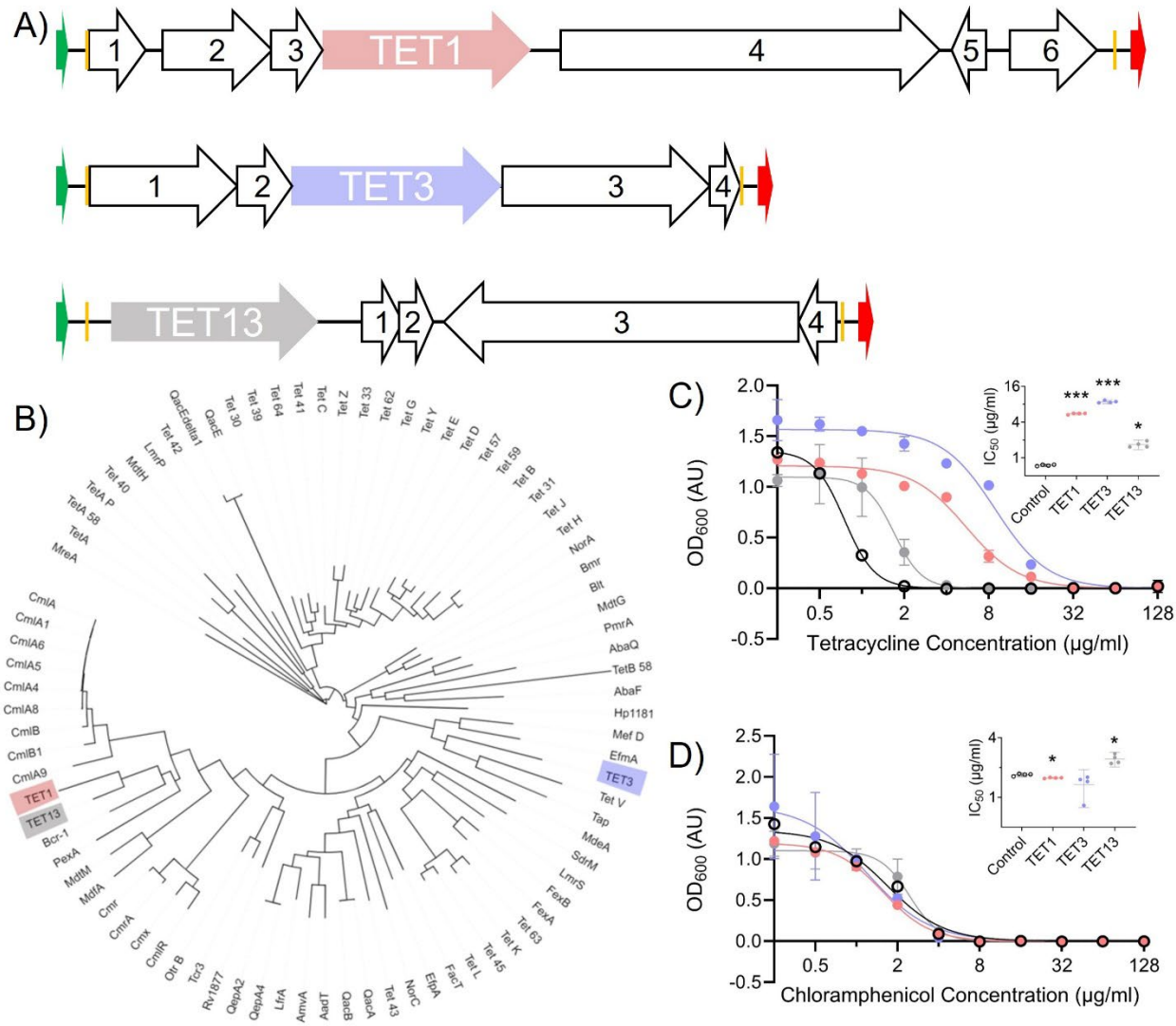

Figure 2. Tetracycline resistance in the Shedd aquarium aquatic microbiome. A) Gene schematics of the TET1, TET3, and TET13 metagenomic DNA fragments with predicted efflux pumps highlighted (TET1 red, TET3 blue, TET13 gray). Other predicted open reading frame are shown as empty arrows and plasmid backbone markers are highlighted as follows: Promoter (green), mosaic end sequence (orange), terminator (red). B) Maximum likelihood phylogenetic tree of CARD MFS efflux pumps with predicted TET1, TET3, and TET13 amino acid sequences highlighted. Microbroth dilution assays performed un quadruplicate for C) tetracycline or D) chloramphenicol resistance with calculated 50% inhibition concentration (IC_{50}) values graphed as insets. For statistical comparisons: * p<0.05, ** p<0.005, and *** p<0.0005.

82.09% identity), *Legionella pneumophila* strain NY23 (30% coverage, 66.87% identity), and *Saccharophagus degradans* strain FZY0027 (32% coverage, 70.26% identity), respectively.

We annotated the metagenomic DNA fragments captured in these three strains (**Supplemental figures 3A, 3B, and 3C**) and found them to each contain predicted MFS family

efflux pumps with low full-length amino acid identity (<30%) to their closest match in The Comprehensive Antibiotic Resistance Database (CARD)[19,20] (**Figure 2A**). When placed on a phylogenetic tree alongside known antibiotic resistance efflux pumps, TET1 and TET13 cluster with each other and a variety of efflux pumps with associated chloramphenicol resistance. TET3 instead clusters with known macrolide and tetracycline efflux pumps (**Figure 2B**). The resistance of all three strains to tetracycline, chloramphenicol, and azithromycin were measured by microbroth dilution. All three strains showed significantly increased resistance to tetracycline, with TET1 and TET3 showing growth at 8 µg/ml tetracycline or higher (**Figure 2C**). The *E. coli* strain expressing the TET3 metagenomic fragment also showed a statistically significant reduction in chloramphenicol susceptibility, while the strain corresponding to TET1 showed a significantly increased susceptibility to the antibiotic (**Figure 2D**). None of the three strains showed significant differences in azithromycin resistance compared to the control strain (**Supplemental figure 3D**).

METa assembly preparation of a functional metagenomic library from a fecal swab

Another area where the ability to prepare a functional metagenomic library using minimal DNA resources could be beneficial is within clinical settings. For example, fecal swabs provide access to the medically important gut microbiome, but extraction of metagenomic DNA from this sample type falls far below what classic functional metagenomic library preparation methods call for[14,37]. To test the possibility of medical swabs being used as input for METa assembly, we turned to the ZymoBIOMICS fecal reference with TruMatrix technology fecal standard. Each aliquot of this standard is expected to contain 10 mg (wet weight) of human fecal material in 1 ml of DNA/RNA shield preservation buffer. We sampled this microbiome by dipping a sterile swab into a thawed tube of the material followed by DNA extraction from the swab, resulting in collection of 100.8 ng of DNA. We prepared and evaluated a functional metagenomic library by

383 METa assembly as above and found it to contain an average insert size of 2.7 kb, 10% empty
384 vectors, for a total expected library size of 1.48 Gb and a library preparation efficiency of 14.7
385 Gb/µg (**Table 1**).

386 The ZymoBIOMICS fecal standard has already been sequenced and annotated for
387 known antibiotic resistance genes. As a result, we chose to investigate a different function in the
388 human gut microbiome, pharmaceutical metabolism. Specifically, we investigated modification
389 of acarbose (**Figure 1D**), a glucosidase inhibitor used in the treatment of diabetes[38,39]. Previous
390 work has established that acarbose can be metabolized by bacteria in the human gut
391 microbiome through modification by phosphorylation[30] and degradation[25–29,29,40] and that
392 acarbose can prevent growth of *E. coli* when it is cultured with maltose as a sole carbon
393 source[41]. This suggested to us that a functional metagenomic approach could be used to
394 investigate acarbose metabolism. We validated this by plating *E. coli* in a lawn on minimal
395 media agar plates containing maltose as a sole carbon and energy source with acarbose
396 concentrations ranging from 0.5 µg/ml to 256 µg/ml. We observed confluent growth until an
397 acarbose concentration of 256 µg/ml at which point almost no colonies were observed
398 (**Supplemental figure 4A**). In contrast, we confirmed growth in the presence of 256 µg/ml
399 acarbose when *E. coli* was plated on minimal media containing glucose as a carbon source
400 (**Supplemental figure 4B**).

401 Based on these observations, we performed a functional metagenomic selection for
402 growth on maltose in the presence of acarbose. An aliquot of the Zymo fecal functional
403 metagenomic library and a similar titer of *E. coli* containing an empty vector were plated on to
404 minimal media plates containing maltose as the sole carbon source and acarbose at a
405 concentration of 256 µg/ml. Inclement weather resulted in the selections incubating at room
406 temperature for multiple days after which both plates showed a lawn of very small colonies.
407 However, the agar plate that was seeded with the functional metagenomic library contained

several larger colonies, the eight largest of which were collected for further study (**Supplemental figure 4C**).

Following whole plasmid sequencing of these eight colonies, we found that four contained identical inserts to each other and refer to the representative strain as ACA1. Protein BLAST analysis of the single predicted open reading frame of ACA1 annotated it as an S-layer homology domain-containing protein (**Figure 3A**). We used the CLEAN enzyme function annotation tool[24] to suggest a potential function. Interestingly, E.C. families 3.2.1.8 and 3.2.1.4 were suggested as potential hits and both belong to the glycosidase family of enzymes. Analysis of the ACA1 metagenomic DNA fragment by nucleotide BLAST failed to find a high-quality hit, with the top predicted match (12% coverage, 77.18% identity) being to a bacteriophage and the top predicted bacterial match (3% coverage, 90.74% identity) being to an *Intestinibacillus* species. Plasmid sequencing of one of the other colonies (termed ACA7) found a metagenomic insert predicted to encode three open reading frames (**Supplemental figure 5A**), one of which was predicted to encode an α-amylase family glycosyl hydrolase gene (**Figure 3A**). Unlike the enigmatic ACA1, the nucleotide sequence associated with ACA7 had a high-quality hit on BLAST, *Alistipes onderdonkii* susbspecies *vulgaris* (100% coverage, 97.18% identity).

To orient the predicted effectors of ACA1 and ACA7 we placed them on a maximum likelihood phylogenetic tree built on the sequences of known acarbose degrading enzymes and known acarbose phosphotransferase (or kinase) enzymes (**Figure 3B**). As expected, these two functions, glycan degradation and phosphorylation, segregate on the phylogenetic tree. The predicted α-amylase ACA7 clusters with the known acarbose degrading enzymes while ACA1 does not appear to cluster with either enzyme group; its exact function is currently under investigation.

To verify that ACA1 and ACA7 counteract the inhibitory activity of acarbose on *E. coli* growth on maltose, we compared the growth of the two corresponding strains to an empty plasmid control *E. coli* strain under a variety of conditions. When cultured in Mueller-Hinton broth, the addition of acarbose resulted in a decreased final optical density of the control *E. coli*, potentially reflecting the mixed carbon sources available in that media. In contrast, the ACA1 and ACA7 strains showed no effect on addition of acarbose **(Supplemental figure 5B**). In

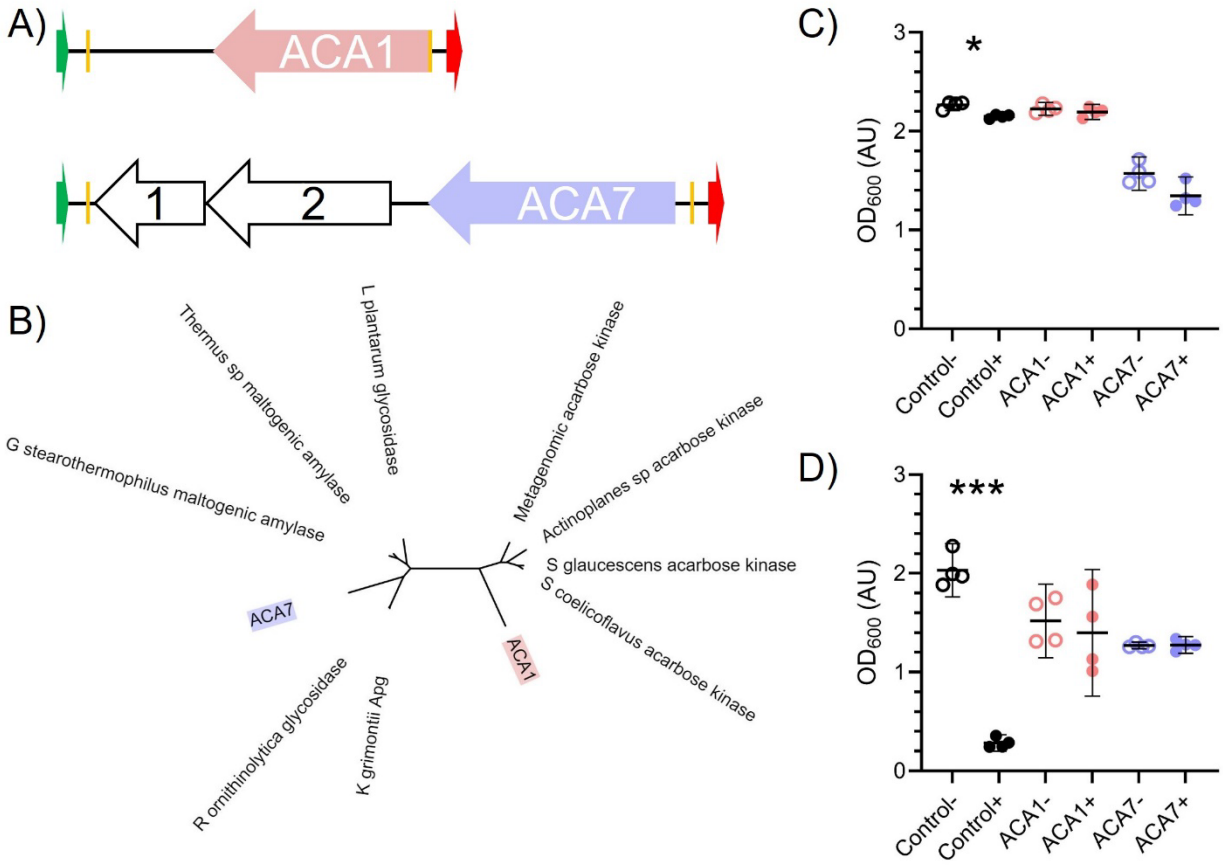

Figure 3. Predicted acarbose degradation enzymes from the human gut microbiome. A) Gene schematics of the ACA1 and ACA7 metagenomic DNA fragments with the predicted effector genes highlighted (ACA1 red and ACA7 blue). Predicted genes captured on the same DNA fragment are shown as empty arrows. Plasmid backbone elements are: Promoter (green), mosaic end sequence (orange), terminator (red). B) Maximum likelihood phylogenetic tree of the predicted proteins for ACA1 (red) and ACA7 (blue) in the context of known acarbose degrading or modifying enzymes (*i.e.,* kinases). C) and D) Culture densities with or without addition of 256 µg/ml acarbose after 24 hours of growth in minimal media with C) 0.4% glucose or D) 0.4% maltose as carbon sources. Pairwise statistical comparisons for the effect of acarbose addition were made for each strain and culture condition: * $p<0.05$, ** $p<0.005$, and *** $p<0.0005$.

minimal media with glucose as a carbon source, all three strains showed essentially no change in growth in the presence of acarbose, although we observed a minor but statistically significant decrease in the OD_{600} reached by the control strain (**Figure 3C**). Finally, when grown in minimal media with maltose as a carbon source, the growth of the empty plasmid *E. coli* strain is nearly entirely inhibited in the presence of acarbose while ACA1 and ACA7 show no difference in growth on the addition of acarbose to the media (**Figure 3D**).

DISCUSSION

Here we report that our METa assembly method for preparing functional metagenomic libraries can be extended to preparing libraries from low biomass and otherwise precious microbiome samples where metagenomic DNA would otherwise be limiting. As an illustration of this potential, we prepared modestly-sized functional metagenomic libraries from metagenomic DNA extracted from an aquarium filtrate and from a simulated fecal swab. We further demonstrated the utility of the resulting functional metagenomic libraries by applying them to identify likely tetracycline efflux pumps and novel acarbose degradation genes.

A key result of our experiments is that using METa assembly to prepare functional metagenomic libraries allows us move the minimal metagenomic DNA input to approximately two orders of magnitude lower than previous standard protocols[9,14]. We show that, across our experiments, 50 ng or lower input metagenomic DNA masses can be used to prepare functional metagenomic libraries encoding near or low Gb libraries (**Table 1**). We propose, and demonstrate, that this opens new sample types to analysis by functional metagenomic selections or screens. These include samples that are intrinsically of low biomass, either due to difficulty in collection or their rarity (*e.g.,* aquatic metagenomes, but also historically banked microbiomes or samples from remote regions). These also include microbiomes sampled using

medical swabs. While our example used Zymo fecal reference material, the mass of the metagenomic DNA extracted from the swab and used to prepare a functional metagenomic library was noticeably less than what has been reported in extractions from other fecal swabs (*i.e.,* ~100 ng vs ~370 ng). Functional metagenomic selections have been proposed to be useful for medical applications[42], and our results support that.

Our previous smallest METa assembly input, 200 ng, resulted in a functional metagenomic library encoding approximately 13.5 Gb with a preparation efficiency of ~68 Gb/µg[8]. Here, our 50 ng, 30.5 ng, and 100.8 ng libraries averaged an efficiency of between 20.5 Gb/µg and 24.1 Gb/µg, depending on the inclusion or exclusion of the library that sparked during transformation, suggesting that lower input DNA masses may come with decreased library preparation efficiency (**Table 1**). It is possible that this is due to DNA losses during the multiple purification steps involved in library preparation. Nevertheless, these levels of efficiency still perform better than our previously estimated average of 1 Gb/µg using blunt ligation-based techniques[8].

While the functional metagenomic libraries prepared here are smaller than our previous high-mass input libraries, their usefulness is demonstrated by their capture of genes for tetracycline efflux pumps and acarbose degradation (**Figures 2 and 3**). The three studied predicted tetracycline efflux pumps show homology to previously identified MFS efflux pumps, with the predicted amino acid sequences having near complete coverage and between 65% and almost 100% identity to hits in the NCBI database. In contrast, they have only low identity to CARD hits, with full length alignments showing only 28% to 29% identity. It is common for proteins identified through functional metagenomic selections for antibiotic resistance to have high identity to known hits, but for those proteins to not previously have been identified as having antibiotic resistance potential[43]. Tetracycline efflux pumps are found within many environments[44–46] but the substrate specificity of novel transporters makes the identification of

potential resistance genes from environmental microbiomes difficult. This is illustrated by the contrast between the apparent clustering of the TET1 and TET13 MFS pumps with amphenicol efflux and TET3 with macrolide efflux (**Figure 2B**) and their respective lack of resistance to chloramphenicol (**Figure 2D**) or azithromycin (**Supplemental figure 3D**). Our work highlights the important role that functional metagenomic selections can play in filling this knowledge gap.

Similarly, one of the predicted proteins from the acarbose/maltose selection, ACA7, has very high coverage and identity to known proteins in the NCBI database that lack an acarbose-modification annotation. On the other hand, the predicted protein from ACA1 only showed homology proteins with limited annotation as an S-layer homology domain-containing protein. Interestingly, while ACA7 appears to clearly cluster with known acarbose degrading proteins (a class which expanded again recently following a functional metagenomic screen for glycan breakdown[40]), ACA1 does not (**Figure 3B**). Instead, ACA1 may represent a new acarbose modifying enzyme. When ACA1 is compared against the CARD database by protein BLAST, the top hit is a chloramphenicol acetyltransferase with an E-value of 0.09. We are currently investigating the possibility that ACA1 may have transferase activity.

While we see the opening of low biomass functional metagenomic libraries as a significant advancement, limitations to the method remain. First, this method relies on *E. coli* cells as the host organism for the functional metagenomic library and therefore is subject to potential limits on what metagenomic genes can be successfully expressed by this host[47]. In theory this can be surmounted using alternative hosts[48–50], and the added efficiency of METa assembly may be more important when using strains with lower transformation efficiency than commercial *E. coli* strains. Second, there are potentially diminishing returns to preparing smaller functional metagenomic libraries even if their preparation efficiency increases. However, the libraries prepared here were shown to still be useful by their capture of potentially novel antibiotic efflux pumps and acarbose degradation enzymes even without covering more than a

fraction of the genetic diversity found in many microbiomes. Tagmentation reactions are regularly performed on metagenomic DNA samples with 5 ng or less mass[12,51,52] suggesting that the limit for functional metagenomic libraries could be pushed even further.

In conclusion, while high-throughput DNA sequencing has helped circumvent the great plate count anomaly and has revolutionized microbiome research, methods for accurately annotating novel genes have not managed to keep pace. Machine-learning tools will likely be able to help with this issue but will also require large amounts of high-quality sequence-function correlation data. Functional metagenomics is well placed to supply this data and, in the meantime, can make significant strides in narrowing the sequence annotation gap. We propose that the fulfillment of this potential by functional metagenomics is significantly aided by the ability to make the necessary libraries more efficiently and using less input DNA, a precondition that we demonstrate is reached here.

Acknowledgements

CRediT contributions: Conceptualization – TSC; Data curation –TSC; Formal analysis – HMA, TSC; Funding acquisition – EMH, TSC; Investigation – HMA, EPB, EF, TSC; Project administration – TSC Resources – FJO EMH, TSC; Supervision – EMH, TSC; Visualization – HMA, EPB, TSC; Writing - original draft – TSC; Writing - reviewing and editing – HMA, EPB, FJO, EMH, TSC. We would like to thank Chrissy Cabay, William Van Bonn, and James Clark for their help in acquiring the Shedd Aquarium sample and Jinglin Hu for the metagenomic DNA. This work was supported by a grant from the Florida State University Council for Research and Creativity and by start-up funds from the Florida State University College of Medicine.

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