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Exometabolomics assisted design and validation of synthetic obligate mutualism

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ABSTRACT

Synthetic microbial ecology has the potential to enhance the productivity and resiliency of biotechnology processes compared to approaches using single isolates. Engineering microbial consortia is challenging; however, one approach that has attracted significant attention is the creation of synthetic obligate mutualism using auxotrophic mutants that depend on each other for exchange or cross-feeding of metabolites. Here, we describe the integration of mutant library fitness profiling with mass spectrometry based exometabolomics as a method for constructing synthetic mutualism based on cross-feeding. Two industrially important species lacking known ecological interactions, *Zymomonas mobilis* and *Escherichia coli,* were selected as the test species. Amino acids exometabolites identified in the spent medium of *Z. mobilis* were used to select three corresponding *E. coli* auxotrophs (*proA, pheA* and *IlvA*), as potential *E. coli* counterparts for the co-culture. A pooled mutant fitness assay with a *Z. mobilis* transposon mutant library was used to identify mutants with improved growth in the presence of *E. coli*. An auxotroph mutant in a gene (ZMO0748) with sequence similarity to cysteine synthase A (*cysK*), was selected as the *Z. mobilis* counterpart for the co-culture. Exometabolomic analysis of spent *E. coli* medium identified glutathione related metabolites as potentially available for rescue of the *Z. mobilis* cysteine synthase mutant. Three sets of co-cultures between the *Z. mobilis* auxotroph and each of the three *E. coli* auxotrophs were monitored by optical density for growth and analyzed by flow cytometry to confirm high cell counts for each species. Taken together, our methods provide a technological framework for creating synthetic mutualisms combining existing screening based methods and exometabolomics for both the selection of obligate mutualism partners and elucidation of metabolites involved in auxotroph rescue.

KEYWORDS: Microbial synthetic biology, synthetic obligate mutualism, exometabolomics, mutant fitness profiling, cross-feeding, mass spectrometry

Microbes in nature are commonly found as part of complex, interdependent microbial communities.[1,2] In these environments, metabolite exchange constitutes a common interspecific interaction[3–6] and represents an exploitable feature for designing mutualistic relationships. While biotechnologies largely use single isolates, it has been demonstrated that mutualistic microbial consortia can offer substantial benefits.[2,7–11] The field of

synthetic ecology focuses on constructing artificial communities to extend synthetic biology approaches to microbial consortia[7,12], and as a means for controlled studies of microbial ecology.[13,14] Of particular interest is the construction of obligate mutualistic relationships, in which each organism requires at least one activity and/or product of one or more other organisms for growth.[5] Typically, mutualism design has relied upon having an adequate understanding of the constituents' genomes and metabolisms in order to make an informed selection of specific autotrophic mutant pairs.

Screening of whole-genome, randomly mutated libraries for rescue[15,16], combined with characterization of metabolite uptake/release (exometabolomics), has the potential to catch more novel metabolic interactions than either approach alone and may be preferable for organisms that are amenable to mutagenesis and have poorly annotated genomes. Mass spectrometry based exometabolomics has been used to examine how cells transform their small molecule environments and is valuable for a range of applications including biofuel development[17], synthetic biology[18], detection of novel metabolites[19,20] and investigating the metabolic interactions and dependencies of cells.[21–23] Mutant fitness profiling[24,25] has also been used extensively for a variety of purposes including the phenotypic and functional characterization of uncharacterized genes[26–28], the improvement of strains used in bio-production methodologies[15], identification of electron transport systems in syntrophic co-cultures[29], and evaluation of gene regulation in relation to metabolic needs.[16] Recently, exometabolomics has been used in combination with mutant library fitness profiling in a high-throughput fashion to identify bacterial mutants that have lost the ability to utilize specific metabolites.[30] However, their combined use in mutualism design represents a novel methodology.

Here, we describe the use of exometabolomics and mutant fitness profiling for the design of obligate synthetic mutualism between two organisms. We demonstrate this approach using *Zymomonas mobilis* and *Escherichia coli,* two bacteria with available mutant libraries[26,31–33] that lack known ecological interaction. Each has been utilized separately in mono and co-culture systems for enhanced lignocellulosic bioethanol production. *Z. mobilis* is of particular interest due to its efficient fermentation of glucose to ethanol[34] while *E. coli* can ferment xylose in addition to glucose (the two main sugars in lignocellulose).[35,36] In fact, previous experiments have demonstrated an inhibitory effect of wild-type *Z. mobilis* on wild-type *E. coli* growth in co-culture (a non-mutualistic interaction)[37], which was not observed in the mutant based synthetic mutualism in our studies. Auxotrophic mutants of each species were selected from existing mutant libraries such that when cultured together in minimal medium, they would rely on metabolite cross-feeding based rescue.
Exometabolomics can be used in consortia studies to generate two useful pieces of information. In consortia design, involving organisms without known mutualistic interactions, exometabolomics based determination (and optional quantification) of the metabolites released by the constituents in isolation can be used to make more informed decisions on mutant selection/construction to force a mutualistic relationship (Figure S1). In consortia analysis, involving organisms with existing or known interactions, exometabolomics can reveal the metabolites responsible for rescue in a cross-feeding based relationship where the metabolite exchange has previously been undefined. In this study, a series of experiments, using mutant fitness profiling for mutant

selection and exometabolomics for both mutant selection and cross-feeding determination, were performed in the construction of synthetic obligate mutualism and validation of metabolite exchange (Figure 1).

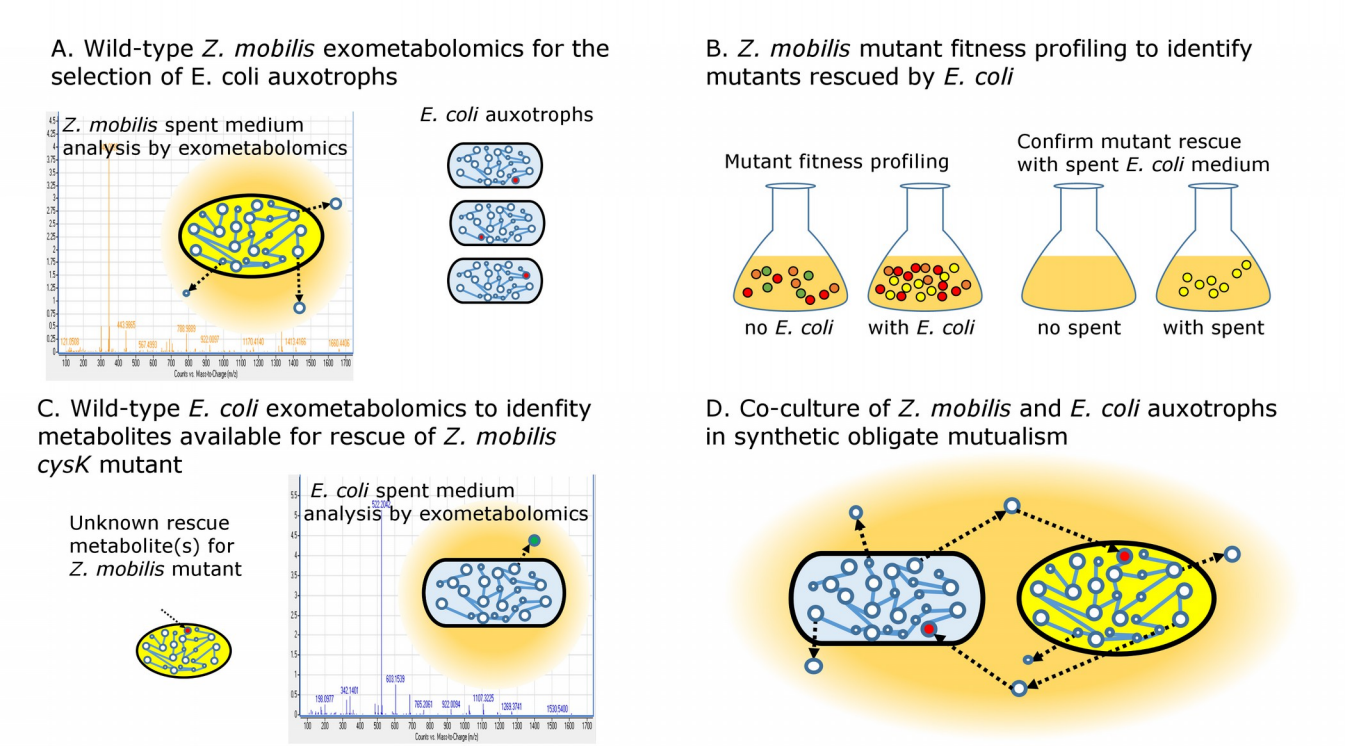

Figure 1. Workflow for synthetic obligate mutualism design with *Z. mobilis* and *E. coli* auxotrophs.

Synthetic obligate mutualism was designed and investigated between one each of three *E. coli* auxotrophs and one *Z. mobilis* auxotroph using a combination of exometabolomics techniques and mutant fitness profiling. The cultures were designed in four steps: exometabolomics analysis of wild-type *Z. mobilis* cultured in ZMMG to determine metabolites available for potential cross-feeding to *E. coli* mutants and for the selection of corresponding *E. coli* auxotrophs (A), *Z. mobilis* mutant library fitness profiling to determine which mutants have a fitness benefit when cultured with *E. coli* (B), exometabolomics of wild-type *E. coli* to determine the metabolites responsible for the *Z. mobilis* rescue in the fitness assay (C) and validation of the design by successful co-culture of the auxotrophs of each species (D).

Exometabolomics analysis of wild-type *Z. mobilis* was used to select *E. coli* mutant auxotrophs that should theoretically be rescued in mutualistic co-culture with *Z. mobilis* (Figure 1a). The spent medium of wild-type *Z. mobilis* (ZM4) cultured in ZMMG was analyzed using hydrophilic interaction liquid chromatography (HILIC) mass spectrometry (MS) to detect metabolites that are synthesized and released by *Z. mobilis*. Eight amino acids (arginine, glutamate, glutamine, isoleucine, leucine, phenylalanine, proline and valine) were detected and used for the selection of corresponding *E. coli* mutants (Tables S1-S3). Using information from the COG database[38] and EcoCyc[39], 3 KEIO deletion mutants[32] in the biosynthetic pathways of proline, phenylalanine and isoleucine (*ΔproA761::kan*, *ΔpheA762::kan*, and *ΔIlvA723::kan*, respectively) that are auxotrophic in defined minimal media[40–42] were selected. It should be noted that while proline has an alternative biosynthetic pathway (using ArgA, ArgB, ArgC and ArgE)[40] and the IlvA isoenzyme TdcB can

catalyze the same reaction as IlvA[41], these do not appear to be functional pathways in the single gene deletion mutants during growth in minimal media (previously only observed as functional in double mutants affecting substrate availability or enzyme expression in the alternative pathways).[40,41] Additionally, PheA has two domains with different enzymatic functions (chorismate mutase and prephenate dehydratase); another enzyme TyrA also has chorismate mutase activity so the *pheA* mutant should still produce tyrosine while having auxotrophy for phenylalanine. The auxotrophic phenotype of each of the three *E. coli* mutants was confirmed by comparing monoculture growth (determined from OD at 600nm) in M9 minimal medium to growth in LB broth (Table S3). To confirm rescue of the mutants by *Z. mobilis* exometabolites, spent medium from wild-type *Z. mobilis* in ZMMG was diluted 1:1 with fresh ZMMG and used for the culture of the *E. coli* mutants. ZMMG supplemented with amino acids additionally confirmed that mutants were rescued as predicted based on their mutations. While none of the mutants were capable of growth in ZMMG, they all successfully grew in 50% spent medium and metabolite supplemented medium (Figure 2a). Growth in 100% spent Z. mobilis medium resulted in decreased growth possibly due to ethanol production or depletion of ZMMG vitamins/minerals required by the E. coli for growth (data not shown).

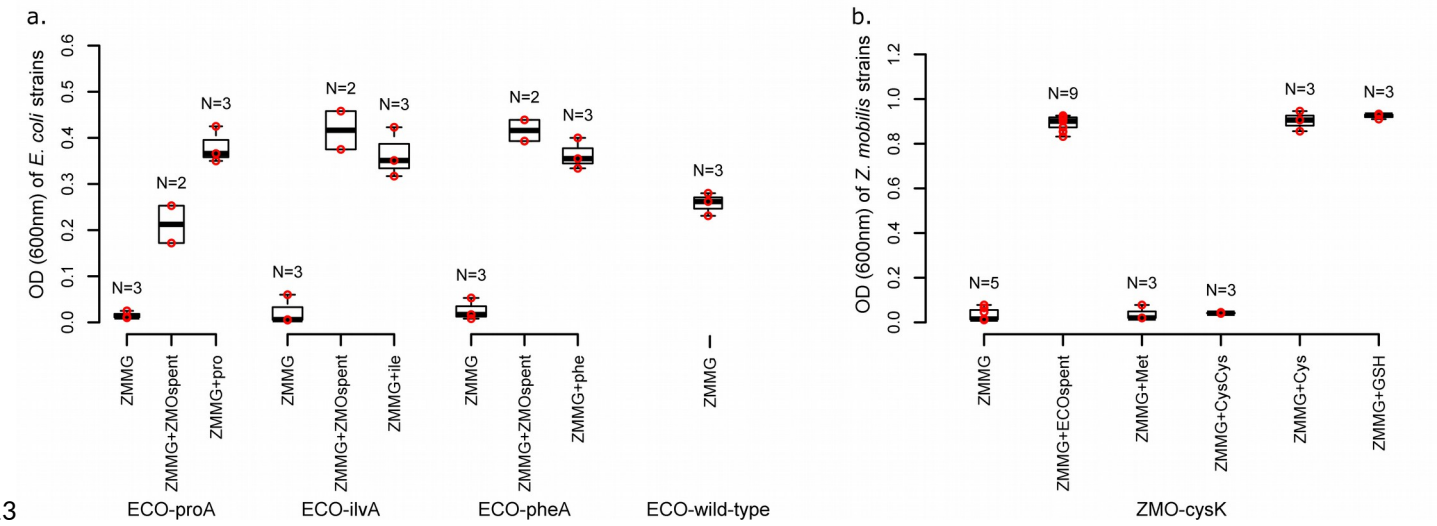

Figure 2. Mutant rescue by supplementation of ZMMG with rescue metabolites or spent medium from the partner strain. Wild-type *Z. mobilis* spent ZMMG was collected and used for the culture of the three *E. coli* auxotrophs for proline, isoleucine and phenylalanine at 50% spent medium in ZMMG; *E. coli* mutants were also cultured in ZMMG supplemented with the rescue metabolite that was used for the strain selection at 10uM each of proline, isoleucine or phenylalanine (a). Wild-type *E. coli* spent ZMMG medium was collected and used for the culture of *Z. mobilis* mutant ZMO0748 at 100% spent medium; *Z. mobilis* mutant ZMO0748 was also cultured in ZMMG that was supplemented with 200uM each of cysteine, cystine, methionine and glutathione (b). Mass spectrometry analysis of uninoculated ZMMG compared with *E. coli* spent ZMMG, indicate that only a small portion of the glucose from ZMMG is consumed by the *E. coli* in ZMMG under these growth conditions, thus *Z. mobilis*, which requires glucose for fermentative growth is capable of growth in the *E. coli* spent ZMMG.

The selection of *Z. mobilis* mutants for rescue by *E. coli* was performed using an established mutant library screening based approach(Figure 1b).[29] Wild-type *Z. mobilis* strain ZM4 was used to generate a DNA-bar coded transposon library consisting of 7432 mutants.[15] We identified *Z. mobilis* mutants with increased fitness in the presence of wild-type *E. coli* using a competitive, genome-wide fitness assay with barcoded transposon mutants. The top ten *E. coli* rescued *Z. mobilis* mutants (when ranked by largest positive difference between fitness with *E. coli* and fitness without *E. coli*) included transposon insertions in the following genes: indole-3-glycerol-phosphate synthase, cysteine synthase, cell division gene *ftsA*, anthranilate phosphoribosyltransferase, anthranilate synthase component I, glutathione S-transferase domain protein, glutamine amidotransferase of anthranilate synthase, cell division gene *ftsW*, and N-acetylglucosamine-6-phosphate deacetylase (Figure 3 and Tables S4 and S5). Of the tryptophan and cysteine synthesis mutants, only mutants in a gene with sequence similarity to cysteine synthase A (ZMO0748; *cysK*) failed to grow in minimal medium (Table S6). Because *Z. mobilis* has a polyploidy genome, genes essential for growth in rich medium may only form mixed/heterozygous mutants under kanamycin selection[15], thus the tryptophan synthesis pathway mutants are likely heterozygotes with at least one copy of the wild-type allele allowing for growth in minimal medium. Cysteine synthase A is likely only essential for growth in minimal medium not in rich resulting in a stable homozygous mutant during library generation.[15] The *ΔcysK*::Tn5 mutation in ZMO0748 would render the mutant unable to synthesize L-cysteine from the precursors O-acetyl-L-serine and sulfide; a strain of this mutant was selected for use as the *Z. mobilis* auxotroph in co-culture.

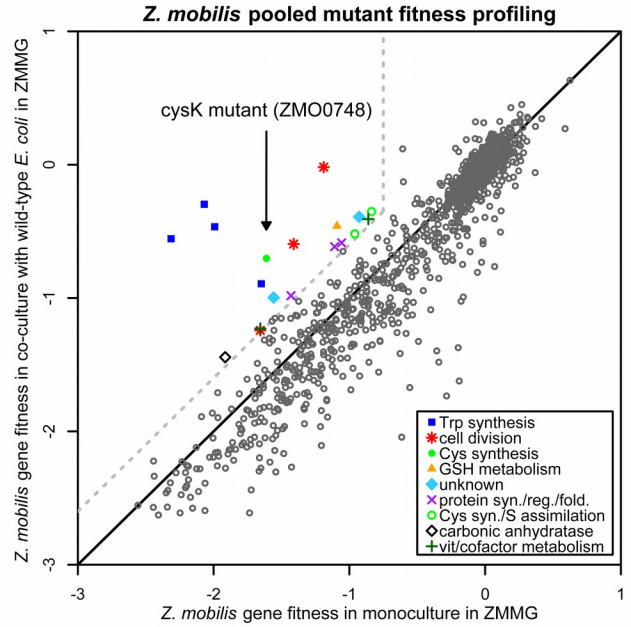

Figure 3. *Z. mobilis* pooled mutant fitness profiling with and without wild-type *E. coli* for selection of mutants. The gene fitness scores for the *Z. mobilis* mutant library when cultured in ZMMG as a monoculture versus a co-culture with *E. coli* are used to determine which mutants have enhanced fitness in the presence of *E. coli*. The gene fitness scores are calculated from the strain fitness values using a previously described method[15] where strain fitness is equal to the log2 ratio of the END mutant tag abundance to the START mutant tag abundance; negative values indicate a decrease in abundance and positive values indicate an

increase in abundance. Each experimental condition was done only once, however each gene typically has multiple unique mutants (Tables S4, S5). A mutant is considered “rescued” when it has poor fitness (decreased abundance) in monoculture (more likely to be an auxotroph) and when the fitness in the co-culture exceeds that in monoculture; the rescue cutoff region, delineated with the grey dashed line, is calculated as follows: gene fitness in monoculture < -0.75 and (gene fitness in co-culture minus gene fitness as a monoculture) > 0.4. Rescued *Z. mobilis* mutants are colored by predicted functional classification. The solid black lines represents monoculture = co-culture fitness. ZMO0748 (cysK), was identified as an auxotroph and selected as the *Z. mobilis* mutant to be used for mutualism construction with *E. coli*.

Exometabolomics analysis of wild-type *E. coli* was performed to check for metabolites capable of rescuing the *Z. mobilis cysK* mutant (Figure 1c). Surprisingly, exometabolomics analysis did not detect cysteine in the spent medium of wild-type *E. coli*. Presumably, a sulfhydryl containing compound or cysteine analog could act as the rescue metabolite(s) in which the sulfide is already incorporated into an organic molecule as a sulfhydryl group. To identify possible cross-feeding metabolites responsible for the observed mutant rescue, we further analyzed the wild-type *E. coli* exometabolomic data for additional sulfur containing metabolites and identified glutathione reduced, glutathione disulfide, adenosylmethionine and gamma-glutamylcysteine (Table S2). Rescue of *Z. mobilis cysK::ΔTn5* by *E. coli* exometabolites was confirmed by culture of the mutant in 100% spent *E. coli* medium (Figure 2b). To determine which sulfur containing compounds are capable of rescuing *Z. mobilis cysK:: ΔTn5*, the mutant was cultured in ZMMG supplemented with methionine, cysteine, cystine or glutathione; we found that of these, both cysteine and glutathione are capable of rescuing *Z. mobilis ΔcysK::Tn5* (Figure 2b). We cannot exclude the possibility that *E. coli* supplied free cysteine may be involved in the rescue and is below the instrument detection limits. Based on homology predictions, genes ZMO1388, ZMO01345/ZMO1776 may be hydrolases, 3.4.19.13 (ggt), 3.4.11.1/2 (pepN), respectively, capable of releasing cysteine from glutathione and related metabolites.[43] We conclude that glutathione and/or gamma-glutamylcysteine from spent *E. coli* medium are capable of and sufficient for the rescue of the *Z. mobilis* cysteine auxotroph.

Using the above described exometabolomic and mutant fitness data that was used for the selection of auxotrophs from each species, we designed 3 sets of synthetic mutualism pairs between *E. coli* and *Z. mobilis.* The *Z. mobilis* cysteine synthase mutant (*ΔcysK*) rescued by *E. coli* released glutathione related metabolites was co-cultured with each of the three *E. coli* amino acid biosynthesis mutants (*ΔproA*, *ΔpheA*, and *ΔIlvA*) rescued by amino acids released by *Z. mobilis.* As expected, auxotrophs of each species, previously shown to be incapable of growth as a monoculture in ZMMG, grew when cultured together in ZMMG as confirmed by OD at 600nm (Figure S2). To further verify that each species was contributing to the biomass abundance (based on optical density) once the culture had reached stationary phase, flow cytometry analysis was used to quantify the cells (Figure 4). Other options for evaluating end-point cell concentrations such as plate counts and counting chambers presented challenges due to the ability to differentiate and/or

isolate the colonies or cells of each species. These co-cultures represent obligate mutualistic growth since they are only capable of growth in minimal medium when cultured together. Based on the selection of auxotrophs, this mutualism appears to be characterized by an exchange of metabolites with each of the three *E. coli* mutants synthesizing and releasing glutathione related metabolites and the *Z. mobilis* mutant synthesizing and releasing proline, isoleucine and phenylalanine. The actual method of metabolite release from the partner cell is unknown; the rescue metabolite may be actively exported, hydrolyzed from larger peptides/proteins outside of the cell and/or made available following cell death and lysis. These released metabolites are then available for use by their respective auxotroph and responsible for the mutant rescue; these may be directly imported or modified by extracellular or periplasmic enzyme(s) prior to uptake.

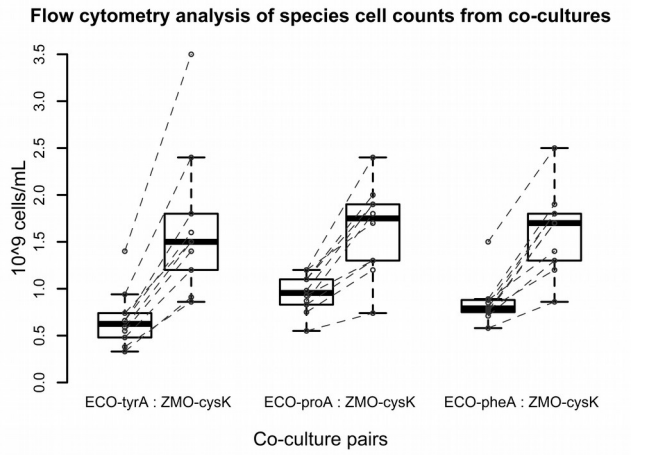

Figure 4. Flow cytometry analysis of cell counts for each species in co-cultures. Cells were inoculated in ZMMG at 0.02 theoretical OD (600nm) at time zero; growth was monitored using OD (600nm) in a plate reader (Figure S1). Cultures were prepared in two sets of five replicates each, collected in stationary phase (at 19.5 and 23.25 hours) and analyzed by flow cytometry to determine cell counts for each mutant within a co-culture pair. N=10.

Community composition may be determined by both spatial and temporal niche partitioning among the community members. Within these communities, a variety of relationships exist[4] which are ubiquitous in nature and of particular interest in understanding the evolution of both the biotic and abiotic factors of the microbial community structure. Mutualism, a bidirectionally beneficial relationship[5] may rely on cross-feeding or the exchange of metabolites between the two organisms that can best be described as 'obligatory mutualistic metabolism'.[6] Low isolation efficiencies from complex natural communities[44], may be due to factors that can be difficult or impractical to screen for in the lab (mineral, nutrient, temperature, moisture, and/or signaling gradients) but may include interspecies interactions such as metabolite exchange. While identifying interspecies relationships is important for understanding microbial communities in nature, it also has potential to be applied in biotechnologies for the design of stable microbial consortia to achieve industrial goals such as consolidated bioprocessing of plant biomass into biofuels.

The importance of incorporating exometabolomics, in addition to screening and engineering approaches, is demonstrated by the rescue of the *Z. mobilis* cysteine synthase mutant by *E. coli*. The combination of techniques resulted in the detection and evaluation of an interaction that would not have been clear using either technique alone. While *Z. mobilis* barcoded mutant library screening identified that the *cysK* mutant was rescued by *E. coli*, exometabolomics data identified glutathione related metabolites, not cysteine, as the probable metabolites responsible for the rescue. Based on *E. coli* exometabolomics data alone, a cysteine synthesis mutant would not likely have been selected as a co-culture partner given that cysteine/cystine were not detected in *E. coli* spent medium. Additionally, reliance on only the fitness profiling for mutant selection, would provide limited information for making informed improvements to the system (for example, with exometabolomics, it can be interred that enhancing expression of exporters, hydrolases, and/or importers important in the exchange of glutathione may be more beneficial than attempting to enhance only cysteine exporters/importers). The fitness of mutants as well as the molecular exchanges can easily be monitored by combining technologies, allowing for fully informed mutant selection for a beneficial microbial partnership.

It is important to note that while we find that the mutants grow together and not in isolation, we anticipate that there are many interactions beyond the few metabolites used for the design of the mutualism and that exchange of other metabolites may contribute to the observed growth (these may include beneficial and inhibitory interactions). Successful optimization of these types of cross-feeding based consortia relies on the ability to determine the nature of these other metabolic interactions. Minimally, cross-feeding based consortia design involves engineering/selecting auxotrophs for different metabolites; however, additional mutations (eg. overproduction of the exchange metabolite and/or transporters, reductions of inhibitors, etc.) may be necessary to ensure the long-term co-culture fitness.[10] Further, while beyond the scope of the current experiment, analysis of species ratios over time as well as live/dead cell ratios may be important for the evaluation of evolved and/or technologically optimized cultures. Additionally, understanding and designing the metabolic behaviors within the environment itself may be useful for determining the nature of the relationship.[45] When used in combination, library screening based approaches and metabolomics analysis provide a broad informative platform for mutualism design with predicted usefulness for improving the efficiency, longevity and stability of synthetic consortia.

METHODS

Strains. *Zymomonas mobilis* strain ZM4 (ATCC 31821) was used as the parent strain for the mutant library and as a wild-type control; construction of the *Z. mobilis* barcoded transposon mutant library and individual *Z. mobilis* mutants has been previously described.[15] *Escherichia coli* strain BW25113, the parental strain of the *E. coli* KEIO Knockout Collection[32] was used as a wild-type control and in fitness assays with the *Z. mobilis* mutant pools. BW25113 and strains JW0233 (*ΔproA761::kan),* JW2580 (*ΔpheA762::kan)* and JW3745 (*ΔilvA723::kan*) were obtained from the *E. coli* KEIO Knockout Collection[32].

Culture media and growth conditions. *Zymomonas* rich medium glucose (ZRMG) was prepared as described previously.[15] *Zymomonas* minimal medium glucose (ZMMG) was prepared as described

previously[46] with the following modifications: 27.85 mg/L FeSO4 * 7$H_2$0, 37.25 mg/L EDTA and 100X final vitamin concentrations. Solid media were prepared with 1.5% agar (final w/v). Luria-Bertani (LB) broth and M9 minimal medium (with glucose) were prepared as per standard protocols. Metabolite supplemented ZMMG was prepared with 200 µM of cystine (ZMMG+CysCys), 200 µM cysteine (ZMMG+Cys), 200 µM methionine (ZMMG+Met), 200 µM glutathione (ZMMG+GSH), 10 µM proline (ZMMG+Pro), 10 µM phenylalanine (ZMMG+Phe) or 10 µM isoleucine (ZMMG+Ile). Frozen aliquots of *Z. mobilis* and *E. coli* mutants were recovered and maintained on/in ZRMG at 30°C and LB at 37°C, respectively; kanamycin was added at a final concentration of 50 µg/ml. Wild-types were recovered and maintained on M9 (*E. coli*) or ZMMG (*Z. mobilis*). Experimental monocultures and co-cultures were washed in minimal medium by centrifugation and resuspension and then inoculated into experiment medium to a starting OD (600nm) of 0.01; cultures were grown in 12 mL culture tubes (BD Falcon, San Jose) and incubated with shaking for up to 24 hours. At the end of the culture period, culture growth was analyzed by measuring OD (600nm), and/or by flow cytometry (described below).

LC-MS Exometabolomics Analysis. After 24 hours of growth in ZMMG, 1 mL of spent medium from cultures and sterile control medium were centrifuged in 1.5 mL microcentrifuge tubes at 1700 RCF for 5 min at 4°C. Supernatants, containing extracellular metabolites, were sterile filtered through a 0.22 µm microcentrifugal filtration device. Filtrates were lyophilized, resuspended in 500 µL LCMS grade methanol and then centrifuged again to pellet salts. Supernatants were dried under vacuum (Savant SpeedVac Plus SC110A) and re-suspended in 200 µL of LCMS grade methanol. The extract was filtered through a 0.22 µm microcentrifugal filtration device and transferred to 1.5mL borosilicate glass vials (Agilent) for LCMS analysis. Samples and controls included 5 replicates each for *Z. mobilis* spent medium, 3 replicates each for *E. coli* spent medium and controls of fresh ZMMG, un-inoculated ZMMG, injection blanks and extraction blanks; quality control mixtures of common metabolites and injection blanks were run at the beginning and ends of each run to ensure no drift in retention times or signal abundances and no signs of column fouling or metabolite carryover. An Agilent 1290 LC system equipped with a ZIC-pHILIC column (150 mm × 1 mm, 3.5 µm 100 Å, Merck Sequant) was used for metabolite separation with the following LC conditions: solvent A – 5mM ammonium acetate; solvent B – 9:1 acetonitrile:H_2O with 5mM ammonium acetate; timetable: 0 min at 100% B, 1.5 min at 100% B, 21 min at 0% B, 27 min at 0% B, 33 min at 100% B, and 45 min at 100% B; 0.8 mL/min; column compartment temperature of 40°C. Mass spectrometry analyses were performed using Agilent 6520 and 6550 Quadrupole Time of Flight Mass Spectrometers. LCMS of pure reference standards of amino acids and other compounds of interest were used to generate an atlas of m/z and retention time values; Agilent software (Santa Clara, CA), including Mass Hunter Qualitative Analysis and Profinder (version 6.0), was used for naïve peak finding, data alignment and compound annotation. Mass spectrometry parameters are defined in Table S1 with metabolite identifications described in Table S2.

Genome-wide *Z. mobilis* mutant fitness assays. *Z. mobilis* mutant pools containing DNA barcodes that enable the quantification of the relative abundances for thousands of transposon mutants in parallel were generated previously.[15] Competitive fitness assays were performed using previously established protocols.[15] In brief, wild-type *E. coli* and the *Z. mobilis* mutant pools were recovered from the freezer (100 µL aliquots

thawed and inoculated into 10 mL ZRMG), washed twice in ZMMG, and inoculated together into a co-culture at a theoretical starting OD (600nm) for each strain of 0.01 in 10 mL of ZMMG. At the start of the co-culture and after reaching saturation, cell pellets were collected for extraction of genomic DNA; DNA barcodes were then amplified and hybridized to a microarray as previously described.[15,26] The mutant fitness of each strain ("strain fitness score") is calculated as the $\log_2$ ratio of its DNA barcode hybridization signal (to a microarray) at the END versus the START of the co-culture incubation. Multiple independent transposon insertions for a single gene are used to calculate a final "gene fitness scores" using a previously described method.[15] Negative values indicate that a mutant has reduced END abundance while positive values are indicative of a mutant with increased END abundance. Mutant rescue is determine using cutoff values for gene fitness: gene fitness in monoculture < -0.75 and (gene fitness in co-culture minus gene fitness as a monoculture) > 0.4.

Identification of lethal auxotroph and confirmation of rescue by released exometabolites. The OD (600nm) of overnight cultures was used to confirm lethal auxotrophs as cultures with an OD (600nm) <0.2 in minimal media (M9 for *E. coli* and ZMMG for *Z. mobilis*) and greater than 1.0 in rich media (LB for *E. coli* and ZRMG for *Z. mobilis*). Rescue by partner species exometabolite(s) was confirmed by culture in the spent minimal medium of the opposing wild-type species. Spent media were collected from 18-24 hour wild-type cultures in ZMMG by centrifugation, followed by sterile filtration of the supernatant through a 0.22 µm PVDF filter to remove cells and cell debris. 100% spent *E. coli* minimal medium (ZMMG+ECOspent) was used to confirm rescue of *Z. mobilis* auxotroph while 50% spent *Z. mobilis* minimal medium in fresh ZMMG (ZMMG+ZMOspent) was used to confirm rescue of *E. coli* auxotrophs; controls included incubation of un-inoculated spent medium.

Flow cytometry analysis of co-culture ratios. Overnight mutant monocultures in LB or ZRMG were washed in minimal medium and resuspended to an OD (600nm) of 0.1; equal volumes of a ZMO0748 transposon mutant (*ΔcysK::Tn5*) and *E. coli* amino acid auxotroph monocultures were combined for co-cultures at a final volume of 250 µL in multi-well plates (50 µL of each washed monoculture suspension plus 150 µL of ZMMG; each mutant at a calculated final OD at 600nm of 0.02). Cultures were incubated in a Tecan Infinite F200-PRO microplate reader at 37°C with shaking to obtain growth curves (OD at 600nm taken every 15 minutes until sample collection). Experiment was performed twice with five replicates each time for 19.5 and 23.25 hours at which point samples were collected for flow cytometry. Co-cultures and monoculture controls were stained with 5 µM SYTO 9 dye (Life Technologies, Carlsbad, CA) for 15 minutes at room temperature according to the manufacturer's instructions. Stained cultures were diluted to approximately 1x10^6 cells/mL in PBS, a calibrated suspension of 6 µm polystyrene microbeads was added to samples at a density of 1x10^6 beads/mL to serve as a counting standard prior to the data acquisition on the flow cytometer. Flow cytometry analyses were performed using a BD FACS Aria II, equipped with a 488-nm solid-state laser and a forward scatter photomultiplier tube (BD Biosciences, San Jose, CA). A 488-nm laser was used as the excitation source for SYTO 9 fluorescence, and emission was collected using a 530/30 nm bandpass filter. For each sample, 10,000 events were collected at a throughput rate of 1000 events/s, using a side scatter threshold of 200 events. All flow cytometry data were analyzed with the FlowJo package (v X.0.7) (TreeStar Inc., Ashland, OR). Forward scatter versus green fluorescence cytogram was used for gating *E. coli* and *Z.*

mobilis cells, and cell concentrations were determined from the ratio of cell events to microsphere events in the cytogram.

ASSOCIATED CONTENT

Supporting Information

Supplementary Tables S1, S2, S3, S4, S5 and S6, and Supplementary Figures S1 and S2 are available free of charge via the Internet at http://pubs.acs.org.

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Notes

These authors declare no competing financial interest(s).

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475**Exometabolomics assisted design and validation of synthetic obligate mutualism**

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477Arkin, Adam M. Deutschbauer, and Trent R. Northen

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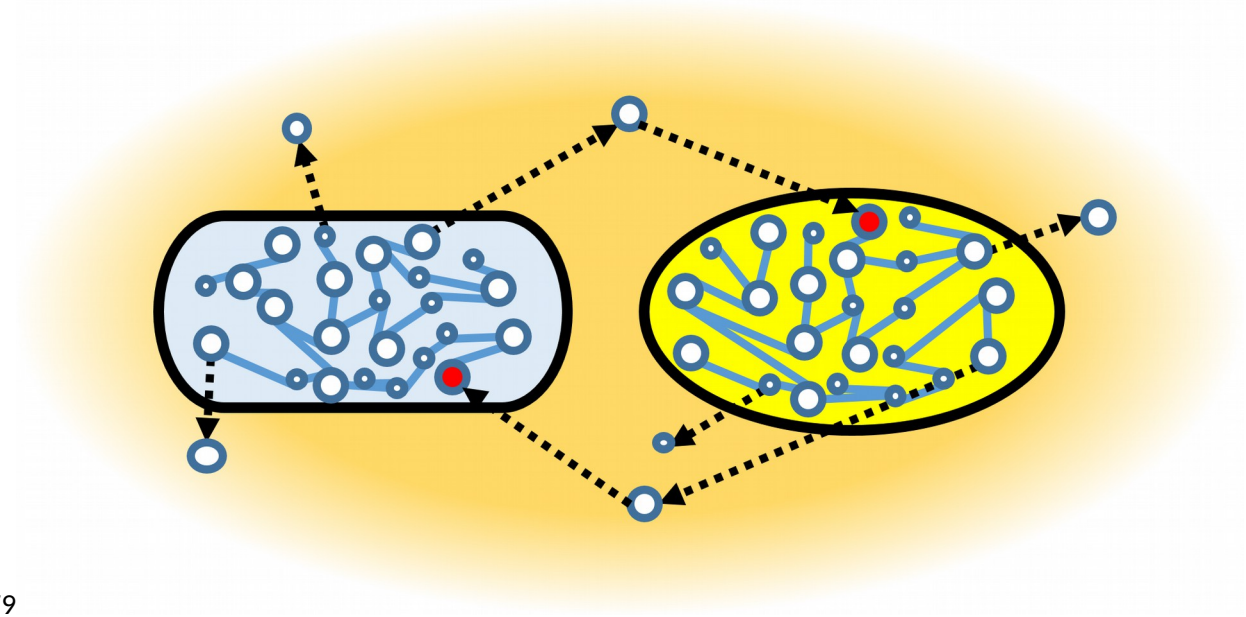

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