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Title

Exometabolomics Assisted Design and Validation of Synthetic Obligate Mutualism.

Permalink

<https://escholarship.org/uc/item/2019585b>

Journal

ACS synthetic biology, 5(7)

ISSN

2161-5063

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Publication Date

2016-07-01

DOI

10.1021/acssynbio.5b00236

Supplemental Material

<https://escholarship.org/uc/item/2019585b#supplemental>

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Peer reviewed

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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 *lvaA*), 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 interaction³⁻⁶ 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

39synthetic ecology focuses on constructing artificial communities to extend synthetic biology approaches to
40microbial consortia^{7,12}, and as a means for controlled studies of microbial ecology.^{13,14} Of particular interest is
41the construction of obligate mutualistic relationships, in which each organism requires at least one activity
42and/or product of one or more other organisms for growth.⁵ Typically, mutualism design has relied upon
43having an adequate understanding of the constituents' genomes and metabolisms in order to make an
44informed selection of specific autotrophic mutant pairs.

45

46Screening of whole-genome, randomly mutated libraries for rescue^{15,16}, combined with characterization of
47metabolite uptake/release (exometabolomics), has the potential to catch more novel metabolic interactions
48than either approach alone and may be preferable for organisms that are amenable to mutagenesis and have
49poorly annotated genomes. Mass spectrometry based exometabolomics has been used to examine how cells
50transform their small molecule environments and is valuable for a range of applications including biofuel
51development¹⁷, synthetic biology¹⁸, detection of novel metabolites^{19,20} and investigating the metabolic
52interactions and dependencies of cells.^{21–23} Mutant fitness profiling^{24,25} has also been used extensively for a
53variety of purposes including the phenotypic and functional characterization of uncharacterized genes^{26–28}, the
54improvement of strains used in bio-production methodologies¹⁵, identification of electron transport systems in
55syntrophic co-cultures²⁹, and evaluation of gene regulation in relation to metabolic needs.¹⁶ Recently,
56exometabolomics has been used in combination with mutant library fitness profiling in a high-throughput
57fashion to identify bacterial mutants that have lost the ability to utilize specific metabolites.³⁰ However, their
58combined use in mutualism design represents a novel methodology.

59

60 Here, we describe the use of exometabolomics and mutant fitness profiling for the design of obligate
61synthetic mutualism between two organisms. We demonstrate this approach using *Zymomonas mobilis* and
62*Escherichia coli*, two bacteria with available mutant libraries^{26,31–33} that lack known ecological interaction. Each
63has been utilized separately in mono and co-culture systems for enhanced lignocellulosic bioethanol
64production. *Z. mobilis* is of particular interest due to its efficient fermentation of glucose to ethanol³⁴ while *E.*
65*coli* can ferment xylose in addition to glucose (the two main sugars in lignocellulose).^{35,36} In fact, previous
66experiments have demonstrated an inhibitory effect of wild-type *Z. mobilis* on wild-type *E. coli* growth in co-
67culture (a non-mutualistic interaction)³⁷, which was not observed in the mutant based synthetic mutualism in
68our studies. Auxotrophic mutants of each species were selected from existing mutant libraries such that when
69cultured together in minimal medium, they would rely on metabolite cross-feeding based rescue.

70Exometabolomics can be used in consortia studies to generate two useful pieces of information. In consortia
71design, involving organisms without known mutualistic interactions, exometabolomics based determination
72(and optional quantification) of the metabolites released by the constituents in isolation can be used to make
73more informed decisions on mutant selection/construction to force a mutualistic relationship (Figure S1). In
74consortia analysis, involving organisms with existing or known interactions, exometabolomics can reveal the
75metabolites responsible for rescue in a cross-feeding based relationship where the metabolite exchange has
76previously been undefined. In this study, a series of experiments, using mutant fitness profiling for mutant

77selection and exometabolomics for both mutant selection and cross-feeding determination, were performed in
 78the construction of synthetic obligate mutualism and validation of metabolite exchange (Figure 1).

A. Wild-type *Z. mobilis* exometabolomics for the selection of *E. coli* auxotrophs

99catalyze the same reaction as IlvA⁴¹, these do not appear to be functional pathways in the single gene
100deletion mutants during growth in minimal media (previously only observed as functional in double mutants
101affecting substrate availability or enzyme expression in the alternative pathways).^{40,41} Additionally, PheA has
102two domains with different enzymatic functions (chorismate mutase and prephenate dehydratase); another
103enzyme TyrA also has chorismate mutase activity so the *pheA* mutant should still produce tyrosine while
104having auxotrophy for phenylalanine. The auxotrophic phenotype of each of the three *E. coli* mutants was
105confirmed by comparing monoculture growth (determined from OD at 600nm) in M9 minimal medium to
106growth in LB broth (Table S3). To confirm rescue of the mutants by *Z. mobilis* exometabolites, spent medium
107from wild-type *Z. mobilis* in ZMMG was diluted 1:1 with fresh ZMMG and used for the culture of the *E. coli*
108mutants. ZMMG supplemented with amino acids additionally confirmed that mutants were rescued as
109predicted based on their mutations. While none of the mutants were capable of growth in ZMMG, they all
110successfully grew in 50% spent medium and metabolite supplemented medium (Figure 2a). Growth in 100%
111spent *Z. mobilis* medium resulted in decreased growth possibly due to ethanol production or depletion of
112ZMMG vitamins/minerals required by the *E. coli* for growth (data not shown).

114**Figure 2. Mutant rescue by supplementation of ZMMG with rescue metabolites or spent medium from**
115**the partner strain.** Wild-type *Z. mobilis* spent ZMMG was collected and used for the culture of the three *E.*
116*coli* auxotrophs for proline, isoleucine and phenylalanine at 50% spent medium in ZMMG; *E. coli* mutants
117were also cultured in ZMMG supplemented with the rescue metabolite that was used for the strain selection
118at 10uM each of proline, isoleucine or phenylalanine (a). Wild-type *E. coli* spent ZMMG medium was
119collected and used for the culture of *Z. mobilis* mutant ZMO0748 at 100% spent medium; *Z. mobilis* mutant
120ZMO0748 was also cultured in ZMMG that was supplemented with 200uM each of cysteine, cystine,
121methionine and glutathione (b). Mass spectrometry analysis of uninoculated ZMMG compared with *E. coli*
122spent ZMMG, indicate that only a small portion of the glucose from ZMMG is consumed by the *E. coli* in
123ZMMG under these growth conditions, thus *Z. mobilis*, which requires glucose for fermentative growth is
124capable of growth in the *E. coli* spent ZMMG.

125 The selection of *Z. mobilis* mutants for rescue by *E. coli* was performed using an established mutant
126 library screening based approach (Figure 1b).²⁹ Wild-type *Z. mobilis* strain ZM4 was used to generate a DNA-
127 bar coded transposon library consisting of 7432 mutants.¹⁵ We identified *Z. mobilis* mutants with increased
128 fitness in the presence of wild-type *E. coli* using a competitive, genome-wide fitness assay with barcoded
129 transposon mutants. The top ten *E. coli* rescued *Z. mobilis* mutants (when ranked by largest positive
130 difference between fitness with *E. coli* and fitness without *E. coli*) included transposon insertions in the
131 following genes: indole-3-glycerol-phosphate synthase, cysteine synthase, cell division gene *ftsA*,
132 anthranilate phosphoribosyltransferase, anthranilate synthase component I, glutathione S-transferase domain
133 protein, glutamine amidotransferase of anthranilate synthase, cell division gene *ftsW*, and N-
134 acetylglucosamine-6-phosphate deacetylase (Figure 3 and Tables S4 and S5). Of the tryptophan and
135 cysteine synthesis mutants, only mutants in a gene with sequence similarity to cysteine synthase A
136 (ZMO0748; *cysK*) failed to grow in minimal medium (Table S6). Because *Z. mobilis* has a polyploidy genome,
137 genes essential for growth in rich medium may only form mixed/heterozygous mutants under kanamycin
138 selection¹⁵, thus the tryptophan synthesis pathway mutants are likely heterozygotes with at least one copy of
139 the wild-type allele allowing for growth in minimal medium. Cysteine synthase A is likely only essential for
140 growth in minimal medium not in rich resulting in a stable homozygous mutant during library generation.¹⁵ The
141 $\Delta cysK::Tn5$ mutation in ZMO0748 would render the mutant unable to synthesize L-cysteine from the
142 precursors O-acetyl-L-serine and sulfide; a strain of this mutant was selected for use as the *Z. mobilis*
143 auxotroph in co-culture.

144 **Figure 3. *Z. mobilis* pooled mutant fitness profiling with and without wild-type *E. coli* for selection of**
145 **mutants.** The gene fitness scores for the *Z. mobilis* mutant library when cultured in ZMMG as a monoculture
146 versus a co-culture with *E. coli* are used to determine which mutants have enhanced fitness in the presence
147 of *E. coli*. The gene fitness scores are calculated from the strain fitness values using a previously described
148 method¹⁵ where strain fitness is equal to the log2 ratio of the END mutant tag abundance to the START
149 mutant tag abundance; negative values indicate a decrease in abundance and positive values indicate an

150increase in abundance. Each experimental condition was done only once, however each gene typically has
151multiple unique mutants (Tables S4, S5). A mutant is considered “rescued” when it has poor fitness
152(decreased abundance) in monoculture (more likely to be an auxotroph) and when the fitness in the co-
153culture exceeds that in monoculture; the rescue cutoff region, delineated with the grey dashed line, is
154calculated as follows: gene fitness in monoculture < -0.75 and (gene fitness in co-culture minus gene fitness
155as a monoculture) > 0.4. Rescued *Z. mobilis* mutants are colored by predicted functional classification. The
156solid black lines represents monoculture = co-culture fitness. ZMO0748 (*cysK*), was identified as an
157auxotroph and selected as the *Z. mobilis* mutant to be used for mutualism construction with *E. coli*.

158

159 Exometabolomics analysis of wild-type *E. coli* was performed to check for metabolites capable of
160rescuing the *Z. mobilis cysK* mutant (Figure 1c). Surprisingly, exometabolomics analysis did not detect
161cysteine in the spent medium of wild-type *E. coli*. Presumably, a sulfhydryl containing compound or cysteine
162analog could act as the rescue metabolite(s) in which the sulfide is already incorporated into an organic
163molecule as a sulfhydryl group. To identify possible cross-feeding metabolites responsible for the observed
164mutant rescue, we further analyzed the wild-type *E. coli* exometabolomic data for additional sulfur containing
165metabolites and identified glutathione reduced, glutathione disulfide, adenosylmethionine and gamma-
166glutamylcysteine (Table S2). Rescue of *Z. mobilis cysK::ΔTn5* by *E. coli* exometabolites was confirmed by
167culture of the mutant in 100% spent *E. coli* medium (Figure 2b). To determine which sulfur containing
168compounds are capable of rescuing *Z. mobilis cysK::ΔTn5*, the mutant was cultured in ZMMG supplemented
169with methionine, cysteine, cystine or glutathione; we found that of these, both cysteine and glutathione are
170capable of rescuing *Z. mobilis ΔcysK::Tn5* (Figure 2b). We cannot exclude the possibility that *E. coli* supplied
171free cysteine may be involved in the rescue and is below the instrument detection limits. Based on homology
172predictions, genes ZMO1388, ZMO01345/ZMO1776 may be hydrolases, 3.4.19.13 (ggt), 3.4.11.1/2 (pepN),
173respectively, capable of releasing cysteine from glutathione and related metabolites.⁴³ We conclude that
174glutathione and/or gamma-glutamylcysteine from spent *E. coli* medium are capable of and sufficient for the
175rescue of the *Z. mobilis* cysteine auxotroph.

176 Using the above described exometabolomic and mutant fitness data that was used for the selection of
177auxotrophs from each species, we designed 3 sets of synthetic mutualism pairs between *E. coli* and *Z.*
178*mobilis*. The *Z. mobilis* cysteine synthase mutant ($\Delta cysK$) rescued by *E. coli* released glutathione related
179metabolites was co-cultured with each of the three *E. coli* amino acid biosynthesis mutants ($\Delta proA$, $\Delta pheA$,
180and $\Delta ilvA$) rescued by amino acids released by *Z. mobilis*. As expected, auxotrophs of each species,
181previously shown to be incapable of growth as a monoculture in ZMMG, grew when cultured together in
182ZMMG as confirmed by OD at 600nm (Figure S2). To further verify that each species was contributing to the
183biomass abundance (based on optical density) once the culture had reached stationary phase, flow cytometry
184analysis was used to quantify the cells (Figure 4). Other options for evaluating end-point cell concentrations
185such as plate counts and counting chambers presented challenges due to the ability to differentiate and/or

186isolate the colonies or cells of each species. These co-cultures represent obligate mutualistic growth since
 187they are only capable of growth in minimal medium when cultured together. Based on the selection of
 188auxotrophs, this mutualism appears to be characterized by an exchange of metabolites with each of the three
 189*E. coli* mutants synthesizing and releasing glutathione related metabolites and the *Z. mobilis* mutant
 190synthesizing and releasing proline, isoleucine and phenylalanine. The actual method of metabolite release
 191from the partner cell is unknown; the rescue metabolite may be actively exported, hydrolyzed from larger
 192peptides/proteins outside of the cell and/or made available following cell death and lysis. These released
 193metabolites are then available for use by their respective auxotroph and responsible for the mutant rescue;
 194these may be directly imported or modified by extracellular or periplasmic enzyme(s) prior to uptake.

195

196**Figure 4. Flow cytometry analysis of cell counts for each species in co-cultures.** Cells were inoculated
 197in ZMMG at 0.02 theoretical OD (600nm) at time zero; growth was monitored using OD (600nm) in a plate
 198reader (Figure S1). Cultures were prepared in two sets of five replicates each, collected in stationary phase
 199(at 19.5 and 23.25 hours) and analyzed by flow cytometry to determine cell counts for each mutant within a
 200co-culture pair. N=10.

201

202 Community composition may be determined by both spatial and temporal niche partitioning among
 203the community members. Within these communities, a variety of relationships exist⁴ which are ubiquitous in
 204nature and of particular interest in understanding the evolution of both the biotic and abiotic factors of the
 205microbial community structure. Mutualism, a bidirectionally beneficial relationship⁵ may rely on cross-feeding
 206or the exchange of metabolites between the two organisms that can best be described as ‘obligatory
 207mutualistic metabolism’.⁶ Low isolation efficiencies from complex natural communities⁴⁴, may be due to
 208factors that can be difficult or impractical to screen for in the lab (mineral, nutrient, temperature, moisture,
 209and/or signaling gradients) but may include interspecies interactions such as metabolite exchange. While
 210identifying interspecies relationships is important for understanding microbial communities in nature, it also
 211has potential to be applied in biotechnologies for the design of stable microbial consortia to achieve industrial
 212goals such as consolidated bioprocessing of plant biomass into biofuels.

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

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

241

242 METHODS

243 **Strains.** *Zymomonas mobilis* strain ZM4 (ATCC 31821) was used as the parent strain for the mutant
244 library and as a wild-type control; construction of the *Z. mobilis* barcoded transposon mutant library and
245 individual *Z. mobilis* mutants has been previously described.¹⁵ *Escherichia coli* strain BW25113, the parental
246 strain of the *E. coli* KEIO Knockout Collection³² was used as a wild-type control and in fitness assays with the
247 *Z. mobilis* mutant pools. BW25113 and strains JW0233 ($\Delta proA761::kan$), JW2580 ($\Delta pheA762::kan$) and
248 JW3745 ($\Delta ilvA723::kan$) were obtained from the *E. coli* KEIO Knockout Collection³².

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

251previously⁴⁶ with the following modifications: 27.85 mg/L FeSO₄ * 7H₂O, 37.25 mg/L EDTA and 100X final
252vitamin concentrations. Solid media were prepared with 1.5% agar (final w/v). Luria-Bertani (LB) broth and M9
253minimal medium (with glucose) were prepared as per standard protocols. Metabolite supplemented ZMMG
254was prepared with 200 µM of cystine (ZMMG+CysCys), 200 µM cysteine (ZMMG+Cys), 200 µM methionine
255(ZMMG+Met), 200 µM glutathione (ZMMG+GSH), 10 µM proline (ZMMG+Pro), 10 µM phenylalanine
256(ZMMG+Phe) or 10 µM isoleucine (ZMMG+Ile). Frozen aliquots of *Z. mobilis* and *E. coli* mutants were
257recovered and maintained on/in ZRMG at 30°C and LB at 37°C, respectively; kanamycin was added at a final
258concentration of 50 µg/ml. Wild-types were recovered and maintained on M9 (*E. coli*) or ZMMG (*Z. mobilis*).
259Experimental monocultures and co-cultures were washed in minimal medium by centrifugation and
260resuspension and then inoculated into experiment medium to a starting OD (600nm) of 0.01; cultures were
261grown in 12 mL culture tubes (BD Falcon, San Jose) and incubated with shaking for up to 24 hours. At the
262end of the culture period, culture growth was analyzed by measuring OD (600nm), and/or by flow cytometry
263(described below).

264 **LC-MS Exometabolomics Analysis.** After 24 hours of growth in ZMMG, 1 mL of spent medium from
265cultures and sterile control medium were centrifuged in 1.5 mL microcentrifuge tubes at 1700 RCF for 5 min
266at 4°C. Supernatants, containing extracellular metabolites, were sterile filtered through a 0.22 µm
267microcentrifugal filtration device. Filtrates were lyophilized, resuspended in 500 µL LCMS grade methanol
268and then centrifuged again to pellet salts. Supernatants were dried under vacuum (Savant SpeedVac Plus
269SC110A) and re-suspended in 200 µL of LCMS grade methanol. The extract was filtered through a 0.22 µm
270microcentrifugal filtration device and transferred to 1.5mL borosilicate glass vials (Agilent) for LCMS analysis.
271Samples and controls included 5 replicates each for *Z. mobilis* spent medium, 3 replicates each for *E. coli*
272spent medium and controls of fresh ZMMG, un-inoculated ZMMG, injection blanks and extraction blanks;
273quality control mixtures of common metabolites and injection blanks were run at the beginning and ends of
274each run to ensure no drift in retention times or signal abundances and no signs of column fouling or
275metabolite carryover. An Agilent 1290 LC system equipped with a ZIC-pHILIC column (150 mm × 1 mm, 3.5
276µm 100 Å, Merck Sequant) was used for metabolite separation with the following LC conditions: solvent A –
2775mM ammonium acetate; solvent B – 9:1 acetonitrile:H₂O with 5mM ammonium acetate; timetable: 0 min at
278100% 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
279mL/min; column compartment temperature of 40°C. Mass spectrometry analyses were performed using
280Agilent 6520 and 6550 Quadrupole Time of Flight Mass Spectrometers. LCMS of pure reference standards of
281amino acids and other compounds of interest were used to generate an atlas of m/z and retention time
282values; Agilent software (Santa Clara, CA), including Mass Hunter Qualitative Analysis and Profinder (version
2836.0), was used for naïve peak finding, data alignment and compound annotation. Mass spectrometry
284parameters are defined in Table S1 with metabolite identifications described in Table S2.

285 **Genome-wide *Z. mobilis* mutant fitness assays.** *Z. mobilis* mutant pools containing DNA barcodes
286that enable the quantification of the relative abundances for thousands of transposon mutants in parallel were
287generated previously.¹⁵ Competitive fitness assays were performed using previously established protocols.¹⁵
288In brief, wild-type *E. coli* and the *Z. mobilis* mutant pools were recovered from the freezer (100 µL aliquots

289thawed and inoculated into 10 mL ZRMG), washed twice in ZMMG, and inoculated together into a co-culture
290at a theoretical starting OD (600nm) for each strain of 0.01 in 10 mL of ZMMG. At the start of the co-culture
291and after reaching saturation, cell pellets were collected for extraction of genomic DNA; DNA barcodes were
292then amplified and hybridized to a microarray as previously described.^{15,26} The mutant fitness of each strain
293("strain fitness score") is calculated as the $\log_2$ ratio of its DNA barcode hybridization signal (to a microarray)
294at the END versus the START of the co-culture incubation. Multiple independent transposon insertions for a
295single gene are used to calculate a final "gene fitness scores" using a previously described method.¹⁵
296Negative values indicate that a mutant has reduced END abundance while positive values are indicative of a
297mutant with increased END abundance. Mutant rescue is determined using cutoff values for gene fitness: gene
298fitness in monoculture < -0.75 and (gene fitness in co-culture minus gene fitness as a monoculture) > 0.4.

299 **Identification of lethal auxotroph and confirmation of rescue by released exometabolites.** The
300OD (600nm) of overnight cultures was used to confirm lethal auxotrophs as cultures with an OD (600nm)
301<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.*
302*coli* and ZRMG for *Z. mobilis*). Rescue by partner species exometabolite(s) was confirmed by culture in the
303spent minimal medium of the opposing wild-type species. Spent media were collected from 18-24 hour wild-
304type cultures in ZMMG by centrifugation, followed by sterile filtration of the supernatant through a 0.22 μm
305PVDF filter to remove cells and cell debris. 100% spent *E. coli* minimal medium (ZMMG+ECOspent) was
306used to confirm rescue of *Z. mobilis* auxotroph while 50% spent *Z. mobilis* minimal medium in fresh ZMMG
307(ZMMG+ZMOspent) was used to confirm rescue of *E. coli* auxotrophs; controls included incubation of un-
308inoculated spent medium.

309 **Flow cytometry analysis of co-culture ratios.** Overnight mutant monocultures in LB or ZRMG were
310washed in minimal medium and resuspended to an OD (600nm) of 0.1; equal volumes of a ZMO0748
311transposon mutant ($\Delta\text{cysK}::\text{Tn5}$) and *E. coli* amino acid auxotroph monocultures were combined for co-
312cultures at a final volume of 250 μL in multi-well plates (50 μL of each washed monoculture suspension plus
313150 μL of ZMMG; each mutant at a calculated final OD at 600nm of 0.02). Cultures were incubated in a Tecan
314Infinite F200-PRO microplate reader at 37°C with shaking to obtain growth curves (OD at 600nm taken every
31515 minutes until sample collection). Experiment was performed twice with five replicates each time for 19.5
316and 23.25 hours at which point samples were collected for flow cytometry. Co-cultures and monoculture
317controls were stained with 5 μM SYTO 9 dye (Life Technologies, Carlsbad, CA) for 15 minutes at room
318temperature according to the manufacturer's instructions. Stained cultures were diluted to approximately
319 1×10^6 cells/mL in PBS, a calibrated suspension of 6 μm polystyrene microbeads was added to samples at a
320density of 1×10^6 beads/mL to serve as a counting standard prior to the data acquisition on the flow cytometer.
321Flow cytometry analyses were performed using a BD FACS Aria II, equipped with a 488-nm solid-state laser
322and a forward scatter photomultiplier tube (BD Biosciences, San Jose, CA). A 488-nm laser was used as the
323excitation source for SYTO 9 fluorescence, and emission was collected using a 530/30 nm bandpass filter.
324For each sample, 10,000 events were collected at a throughput rate of 1000 events/s, using a side scatter
325threshold of 200 events. All flow cytometry data were analyzed with the FlowJo package (v X.0.7) (TreeStar
326Inc., Ashland, OR). Forward scatter versus green fluorescence cytogram was used for gating *E. coli* and *Z.*

327*mobilis* cells, and cell concentrations were determined from the ratio of cell events to microsphere events in
328the cytogram.

329**ASSOCIATED CONTENT**

330**Supporting Information**

331Supplementary Tables S1, S2, S3, S4, S5 and S6, and Supplementary Figures S1 and S2 are available free
332of charge via the Internet at <http://pubs.acs.org>.

333

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340

341**Notes**

342These authors declare no competing financial interest(s).

343

344**ACKNOWLEDGEMENTS**

345This material by ENIGMA- Ecosystems and Networks Integrated with Genes and Molecular Assemblies
346(<http://enigma.lbl.gov>), a Scientific Focus Area Program at Lawrence Berkeley National Laboratory is based
347upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological &
348Environmental Research under contract number DE-AC02-05CH11231.

349

350We thank Morgan N. Price (Lawrence Berkeley National Laboratory) for processing the *Z. mobilis* mutant
351fitness assay data.

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

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