ACS Synthetic Biology
Cite This: ACS Synth. Biol. 2019, 8, 2174−2185
Research Article
pubs.acs.org/synthbio

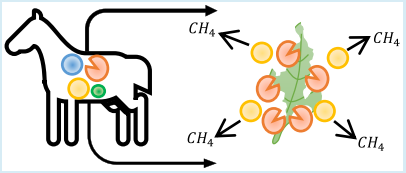
Top-Down Enrichment Guides in Formation of Synthetic Microbial Consortia for Biomass Degradation

Sean P. Gilmore,[†,#] Thomas S. Lankiewicz,[†,‡,#] St. Elmo Wilken,[†] Jennifer L. Brown,[†] Jessica A. Sexton,[†] John K. Henske,[†] Michael K. Theodorou,[§] David L. Valentine,[∥] and Michelle A. O'Malley*[,†]

[†]Department of Chemical Engineering, University of California Santa Barbara, Santa Barbara, California 93106, United States
[‡]Department of Ecology, Evolution, and Marine Biology, University of California Santa Barbara, Santa Barbara, California 93106, United States
[§]Harper Adams University, Agriculture Centre for Sustainable Energy Systems, Newport, Shropshire TF10 8NB, United Kingdom
[∥]Department of Earth Science and Marine Science Institute, University of California Santa Barbara, Santa Barbara, California 93106, United States

Supporting Information

ABSTRACT: Consortium-based approaches are a promising avenue toward efficient bioprocessing. However, many complex microbial interactions dictate community dynamics and stability that must be replicated in synthetic systems. The rumen and/or hindguts of large mammalian herbivores harbor complex communities of biomass-degrading fungi and bacteria, as well as archaea and protozoa that work collectively to degrade lignocellulose, yet the microbial interactions responsible for stability, resilience, and activity of the community remain largely uncharacterized. In this work, we demonstrate a "top-down" enrichment-based methodology for selecting a minimal but effective lignocellulose-degrading community that produces methane-rich fermentation gas (biogas). The resulting enrichment consortium produced 0.75−1.9-fold more fermentation gas at 1.4−2.1 times the rate compared to a monoculture of fungi from the enrichment. Metagenomic sequencing of the top-down enriched consortium revealed genomes encoding for functional compartmentalization of the community, spread across an anaerobic fungus (*Piromyces*), a bacterium (*Sphaerochaeta*), and two methanogenic archaea (*Methanosphaera* and *Methanocorpusculum*). Guided by the composition of the top-down enrichment, several synthetic cocultures were formed from the "bottom-up" using previously isolated fungi, *Neocallimastix californiae* and *Anaeromyces robustus* paired with the methanogen *Methanobacterium bryantii*. While cross-feeding occurred in synthetic co-cultures, removal of fungal metabolites by methanogens did not increase the rate of gas production or the rate of substrate deconstruction by the synthetic community relative to fungal monocultures. Metabolomic characterization verified that syntrophy was established within synthetic co-cultures, which generated methane at similar concentrations compared to the enriched consortium but lacked the temporal stability (resilience) seen in the native system. Taken together, deciphering the membership and metabolic potential of an enriched gut consortium enables the design of methanogenic synthetic co-cultures. However, differences in the growth rate and stability of enriched versus synthetic consortia underscore the difficulties in mimicking naturally occurring syntrophy in synthetic systems.



KEYWORDS: *microbial consortia, anaerobic fungi, lignocellulose, metagenomics, bioprocessing*

Natural and synthetic consortia hold the potential to revolutionize bioprocessing, because of their increased efficiency by distributing difficult processes across the individual members.[1] Microbial consortia are currently used in a limited number of bioprocessing schemes, like anaerobic digestion, where an undefined mixture of microbes convert cellulosic waste into biogas. Typically, these communities are enriched or formed from nature in a "top-down" approach, resulting in mixtures of unknown microbes with cryptic metabolic and functional interactions. However, when synthetic consortia are formed by combining microbial cultures from the "bottom-up", wieldy microbes compete for the same resources often resulting in the dominance of one microbe that outperforms the others.[1] Identifying key factors that both connect and stabilize microbial consortia is critical to overcome efficiency and stability limitations currently inherent in using microbial communities for bioprocessing.[2]

Diverse microbial communities from nature participate in many different types of interactions, ranging from favorable to benign and even unfavorable, which can be leveraged to enhance the productivity of bioprocessing consortia.[3] These interactions help regulate community dynamics, resulting in stable community membership and metabolic activity. Syntrophy is one possible interaction that helps metabolically tether mutually beneficial microorganisms in communities.[4] Syntrophy, or cross-feeding, occurs when one microbe consumes metabolites produced by a second microbe, resulting

Received: June 30, 2019
Published: August 28, 2019

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

in interwoven and interdependent metabolisms that benefit both microbes.[5] In the context of bioprocessing, syntrophic relationships have the potential benefit of removing inhibitory metabolic products, thus facilitating processes of interest[5] like biomass deconstruction or biogas production. For example, cross-feeding occurs between hydrogenic anaerobic fungi and methanogenic archaea in the herbivore gut, and many published reports hypothesize that the exchange of metabolites between these organisms increases the rate of substrate deconstruction by the fungus.[6−11]

One route to identify mechanisms that regulate community formation, diversity, and stability is to investigate "minimal" native consortia, whereby key microbial players have been enriched from a complex ecosystem. The rumen and/or hindguts of large mammalian herbivores are ecosystems of key biotechnological interest, where lignocellulosic biomass is routinely and rapidly degraded by such consortia.[12] While anaerobic gut fungi found in large herbivores are responsible for the primary colonization and disruption of fibrous lignocellulosic substrates,[13] they are typically isolated and cultured axenically.[13,14] These cultures do not mimic their native environment, which are rich in archaea, bacteria, and protozoa. In particular, methanogens siphon hydrogen, carbon dioxide, formate, and other metabolites from the environment, allowing the fungi to avoid feedback inhibition, enabling them to produce energy more efficiently by increasing flux through their hydrogenosomes.[6] Since fungus-methanogen co-cultures are capable of producing methane directly from crude lignocellulose, exploiting this syntrophic interaction in native and engineered consortia could enable new strategies for biogas production *via* anaerobic digestion.[6] While previous studies have characterized fungus-methanogen co-cultures,[6,9−11,15−17] methodologies for community assembly have not been compared, and therefore potential differences in degradation efficiency, productivity, resilience, and stability remain unclear.

In this study, we use "top-down" enrichment to inform "bottom-up" reconstruction of a synthetic biomass-degrading microbial consortium (Figure 1). Using next generation sequencing, we identify potential interactions between microbes in a hind-gut consortium enriched from nature, while measuring methane-rich fermentation gas accumulation. Guided by this natural consortium, we then demonstrate that the syntrophic mechanisms identified in the native community can be used to design a synthetic set of methane-generating co-cultures that degrade lignocellulose. We further quantify the fermentation gas generation, growth rate, and biomass deconstruction behavior of the native and synthetic systems in comparison to fungal monocultures. Overall, this study provides an enabling approach and informs design of minimal consortia for value-added chemical production with increased potential for lignocellulolytic activity, methanogenic activity, or both.

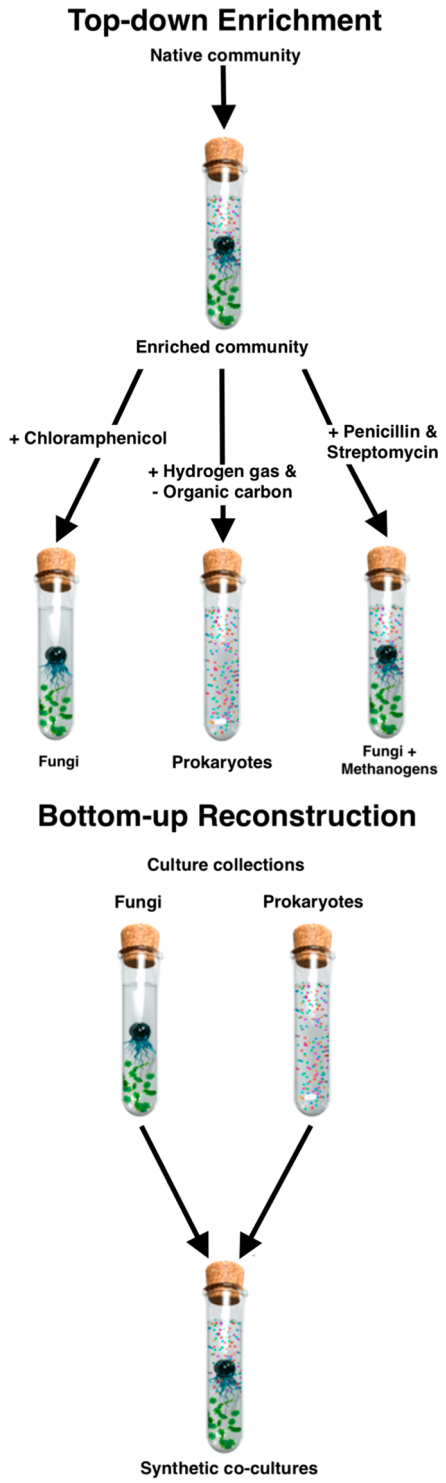


Figure 1. Complementary "top-down" and "bottom-up" methods establish enriched biomass-degrading consortia from source microbiomes and synthetic co-cultures from isolates. For top-down enrichment, native microbial communities are selected by antibiotic treatment and serial cultivation. Fungi from the native community can be subsequently isolated by treatment with chloramphenicol. Through incubation with hydrogen and removal of a supplemental carbon source, fungi can be selected against to yield a prokaryotic consortium. In this case, fungi and methanogens can be further separated from bacteria via treatment with penicillin and streptomycin. Bottom-up reconstruction of co-cultures involves combining separate, isolated microbial cultures to form a co-culture based on interactions predicted by genomics and metabolic potential.

RESULTS AND DISCUSSION

Selective Enrichment Enables Isolation of a Stable Native Consortium. In order to capture a consortium from the herbivore digestive tract microbiome, a selective enrichment process was developed to obtain a minimal system of microbes (Figure 1). Methanogens and anaerobic fungi were successfully enriched on a lignocellulosic reed canary grass substrate, as determined by periodic sampling of methane in the headspace of cultures (Figure S1), fungal internal

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

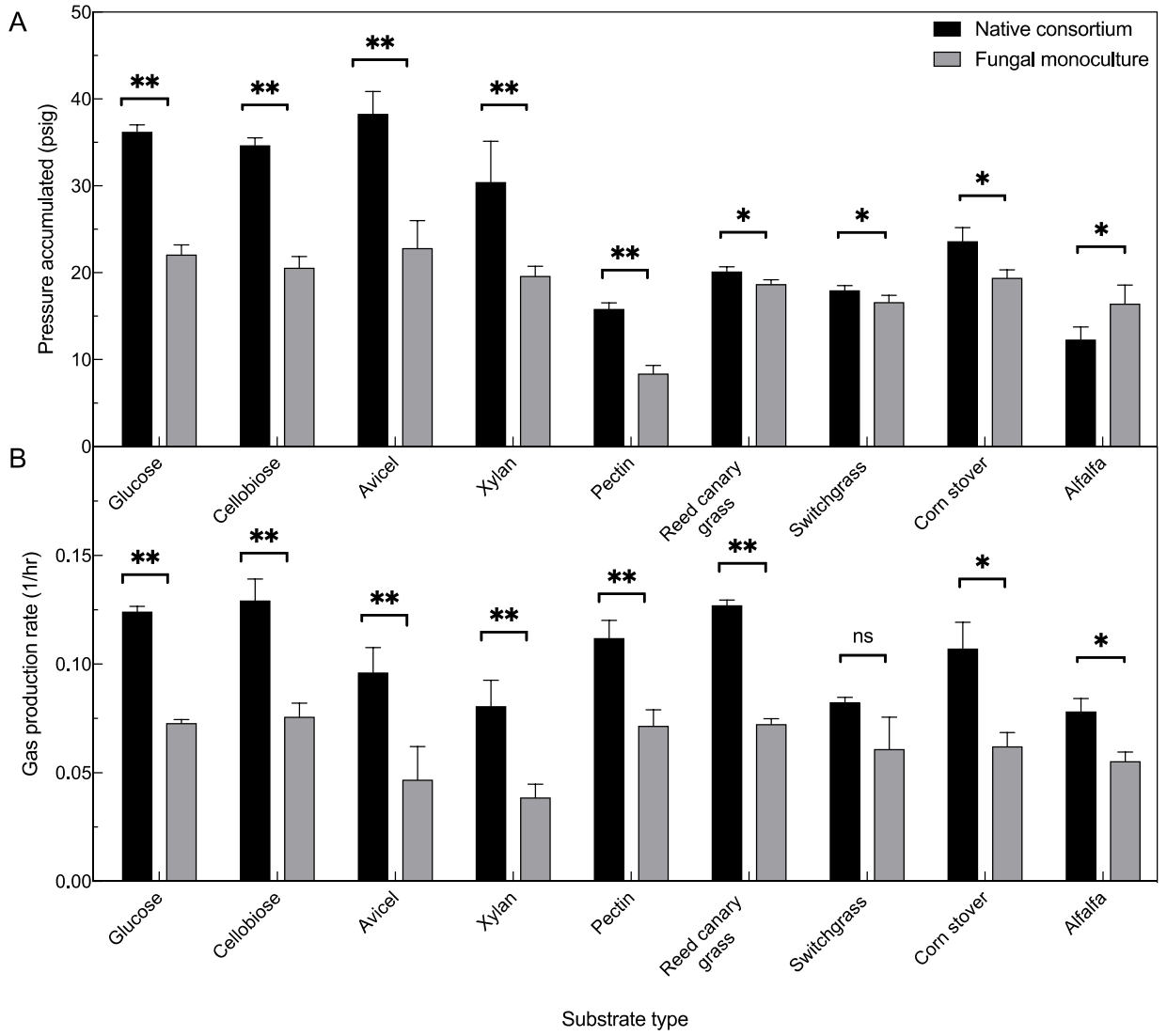


Figure 2. An enriched anaerobic consortium leads to rapid, elevated gas production during substrate hydrolysis compared to fungal monocultures. Panel A compares gas production between the top-down, natural consortium and the fungal component of that consortium in monoculture on the indicated substrates. Panel B compares total gas production rates between the fungal monoculture and native consortium on the indicated substrates. Across all substrates tested, the top-down consortium displayed a higher fermentation gas production rate that was 1.4−2.1 times greater than the rate of gas production by the fungal monoculture. The total amount of fermentation gas produced was also increased in all but one instance (A), generating up to 0.75−1.9 times the amount of the fungal monoculture. Error bars represent the standard deviation of three replicates (n = 3). Statistical significance was tested in Prism 8.1.1 using the Holm−Šídák method of multiple comparisons, where ^{ns} represents not significant, * represents $p < 0.05$, ** represents $p < 0.01$. The cultures that produced these data were incubated with the specified substrate for 90−140 h, and monitoring was discontinued when cultures stopped producing fermentation gas.

transcribed spacer (ITS) and methanogen-specific 16S primers (Table S1). An estimate of microbial diversity in the consortium was calculated by cloning ITS or 16S amplicons into vectors and selecting individual colonies for Sanger sequencing. The ITS sequences derived from the enriched community revealed that a single fungus was isolated with only slight variations in sequence identity, likely due to the technical challenge of sequencing DNA with extremely high AT content, or sequence variation among gene copies.[14,18]

The closest cultured match to the enriched fungus was a *Piromyces* isolate, with only 86% identity matching a cultured strain across the entire ITS and 5.8S region. The sequences clustered distinctly from other fungal genera (Figure S2), grouping into a clade with an uncultured isolate. Light microscopy of the isolate revealed abundant tapering rhizoids and multinucleated sporangia that are general characteristics of *Piromyces* (Figure S3). Methanogen-specific primers amplified two distinct sequences most similar to *Methanosphaera* and *Methanocorpusculum* isolates (Figure S2). The phylogenetic identity (Figure S2b) of the two methanogens was somewhat surprising, as the digestive tract of horses and other large herbivores is often dominated by *Methanobrevibacter* methanogens.[19,20] However, it is possible that cultivation conditions including specific fungus-methanogen or bacteria-methanogen interactions selected for isolation of the *Methanosphaera* and *Methanocorpusculum* over the more abundant *Methanobrevibacter*.

Metagenomic sequencing was performed to characterize the prokaryotic component of the enriched consortium, which was not done for the eukaryotic population due to present technical limitations in assembling fungal genomes.[13,21] DNA was isolated from the natural consortium after 4 days of growth

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

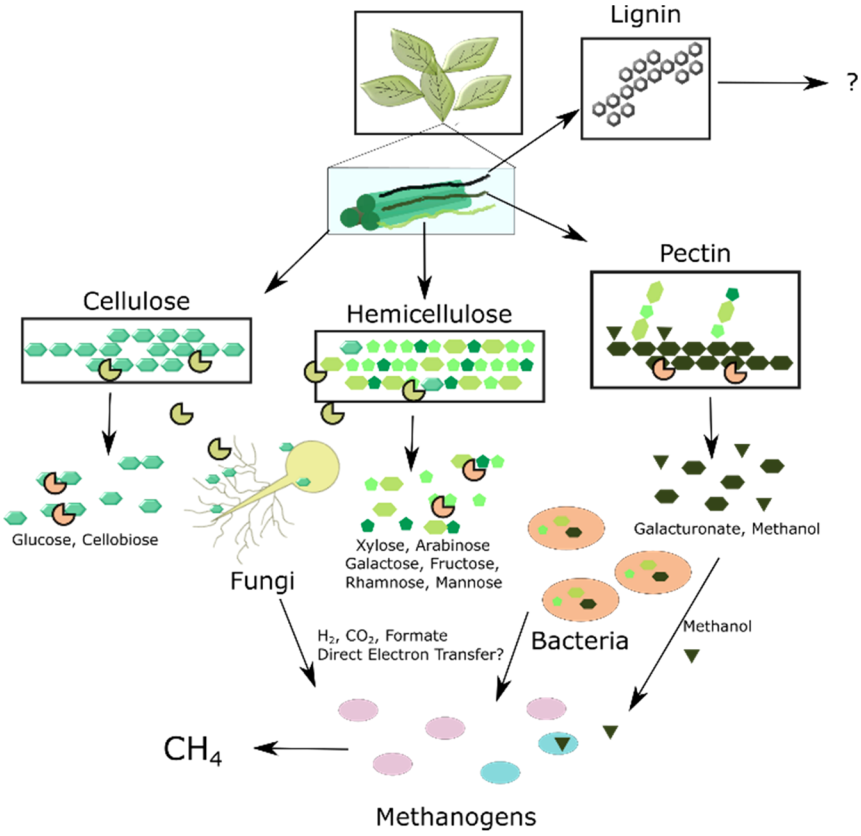


Figure 3. Metagenomic analysis of the enriched consortium reveals potential for compartmentalized substrate degradation and metabolism. A schematic overview of predicted metabolic interchange between microbes, based on the annotated genomic content of the metagenome assembled genome (MAG), of each member is displayed. The fungi are predicted to be primary degraders of biopolymers, deconstructing the linear polymer chains of cellulose and branched polymers of hemicellulose. The *Sphaerochaeta* bacterial MAG contains genes to degrade shorter cellulose and hemicellulose fragments, as well as genes to metabolize sugars that remain unused by the fungi. The *Methanosphaera* archaeal MAG contains genes to consume methanol liberated from pectin degradation as well as H_2. Finally, the *Methanocorpusculum* archaeon contains genes to consume H_2, CO_2, and formate.

to capture initial relative microbial abundances for the enriched community. A summary of the metagenomic sequencing is detailed in Table S2. An initial search for 16S sequences revealed the two methanogens previously detected by methanogen-specific primers, but also a third unique sequence, most similar to the bacterium *Sphaerochaeta* sp. The phylogenetic placement of each 16S sequence is shown in Figure S2. The assembled contigs were checked for completion and contamination and each bin was at least 93% complete with less than 2% contamination (Figure S4). Completeness and contamination in these ranges qualifies each MAG as a high-quality draft according to the Minimum Information about a Metagenome Assembled Genome.[22]

A Natural Gut Consortium Accelerates Biogas Production Relative to a Fungal Monoculture. To compare the degradation capabilities of the enriched anaerobic consortium versus its individual microbial constituents, a novel separation scheme was designed to separate the fungal and prokaryotic components of the consortium (Figure 1). While methanogens are resistant to penicillin and streptomycin, they are susceptible to chloramphenicol. As such, pure fungal monocultures were obtained by treatment of the enriched community with chloramphenicol, and a methanogenic top-down consortium was obtained in parallel by treatment with penicillin and streptomycin. Through incubation with hydrogen and removal of a supplemental carbon source, fungi were selected against to yield a prokaryotic consortium. Through this enrichment and antibiotic selection scheme, only cultures that contained anaerobic fungi were able to hydrolyze cellulosic and lignocellulosic substrates (Figure S5), though slight growth was observed for the prokaryotic consortium on xylan and more so on pectin (Figure S5). The top-down enriched natural consortium of fungi, methanogens, and antibiotic resistant bacteria was tested for growth on a wide variety of substrates ranging from simple sugars to complex lignocellulose, and then compared to the chloramphenicol treated fungal monocultures from the parallel isolation (Figure 2).

The fermentation gas production rate of the natural consortium and the coderived fungal monoculture were measured across a variety of substrates as shown in Figure 2. The enriched microbial consortium outperformed the fungal monocultures significantly across all substrates tested with the exception of switchgrass which was within statistical variation (Figure 2). No significant gas production was observed by the isolated prokaryotic component of the consortium on any substrate, with the exception of xylan and pectin (Figure S5). The fermentation gas production rate of the enriched anaerobic consortium was between 1.4 and 2.1 times faster than that of the fungal monoculture alone on the same substrate (Figure 2A) (ANCOVA, $p < 0.05$). Additionally, the total amount of fermentation gas produced by the fungus-

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

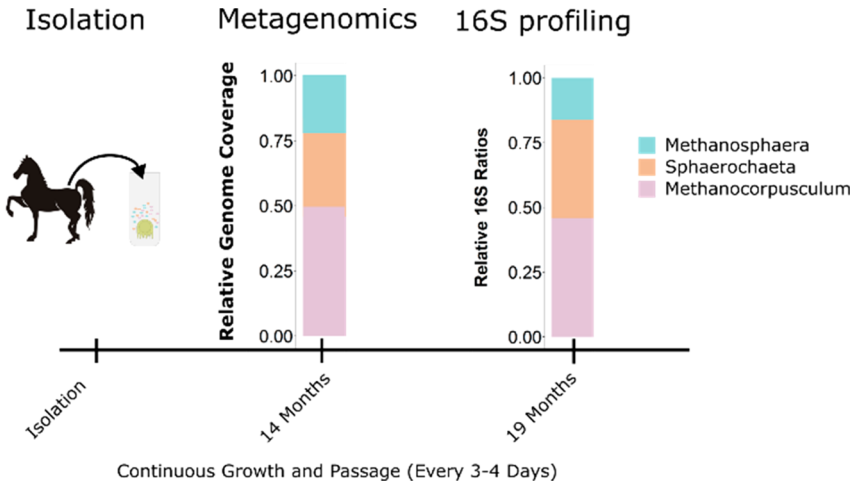


Figure 4. Community composition in the enriched consortium remains stable despite continuous growth and culture transfer. Relative ratios of the prokaryotic members are displayed, determined from whole genome metagenomics 14 months after isolation and reassayed by marker gene metagenomics 19 months after isolation. As displayed, the top-down consortium demonstrated remarkable stability, with the relative abundances remaining the same across two measured time points, despite serial subcultivation at 3−4 day intervals.

containing consortium was enhanced for all substrates except alfalfa stems (Figure 2A) (Student's *t* test, $p < 0.05$), where the consortium produced between 0.75 and 1.9 times more total gas compared to fungal monocultures. However, since anaerobic methanogenesis reduces the total number of moles of gas released, the total gas measurement likely underestimates the production capacity of consortia.[17] Across complex lignocellulosic substrates (reed canary grass, alfalfa stems, corn stover, and switchgrass) the gas production rate was greatest on reed canary grass for both the enriched consortium and the isolated fungi (Figure 2B). Moreover, the gas production rates on reed canary grass and on the simple soluble sugars, like glucose and cellobiose, were comparable (Figure 2B). As the community was isolated using reed canary grass, it is likely that enrichment conditions selected for community members adapted for growth on that substrate.

Metagenomic Analysis Suggests that Syntrophy and Compartmentalized Metabolism Drive Stability of an Enriched Consortium. Metagenomic sequencing of the top-down enriched natural consortium revealed genomes coding for a tightly interwoven community metabolism, despite some redundant capabilities, which is schematically depicted in Figure 3. Syntrophy occurs between the methanogens and fungi, as well as methanogens and anaerobic bacteria in ruminants *via* hydrogen exchange.[6−11] From previous work, it is known that anaerobic fungi are enriched in biomass degrading enzymes,[13,14,21,23] but these enzymes are primarily exo- and endoacting cellulases. They also contain some enzymes for degradation of smaller cellodextran fragments, as well as transporters capable of taking up these carbohydrate fragments.[24] When grown in isolation, excess sugars are released from fungal degradation of biomass,[25] which likely supports the growth and proliferation of "sugar cheaters" often seen in biomass-degrading consortia.[26] We hypothesize that the *Sphaerochaeta* identified in our metagenomic analysis likely serves such a role in the natural consortium enriched here. Thus, in the enriched anaerobic consortium, the fungi likely act as the primary degraders of the plant biomass; they assimilate some sugar-rich hydrolysates, but leave behind ample sugars for other opportunistic microbes that were not directly selected for in the enrichment.

Further analysis of the bacterial *Sphaerochaeta* genome bin revealed very few enzymes that act on crystalline cellulose or other components of plant biomass (Supplementary Database S1); however, the analysis exposed many enzymes that further degrade the small fragments of carbohydrates that are released by primary biomass degraders. The *Sphaerochaeta* member also contained several enzymes for pectin degradation, which likely explains the enhanced gas production rate on pectin seen by the enriched consortium (Figure 2), as well as the modest pectin-degrading ability of the enriched prokaryotic component of consortium (Figure S5). The role of *Spirochaetes* in pectin degradation was recently hypothesized from a moose gut metagenomic survey,[27] lending further credence to the idea that these microbes have the potential to degrade pectin. Furthermore, genomic analysis of the transporters and metabolism in *Sphaerochaeta* showed that it is capable of taking up and using a wide array of sugars and sugar oligomers like glucose, maltose, mannose, galactose, xylan, and alginic acid (Supplementary Database S2). Finally, a beta-lactamase present in the *Sphaerochaeta* genome likely explains its resistance to penicillin and streptomycin, which permitted it be enriched within the fungal-methanogen dominated consortium.

The two MAGS representing methanogens from the *Methanocorpusculum* and *Methanosphaera* genera played similar roles in the consortium, as both act as the terminal electron acceptors in the anaerobic community. However, genomic analysis of the *Methanocorpusculum* member revealed a pathway for formate utilization, potentially allowing for growth on either H_2/CO_2 or H_2/formate. The *Methanosphaera* contained all genes for growth on H_2/CO_2 and H_2/methanol, as previously shown for a different species in this genus.[28] However, *Methanosphaera* typically require a combination of H_2 and methanol for growth despite their ability to metabolize CO_2.[29] Methanol is released during the degradation of pectin and other plant cell wall polymers,[30,31] which could explain the functional role of the *Methanosphaera* in this enriched natural consortium. Previous analyses have demonstrated the importance of compartmentalized nitrogen metabolism,[4] however our genomic analysis suggests that compartmentalized

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

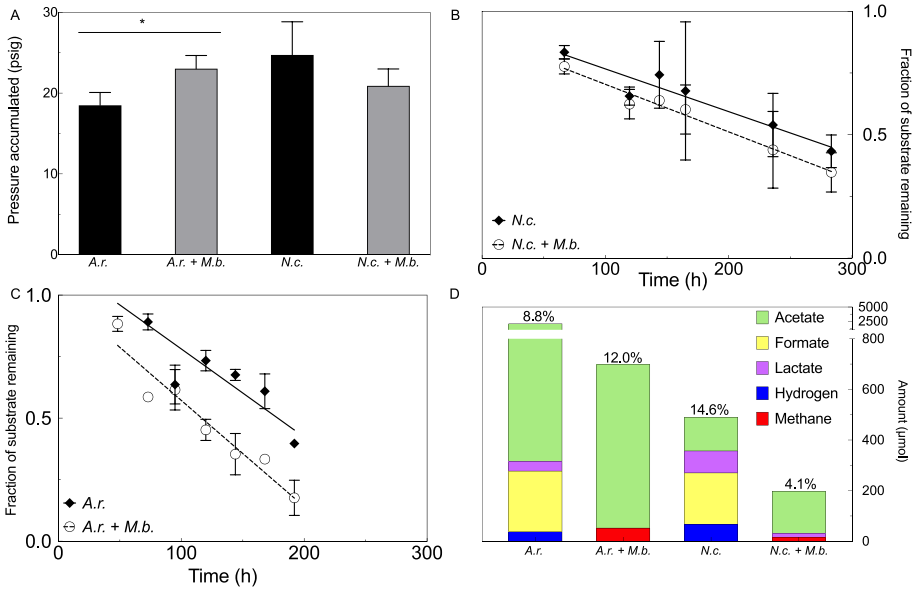


Figure 5. Synthetic co-cultures of fungi and methanogens exhibit syntrophy but do not increase rates of cellulose deconstruction by fungus. Panel A shows total accumulated pressure for cultures of the fungi *Anaeromyces robustus* (*A.r.*) and *Neocallimastix californiae* (*N.c.*) cultured in isolation for 10 days in a sealed Hungate tube as well as parallel synthetic co-cultures that additionally contained *Methanobacterium bryantii* (*M.b.*); * designates a statistically significant Student's *t* test ($p < 0.05$). Panels B and C show the rate of cellulose solubilization by *N.c.* (B) and *A.r.* (C), with and without the cocultivated methanogen *M.b.* Lines represent linear regression analyses used for analysis of covariance (ANCOVA) calculations. No significant difference was found between the slope of cellulose deconstruction in fungal monoculture (*N.c.* = −1.7 ± 0.4 mg/h, *A.r.* = −3.6 ± 0.4 mg/h) *versus* fungal-methanogen co-culture (*N.c* + *M.b.* = −1.9 ± 0.3 mg/h, *A.r.* + *M.b.* = −4.3 ± 0.4 mg/h) *via* ANCOVA. Panel D displays each metabolite as the result of averaging three replicates after cultures were allowed to grow for 10 days in sealed Hungate tubes. Standard deviations are not shown for clarity; however, values obtained from liquid chromatography have standard deviations less than 10% of the mean, and values obtained from gas chromatography have standard deviations less than 17% of the mean. Percentages indicated at the top of each bar-stack in Panel D refer to the mean percentage of headspace gas that was measured as hydrogen in monocultures or as methane in co-cultures. For all panels error bars represent the standard deviation of three biological replicates ($n = 3$).

carbon metabolism is likely a key factor determining composition and stability of the enriched community.

Since the enriched consortium was continuously passaged every 3 to 4 days, we used 16S and ITS metagenomic profiling to assay stability of consortium membership five months after initial shotgun metagenomic sequencing. The ITS data confirmed that the chloramphenicol-treated fungal monoculture had the same ITS sequence as the penicillin streptomycin selected consortium (Table S1). Furthermore, as shown in Figure 4, prokaryotic membership in the enriched consortium was stable across both time points. However, we observed that rumen fluid in the culture medium produced a background 16S signal. Our analysis, therefore, only contains sequences significantly enriched compared to the background of an uninoculated control (>1% of the community), and it is possible that the presence of low abundance community members was masked by the contaminating DNA from clarified rumen fluid.

Synthetic Biomass-Degrading Co-cultures Produce Methane but Lack the Stability and Productivity of Natural Consortia. Given the compartmentalization observed in the minimal consortium enriched from herbivore feces, we formed synthetic anaerobic co-cultures composed of microbes with interdependent metabolisms and characterized their biomass-degrading activities, metabolite generation, gas production rate, and stability of membership (resilience) in batch culture. While several previous studies have probed the behavior of fungus-methanogen co-cultures to suggest that methanogen addition accelerates biomass deconstruction,[6−11] none have compared the activity, stability, and productivity of synthetic co-cultures with enriched anaerobic consortia. To assemble synthetic co-cultures, we employed the fungal isolates *Anaeromyces robustus* and *Neocallimastix californiae*. These are relatively well-studied members of the Neocallimastigomycota that are capable of cryopreservation and have high-quality sequenced genomes.[14,32] These fungal testbed strains were paired separately with the model methanogen *Methanobacterium bryantii* (DSM 863) to establish synthetic co-cultures from the "bottom-up" (Figure 1), whereby syntrophy would establish interaction and stability of cultures.

All the synthetic co-cultures we established exhibited syntrophy, degraded lignocellulose, were propagated, and successfully produced methane (Figure 5, Figure S1). Furthermore, the *A. robustus* co-culture even outperformed its matching monoculture, producing significantly more total fermentation gas (Student's *t* test $p < 0.05$, Figure 5A). Metabolite measurements in Figure 5D show that the addition of methanogens altered the metabolic end-point product profile of the fungal mono- *vs* co-cultures, indicating that the predicted syntrophy had been established. Specifically, both formate and hydrogen are metabolized by methanogens in co-culture, resulting in the near total absence of these metabolites under co-culture conditions (Figure 5D). Furthermore, synthetic co-cultures produced similar headspace concentrations of methane (4−12% of total culture headspace) compared with the enriched native community (Figure S1). When grown on 0.1 g of Whatman filter paper, synthetic co-cultures of *M. bryantii* paired with *N. californiae* yielded 0.02 mM methane, while the synthetic co-culture of *M. bryantii* and *A. robustus* yielded 0.05 mM methane. Interestingly, the

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

quantities of methane produced when the co-cultures were grown on lignocellulosic reed canary grass were similar (0.02 and 0.06 mM for *N. californiae* and *A. robustus* co-cultures, respectively) despite the higher recalcitrance of lignocellulose relative to purified cellulose.

For both *N. californiae* and *A. robustus* synthetic co-cultures with *M. bryantii*, there were statistically significant differences between the *y*-intercepts of the linear regression models describing cellulose deconstruction relative to monoculture (Figure 5A−C), indicating that the co-cultures experienced a decrease in lag phase relative to monocultures. It has been demonstrated in both bacteria and yeasts that lag phase is a distinct, and vastly understudied, phase of growth with its own signature of transcriptomic regulation.[33−35] Additionally, in the biotechnologically relevant *Aspergillus* fungi, reductions in lag phase under co-culture conditions have been proposed as driving factor to increase product output.[36] Since lag phase is broadly defined as a phase of growth where organisms adapt to their new environment, reductions in lag phase in our synthetic co-cultures likely arose from the established fungus-methanogen syntrophy indirectly *via* alterations to concentrations of chemical species in the environment. Both formate and hydrogen are metabolized by the methanogens in the synthetic co-cultures we describe here, resulting in reduction or elimination of fungal waste compounds under co-culture conditions (Figure 5D). The removal of such waste products likely reduces inhibitory effects on the ATP-generating hydrogenosomes of the anaerobic fungi allowing them to establish and maintain catabolic activity without hindrance.[37] Similar to effects seen in enriched consortia, hydrogen conversion to methane through methanogenesis also likely contributes to the different effective gas productions by synthetic co-cultures *versus* their respective monocultures (Table S3). Methanogenesis by *M. bryantii* consumes five moles of gas for every one mole of methane produced ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$), and therefore the total pressures measured in methanogenic cultures represent underestimates of total gas production. This downward shift in total moles of gas, as well as a shift from hydrogen mole fraction solubility ($1.332 \times 10^{-5} \pm 7.20 \times 10^{-8}$ mol/atm) to that of methane solubility ($2.069 \times 10^{-5} \pm 1.16 \times 10^{-8}$ mol/atm), helps to explain why synthetic *Neocallimastix californiae* (*N.c.*) + *Methanobacterium bryantii* (*M.b.*) co-cultures failed to produce more total pressure than their matching *N.c.* monocultures despite the shortened lag phase of the co-culture relative to the monoculture (Figure 5A,B).

Both liquid and gaseous metabolites reflected an effective syntrophy in synthetic fungal-methanogen co-cultures (Figure 5D and Table S3), but this cross-feeding did not impact the rates of substrate deconstruction or fermentation gas production relative to fungal monoculture (Figure 5B,C and Figure S6), as determined by ANCOVA ($p > 0.05$). We were surprised to note the lack of differences in substrate degradation rates between fungal mono- and co-cultures since this phenomenon had been widely reported throughout published literature for several decades.[6−11] However, upon thorough reanalysis of literature data we found that differences in substrate deconstruction rate reported were qualitative assessments lacking statistical support (Supplementary Discussion, Table S4). Nevertheless, it is important to note that other outcomes of synthetic co-culture are possible beyond accelerated gas production or substrate deconstruction. For example, cocultivation of *M. bryantii* with *A. robustus* was recently shown to selectively elevate transcription of several key CAZymes and components of cellulosomes in the fungus.[17] Thus, fungus-methanogen cocultivation may shift metabolic energy to amplify production of enzymes that liberate specific sugars from lignocellulose without affecting the rate of total substrate degradation.

Though the synthetic co-cultures formed a successful association to yield methane, their primary limitation when compared to the native enriched consortium, was stability. Although the synthetic co-cultures of *M. bryantii* paired with *N. californiae* or *A. robustus* were cultivatable for several culture transfers, they never reached the >2 years (∼204 transfers) of stability achieved by the native community (Figure 4), often losing the associated methanogens within 7−10 culture transfers of formation. Methanogens typically grow much more slowly than anaerobic fungi,[25,38] such that the paired methanogens may be lost due to dilution from consecutive culture transfers. The extreme oxygen sensitivity of methanogens might lead to culture instability in the synthetic co-cultures through brief oxygen exposure during transfer, which is largely avoided in the natural consortium enriched here due to the oxygen scrubbing activity of the *Sphaerochaeta* bacterium, which is a facultative anaerobe. Additionally, some methanogens may physically "attach" to their syntrophic partner fungi to form a biofilm-like association,[28,39] and it is possible that *M. bryantii* does not form such an association with *N. californiae* or *A. robustus*, leading to instability of synthetic co-cultures.

CONCLUSIONS

Here, we have compared two complementary methods to establish a lignocellulose-degrading microbial community—"top-down" enrichment of native communities, and "bottom-up" formation of synthetic co-cultures. The top-down approach resulted in a highly stable consortium that produced more fermentation gas at a faster rate than its fungal component in isolation. Culture conditions likely selected for coevolved microbes, which increased the stability of the enriched consortium (>2 years, ∼204 transfers) compared to separately isolated methanogen and fungus in the synthetic co-cultures (∼1 month, 7−10 transfers).[8] Additionally, the resilience of the naturally enriched microbial consortium may also stem from the presence of low-abundance, facultative "sugar cheaters" that scavenge oxygen and aid in pectin-rich biopolymer degradation.

Metagenomic characterization of the enriched anaerobic community was integral to the design of synthetic co-cultures that successfully established a syntrophic interaction to generate methane directly from lignocellulose. While the synthetic co-cultures in this study did not capture the growth rate or productivity seen in naturally derived systems, the flexibility of this approach enables additional microbes to be added that would help control carbon flow in the system.[40,41] Importantly, these co-cultures required no genetic engineering or involved methods such as synthetic signaling[42] or engineered auxotrophy,[1] and therefore are achievable using microbes lacking genetic tools. The stability seen from the synthetic co-cultures is still an improvement over consortia formed between competing microbes, where competition for resources often leads to consortia instability.[43] While lignocellulosic bioconversion for methane capture often takes place in anaerobic digesters with an array of hundreds of different species,[44] here we have demonstrated that signifi-

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

cantly simpler two-microbe systems could be a feasible alternative. Overall, both "top-down" and "bottom-up" strategies for consortia assembly are promising paths toward implementing microbial consortia for bioconversion of lignocellulosic biomass. Characterization of both types of communities are critical to unravel and engineer syntrophic relationships that drive the synergistic activity of consortia.

METHODS

Enrichment and Assembly of Microbial Consortia. Native anaerobic consortia were obtained from equine fecal materials collected from the UCSB West Campus Equine Stables, suspended in Medium C,[13] serially diluted, and inoculated into seed cultures containing 1% (w/v) lignocellulose (dried and ground reed canary grass). Cultures positive for fungal growth, evidenced by clumped floating grass and increased gas pressure in closed Hungate tubes, were transferred to fresh growth medium every 3 to 4 days while incubated anaerobically at 39 °C. The presence of methanogens was verified by periodic determination of methane in the headspace of cultures using gas chromatography (Figure S1). In addition, methanogen-specific 16S[45] and fungal ITS[46] primers were used to confirm the presence of both members (Table S1). A rough estimate of microbial diversity was achieved by cloning the ITS or 16S amplicons into vectors and selecting 10 individual colonies for Sanger sequencing.

The enriched consortium was split into the fungal component, the prokaryotic component, and the fungal-prokaryotic component according the "top-down" schematic in Figure 1. Chloramphenicol was added to the fungal Medium C at 50 μg/mL, and likewise penicillin (1000 U/mL) and streptomycin (1000 U/mL) enabled acquisition of a fungus-methanogen consortium. The consortium was inoculated into M2 medium with 80%/20% H_2/CO_2 headspace, supplemented with methanol (1% v/v) to obtain the prokaryotic part of the consortium. The initial enrichment culture was separated into its fungal and prokaryotic components after 14 months, and approximately 56 transfers, after the original isolation just prior to metagenomic sequencing.

"Bottom-up" consortia were formed in 40 mL culture volumes by inoculating 1 mL of the fungal member after 3 days of growth in its previous culture tube, plus 1 mL of the methanogen member after 7 days of growth in its previous culture tube into 38 mL fresh medium. In parallel, fungal monoculture medium was supplemented with 1 mL of unused methanogen M2 medium and inoculated with 1 mL of the fungal culture to establish fungal monocultures for comparison to synthetic co-cultures. The 40 mL seed cultures were used after 4 days of growth to inoculate the culture tubes for fermentation studies on varying substrates. Both types of cultures were allowed to grow for 4 days and were then used to inoculate (10% v/v) 10 mL cultures in Hungate tubes for fermentation studies. For synthetic co-cultures, the previously isolated fungi *Anaeromyces robusus* and *Neocallimastix californiae*[13] were used, as well as *Methanobacterium bryantii* strain M.o.H. (DSM 863), which was obtained from the Leibniz Institute DSMZ − German Collection of Microorganisms and Cell Culture (https://www.dsmz.de/).

Microbial Cultivation and Characterization. Fungi and enriched consortia were grown in anaerobic fungal Medium C as previously described,[13] supplemented with penicillin (1000 U/mL), streptomycin (1000 U/mL), nickel sulfate (0.2 μg/mL), and sodium 2-mercaptoethanesulfonate (40 μg/mL) in 10 mL culture volume (9 mL fresh medium plus 1 mL inoculum), unless otherwise stated. Cultures were routinely maintained on 1% (w/v) reed canary grass. Methanogens were cultured as previously described,[28] in M2 medium with 80%/20% H_2/CO_2 headspace, supplemented with methanol (1% v/v) in 10 mL culture volume. All cultures were grown at 39 °C without shaking.

Fermentation experiments altered the supplied substrate from reed canary grass to the following at 1% (w/v) prior to autoclaving: Avicel PH-101 (Cat. No. 11365, Sigma-Aldrich, Kansas City, MO), xylan (Cat. No. X0078, TCI Chemicals, Portland, OR), pectin (Cat. No. 0215605780, MP Biomedicals, Santa Ana, CA), reed canary grass, corn stover, switchgrass, and alfalfa stems (4 mm particle size, obtained from USDA-ARS Research Center, Madison, WI). Additionally, glucose and cellobiose were tested by dissolving in water, sterile-filtering, and adding to media post autoclaving at 0.5% (w/v). Total pressure accumulation was quantified by measuring and venting the headspace pressure using a pressure transducer (Part No. PX119-015GI, Omega Engineering Inc., Norwalk, CT).[37,47] This pressure data was fit to a standard logistic growth equation ($dX/dt = \mu_{max} \cdot X \cdot (K - X)/K$ to estimate the gas production rate, where X signifies net gas pressure produced (psi), t represents time (hours), μ_{max} is the maximum pressure production rate (1/h), and K is the maximum total pressure produced (psi). Incubation times of these fermentation experiments varied from 90 to 140 h and incubations were stopped when each cultures ceased production of fermentation gases. Mean total gas produced and rate of gas production were compared using Holm−Šídák method of multiple comparisons test in Prism 8.1.1. To account for any abiotic pressure increases from the medium alone, pressure accumulation from blank media tubes lacking inoculum were subtracted.

Determination and Quantification of Culture Metabolites. This study further compared the ability of fungal monocultures and synthetic co-cultures to deconstruct 1% (w/v) Whatman filter paper and reed canary grass while tracking accumulation of major microbial products. Samples of the gaseous headspace and culture supernatant were obtained after 10 days of growth in sealed Hungate tubes. For this analysis, monocultures and co-cultures were grown in a minimalist recipe of Medium C called "MC−" with reduced rumen fluid (1/20), yeast extract (1/10), and Casitone (1/20) compared to the standard recipe. The altered MC− recipe allowed for subsequent HPLC analysis of short-chain fatty acids in the culture supernatant whereas the complexity of standard Medium C masks signals for many metabolites of interest. All other cultivation conditions were as described previously, and total fermentation gases were assayed using the pressure transducer method.

Metabolites in liquid media were measured using a high-performance liquid chromatograph (HPLC) and an assay for total reducing sugars. An Agilent 1260 Infinity HPLC (Aligent, Santa Clara, CA) equipped with a Bio Rad Aminex HPX-87H analytical column (Part No. 1250140, Bio Rad, Hercules, CA) was used to quantify volatile fatty acids. Samples were run in a 5 mM sulfuric acid mobile phase at 0.5 mL/min and at a column temperature of 35 °C. A 0.22 μm inline filter (Part No. 50671551, Agilent) followed by a polyether ether ketone guard cartridge (Part No. ANX993515, Transgenomic, San Jose, CA) were included before the analytical column. Signals were measured using a variable wavelength detector set to 210 nm.

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

Samples were acidified with a 1:10 volume of 50 mM sulfuric acid, vortexed, incubated at room temperature for 5 min, centrifuged at 21 000*g* for 5 min, and then 0.22 μm filtered into HPLC vials. Standards for acetate, butyrate, formate, lactate, and propionate were prepared in uninoculated culture medium using the same procedure described for samples, and standards for each short-chain fatty acid were prepared at 0.1% and 0.01% (w/v). In addition to short chain fatty acids, the total reducing sugars in culture supernatants were quantified using a microplate adapted 3,5-dinitrosalicylic acid method with a 300 μL total reaction volume with a sample:reagent ratio of 1:1.[48]

The percentage compositions of methane and hydrogen in the headspace of each culture tube were assayed using a Thermo Fisher Scientific TRACE 1300 Gas Chromatograph (Thermo Fisher Scientific, Waltham, MA) with a TRACE TR-5 GC Column (Part No. 260E113P, Thermo Fisher Scientific) or a Shimadzu GC 14A (Shimadzu Corp., Kyoto, Japan) equipped with an *N*-octane on Res-Sil C column (Part No. 80436800, Restek, Bellefonte, PA). Headspace samples of 100 μL were taken from sealed cultures using a 500 μL gastight syringe and injected immediately. Samples were run at a constant oven temperature of 30 °C using high purity He as a carrier gas, and signals were measured using an Instant Connect Pulsed Discharge Detector (PDD) (Part No. 19070014, Thermo Fisher Scientific), when using the Thermo Fisher TRACE 1300 GC. For the Shimadzu GC 14A, oven temperature was set to 50 °C, high purity N_2 was used as carrier gas, and an FID detector was used to measure signals. Supplier-mixed standards of 1%, 3%, 5%, 10%, and 20% methane and hydrogen were run before and after injecting samples. To ensure accuracy of gas calculations, separate standard curves were used to calculate concentrations from 1 to 5% and 5 to 20%.

Quantification of Cellulose Degradation Rates in Monocultures and Synthetic Co-culturess. To assay the rate of substrate solubilization by fungal monocultures and synthetic co-cultures, experiments were conducted in parallel to those described above for metabolite quantification. Culture conditions were identical to those previously described for metabolite analysis. Briefly, 30 10 mL Hungate tubes were inoculated with each fungal monoculture or co-culture, where each tube contained 1% (w/v) premeasured Whatman filter paper in MC- medium. Cultures were monitored for pressure increase daily and three replicate cultures were harvested periodically for analysis over a time period of 15 days. Residual substrate mass remaining was determined by lyophilizing (FreeZone 4.5 Liter Benchtop Freeze-Dry System, Part No. 7750020, Labconco Corp., Kansas City MO) the remaining Whatman paper for 48 h following culture harvest and weighing the dried sample. Linear regressions were used to describe the portion of gas production or cellulose draw-down corresponding to the maximum activity of the culture, while lag and stationary phases were excluded. Analysis of covariance (ANCOVA), performed in Prism 8.1.1, determined whether the slopes and intercepts of these linear regression models were different. Significantly different slopes of linear regressions ($p < 0.05$) indicate a statistically significant difference in the rate of substrate deconstruction or gas production between two cultures, while significantly different *y*-intercepts represent a difference in lag phase between the monocultures and co-cultures.

Metagenomic Library Preparation and Sequencing. To isolate genomic DNA (gDNA), cultures were grown in 40 mL of media in 60 mL Wheaton serum bottles until stationary phase and then harvested by centrifugation for 30 min at 10 000*g* at 4 °C. Cell pellets were resuspended in 0.5 mL TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Sodium dodecyl sulfate was added to a final concentration of 0.5%, proteinase K (New England BioLabs, Ipswitch, MA) was added to 100 μg/mL, and RNaseA (MoBio Laboratories, Carlsbad, CA) was added to 100 μg/mL. The mixture was incubated at 37 °C for 1 h. NaCl was added to 0.5 M, and 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was mixed and then centrifuged at 13 000*g* for 10 min at 4 °C. The aqueous phase was transferred to a new tube and 0.6 mL of isopropyl alcohol was added. The mixture was incubated at −20 °C for ∼16 h and then centrifuged at 13 000*g* for 5 min at 4 °C. The pellet was washed with 70% ethanol, centrifuged at 13 000*g* for 5 min at 4 °C, and finally resuspended in 10 mM Tris buffer pH 8.0 and stored at −20 °C.[49]

Genomic DNA (gDNA) was prepared for high throughput sequencing (HTS) using the TruSeq DNA PCR-Free library prep kit supplied by Illumina, Inc. (San Diego, CA). Briefly, purified gDNA were first fragmented using a M220 Focused Ultrasonicator (Covaris, Woburn, Massachusetts) followed by end repairs, size selection (∼330 bp), end adenylation and paired-end adapters ligation using the kit. Prepped libraries were then quantified using Qubit 2.0 (Part no. Q32866, Life Technologies, Carlsbad, CA,) and TapeStation (Part no. G2991AA, Agilent, Santa Clara, CA) before pooling. HTS was performed with an Illumina NextSeq500 sequencer using a 150 cycle, mid output kit (2 × 75 paired-end).

Metagenomic Binning and Analysis. Metagenomic reads were assembled using Megahit v1.1.[50] Assembled contigs were binned using MetaBAT v2.12.1[51] and CONCOCT v0.4.1,[52] with BLAST used to manually curate unbinned contigs. Binned genomes were annotated with the Department of Energy Systems Biology Knowledgebase (KBase, http://kbase.us) automated pipeline. Genomic features including ORFs, large repeat regions, rRNAs, CRISPRs, and tRNAs were identified and annotated with the Rapid Annotations using Subsystems Technology toolkit (RASTtk).[53] These gene annotations were combined with biochemical information from the Kyoto Encyclopedia of Genes and Genomes (KEGG)[54] to reconstruct the metabolism of each genome bin. Metagenome assembled genome (MAG) completion was determined utilizing CheckM v1.0.7.[55] Metagenomic relative abundance for each bin was calculated by mapping reads to the assembled MAGs using Bowtie2 v2.3.2.[56] Transporters were classified using the Transporter Classification DataBase[57] downloaded on June 18, 2019. Results were filtered to only include hits that covered 70% of both query and subject with an *E*-value less than 10^{-3}. Hits were then manually curated to classify the most likely sugar specificity. CAZymes were predicted using dbCAN2[58] v4 accessed June 18, 2019.

16S and ITS Profiling. For assessment of community diversity after successive cultivation, cultures were grown for 4 days and harvested by centrifugation at 3220*g* for 20 min at 4 °C. DNA was extracted using the FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the instructions. Primers were designed for the V5 16S region using the Ribosomal Database Project.[59] ITS primers were as previously described.[21] Primers had overhangs compatible with Nextera XT primers (P5 for forward and P7 for reverse). The sequences of all primers used are in Table S1. Amplification

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

was performed in 50 μL reactions composed of 1 μL of extracted DNA, 10 μL of 5× Phusion GC Buffer, 1 μL of 10 mM dNTPs, 2.5 μL of 10 μM forward primer, 2.5 μL of 10 μM reverse primer, 0.5 μL of Phusion DNA Polymerase (New England BioLabs, Ipswitch, MA), and 32.5 μL of DNase-free H_2O. Amplification occurred with an initial 30 s denaturation at 98 °C; followed by 30 cycles of 10 s at 98 °C, 30 s at 57 °C, and 30 s at 72 °C; a final extension of 5 min at 72 °C; and a hold at 4 °C. Prepped libraries were then quantified using Qubit and TapeStation, before pooling. HTS was performed with an Illumina NextSeq500 sequencer using a 150 cycle, mid output kit (2 × 75 paired-end). Both 16S and ITS reads were analyzed using QIIME[60] version 1.9.1. OTUs were picked using UCLUST[61] version 1.2.22q. The Greengenes[62] database version 13.8 was used to classify 16S reads, and the UNITE[63] database version 7 was used to classify ITS reads.

Data Availability. Raw reads from whole genome metagenomic sequencing and from amplicon metagenomic sequencing have been deposited under the accession number PRJNA471522.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00271.

Discussion S1: Literature analysis of fungus-methanogen syntrophy results purporting to demonstrate increased substrate deconstruction by fungal-methanogen co-cultures; Table S1: Primers used and 16S and ITS sequences of enriched organisms; Table S2: Metagenomic assembly statistics; Table S3: Metabolites with associated measurement error from synthetic co-culture experiments; Table S4: ANCOVA analysis of data form publications asserting that methanogens increase deconstruction rate of cellulosic substrate by cocultivated anaerobic fungus; Figure S1: Methane production during enrichment cultivation; Figure S2: Phylogeny of enrichment consortium members; Figure S3: Morphological features of *Piromyces sp.* H1B2; Figure S4: Metagenome assembled genome completion by CheckM; Figure S5: Comparison of total fermentation gas production by different consortia isolated from the same enrichment; Figure S6: Supernatant metabolites measurements from co-culture experiments on both cellulose and lignocellulose; Figure S7: Comparisons of gas production rates between mono- and co-cultures of *Neocallimastix californiae* and *Anaeromyces robustus* (PDF)

Supplemental Database S1: CAZyme analysis of metagenomes; Supplemental Database S2: Transporter analysis of metagenomes (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: momalley@engineering.ucsb.edu.

ORCID

David L. Valentine: 0000-0001-5914-9107

Michelle A. O'Malley: 0000-0002-6065-8491

Author Contributions

#SPG and TSL contributed equally. SPG, JAS, JKH, and MKT carried out enrichment and fermentative experiments with the native consortium. SPG and JKH performed the sequencing, and SPG and TSL conducted the bioinformatics analyses. TSL, SW, and JLB performed experiments with co-cultures. SPG, JAS, MKT, DLV, TSL, JLB, SW, and MAO planned the experiments. TSL, SPG, JLB, SW, and MAO wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the use of the Biological Nanostructures Laboratory within the California NanoSystems Institute, supported by the University of California, Santa Barbara and the University of California, Office of the President. Computational resources were provided by the Center for Scientific Computing at UCSB and NSF Grant CNS-0960316. Portions of this manuscript have been previously included in the PhD Thesis of SPG, and are reproduced here with permission.[64] The authors are grateful to funding sources from the Office of Science (BER), U.S. Department of Energy (DE-SC0010352), the National Science Foundation (MCB-1553721), the Institute for Collaborative Biotechnologies through grant W911NF-09-0001 from the U.S. Army Research Office, and the Camille Dreyfus Teacher-Scholar Awards Program. All authors acknowledge support from the California NanoSystems Institute (CNSI) Challenge Grant Program, supported by the University of California, Santa Barbara and the University of California, Office of the President. SPG also acknowledges support from the National Science Foundation Graduate Research Fellowship Program under Grant DGE 1144085. This work was part of the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy.

■ ABBREVIATIONS

ITS, internal transcribed spacer; CAZyme, carbohydrate active enzyme.

■ REFERENCES

(1) Mee, M. T., Collins, J. J., Church, G. M., and Wang, H. H. (2014) Syntrophic exchange in synthetic microbial communities. *Proc. Natl. Acad. Sci. U. S. A. 111* (20), E2149−E2156.

(2) Wilken, S. E., Saxena, M., Petzold, L. R., and O'Malley, M. A. (2018) In Silico Identification of Microbial Partners to Form Consortia with Anaerobic Fungi. *Processes 6* (1), 7.

(3) Peng, X. N., Gilmore, S. P., and O'Malley, M. A. (2016) Microbial communities for bioprocessing: lessons learned from nature. *Curr. Opin. Chem. Eng. 14*, 103−109.

(4) Embree, M., Liu, J. K., Al-Bassam, M. M., and Zengler, K. (2015) Networks of energetic and metabolic interactions define dynamics in microbial communities. *Proc. Natl. Acad. Sci. U. S. A. 112* (50), 15450−5.

(5) Morris, B. E., Henneberger, R., Huber, H., and Moissl-Eichinger, C. (2013) Microbial syntrophy: interaction for the common good. *FEMS microbiology reviews 37* (3), 384−406.

(6) Bauchop, T., and Mountfort, D. O. (1981) Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl. Environ. Microbiol. 42* (6), 1103−1110.

(7) Hungate, R. E. (1966) *The Rumen and Its Microbes*, Academic Press.

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

(8) Li, Y., Jin, W., Mu, C., Cheng, Y., and Zhu, W. (2017) Indigenously associated methanogens intensified the metabolism in hydrogenosomes of anaerobic fungi with xylose as substrate. *J. Basic Microbiol. 57* (11), 933−940.
(9) Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C., and Prins, R. A. (1990) Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Appl. Environ. Microbiol. 56* (12), 3793−3797.
(10) Mountfort, D. O., Asher, R. A., and Bauchop, T. (1982) Fermentation of Cellulose to Methane and Carbon Dioxide by a Rumen Anaerobic Fungus in a Triculture with Methanobrevibacter sp. Strain RA1 and Methanosarcina barkeri. *Appl. Environ. Microbiol. 44* (1), 128−134.
(11) Teunissen, M. J., Kets, E. P. W., Op den Camp, H. J. M., Huis in't Veld, J. H. J., and Vogels, G. D. (1992) Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. *Arch. Microbiol. 157* (2), 176−182.
(12) Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N., and Kelly, W. J. (2018) Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechnol. 36* (4), 359−367.
(13) Solomon, K. V., Haitjema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., Brewer, H. M., Purvine, S. O., Wright, A. T., Theodorou, M. K., Grigoriev, I. V., Regev, A., Thompson, D. A., and O'Malley, M. A. (2016) Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes. *Science (Washington, DC, U. S.) 351* (6278), 1192−5.
(14) Haitjema, C. H., Gilmore, S. P., Henske, J. K., Solomon, K. V., de Groot, R., Kuo, A., Mondo, S. J., Salamov, A. A., LaButti, K., Zhao, Z., Chiniquy, J., Barry, K., Brewer, H. M., Purvine, S. O., Wright, A. T., Hainaut, M., Boxma, B., van Alen, T., Hackstein, J. H. P., Henrissat, B., Baker, S. E., Grigoriev, I. V., and O'Malley, M. A. (2017) A parts list for fungal cellulosomes revealed by comparative genomics. *Nat. Microbiol. 2*, 17087.
(15) Joblin, K. N., Matsui, H., Naylor, G. E., and Ushida, K. (2002) Degradation of fresh ryegrass by methanogenic co-cultures of ruminal fungi grown in the presence or absence of Fibrobacter succinogenes. *Curr. Microbiol. 45* (1), 46−53.
(16) Joblin, K. N., Naylor, G. E., and Williams, A. G. (1990) Effect of Methanobrevibacter smithii on Xylanolytic Activity of Anaerobic Ruminal Fungi. *Appl. Environ. Microbiol. 56* (8), 2287−2295.
(17) Swift, C. L., Brown, J. L., Seppala, S., and O'Malley, M. A. (2019) Co-cultivation of the anaerobic fungus Anaeromyces robustus with Methanobacterium bryantii enhances transcription of carbohydrate active enzymes. *J. Ind. Microbiol. Biotechnol.*, DOI: 10.1007/s10295-019-02188-0.
(18) Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., Chang, J., Kittelmann, S., Fliegerova, K., Puniya, A. K., Henske, J. K., Gilmore, S. P., O'Malley, M. A., Griffith, G. W., and Smidt, H. (2017) PCR and Omics Based Techniques to Study the Diversity, Ecology and Biology of Anaerobic Fungi: Insights, Challenges and Opportunities. *Front. Microbiol. 8*, 1657.
(19) Ericsson, A. C., Johnson, P. J., Lopes, M. A., Perry, S. C., and Lanter, H. R. (2016) A Microbiological Map of the Healthy Equine Gastrointestinal Tract. *PLoS One 11* (11), No. e0166523.
(20) Seedorf, H., Kittelmann, S., and Janssen, P. H. (2015) Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. *Appl. Environ. Microbiol. 81* (3), 986−95.
(21) Youssef, N. H., Couger, M. B., Struchtemeyer, C. G., Liggenstoffer, A. S., Prade, R. A., Najar, F. Z., Atiyeh, H. K., Wilkins, M. R., and Elshahed, M. S. (2013) The genome of the anaerobic fungus Orpinomyces sp. strain C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. *Appl. Environ. Microbiol. 79* (15), 4620−34.
(22) Bowers, R. M., Kyrpides, N. C., Stepanauskas, R., Harmon-Smith, M., Doud, D., Reddy, T. B. K., Schulz, F., Jarett, J., Rivers, A. R., Eloe-Fadrosh, E. A., Tringe, S. G., Ivanova, N. N., Copeland, A., Clum, A., Becraft, E. D., Malmstrom, R. R., Birren, B., Podar, M., Bork, P., Weinstock, G. M., Garrity, G. M., Dodsworth, J. A., Yooseph, S., Sutton, G., Glockner, F. O., Gilbert, J. A., Nelson, W. C., Hallam, S. J., Jungbluth, S. P., Ettema, T. J. G., Tighe, S., Konstantinidis, K. T., Liu, W. T., Baker, B. J., Rattei, T., Eisen, J. A., Hedlund, B., McMahon, K. D., Fierer, N., Knight, R., Finn, R., Cochrane, G., Karsch-Mizrachi, I., Tyson, G. W., Rinke, C., Lapidus, A., Meyer, F., Yilmaz, P., Parks, D. H., Eren, A. M., Schriml, L., Banfield, J. F., Hugenholtz, P., and Woyke, T. (2017) Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. *Nat. Biotechnol. 35* (8), 725−731.
(23) Henske, J. K., Gilmore, S. P., Knop, D., Cunningham, F. J., Sexton, J. A., Smallwood, C. R., Shutthanandan, V., Evans, J. E., Theodorou, M. K., and O'Malley, M. A. (2017) Transcriptomic characterization of Caecomyces churrovis: a novel, non-rhizoid-forming lignocellulolytic anaerobic fungus. *Biotechnol. Biofuels 10*, 305.
(24) Seppala, S., Solomon, K. V., Gilmore, S. P., Henske, J. K., and O'Malley, M. A. (2016) Mapping the membrane proteome of anaerobic gut fungi identifies a wealth of carbohydrate binding proteins and transporters. *Microb. Cell Fact. 15* (1), 212.
(25) Henske, J. K., Wilken, S. E., Solomon, K. V., Smallwood, C. R., Shutthanandan, V., Evans, J. E., Theodorou, M. K., and O'Malley, M. A. (2018) Metabolic characterization of anaerobic fungi provides a path forward for bioprocessing of crude lignocellulose. *Biotechnol. Bioeng. 115* (4), 874−884.
(26) Jimenez, D. J., Dini-Andreote, F., and van Elsas, J. D. (2014) Metataxonomic profiling and prediction of functional behaviour of wheat straw degrading microbial consortia. *Biotechnol. Biofuels 7*, 92.
(27) Svartstrom, O., Alneberg, J., Terrapon, N., Lombard, V., de Bruijn, I., Malmsten, J., Dalin, A. M., El Muller, E., Shah, P., Wilmes, P., Henrissat, B., Aspeborg, H., and Andersson, A. F. (2017) Ninety-nine de novo assembled genomes from the moose (Alces alces) rumen microbiome provide new insights into microbial plant biomass degradation. *ISME J. 11* (11), 2538−2551.
(28) Gilmore, S. P., Henske, J. K., Sexton, J. A., Solomon, K. V., Seppala, S., Yoo, J. I., Huyett, L. M., Pressman, A., Cogan, J. Z., Kivenson, V., Peng, X., Tan, Y., Valentine, D. L., and O'Malley, M. A. (2017) Genomic analysis of methanogenic archaea reveals a shift towards energy conservation. *BMC Genomics 18* (1), 639.
(29) Fricke, W. F., Seedorf, H., Henne, A., Kruer, M., Liesegang, H., Hedderich, R., Gottschalk, G., and Thauer, R. K. (2006) The genome sequence of Methanosphaera stadtmanae reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. *Journal of bacteriology 188* (2), 642−58.
(30) Dongowski, G., Lorenz, A., and Anger, H. (2000) Degradation of pectins with different degrees of esterification by Bacteroides thetaiotaomicron isolated from human gut flora. *Applied and environmental microbiology 66* (4), 1321−7.
(31) Ndeh, D., Rogowski, A., Cartmell, A., Luis, A. S., Basle, A., Gray, J., Venditto, I., Briggs, J., Zhang, X., Labourel, A., Terrapon, N., Buffetto, F., Nepogodiev, S., Xiao, Y., Field, R. A., Zhu, Y., O'Neil, M. A., Urbanowicz, B. R., York, W. S., Davies, G. J., Abbott, D. W., Ralet, M. C., Martens, E. C., Henrissat, B., and Gilbert, H. J. (2017) Complex pectin metabolism by gut bacteria reveals novel catalytic functions. *Nature 544* (7648), 65−70.
(32) Solomon, K. V., Henske, J. K., Theodorou, M. K., and O'Malley, M. A. (2016) Robust and effective methodologies for cryopreservation and DNA extraction from anaerobic gut fungi. *Anaerobe 38*, 39−46.
(33) Brejning, J., Arneborg, N., and Jespersen, L. (2005) Identification of genes and proteins induced during the lag and early exponential phase of lager brewing yeasts. *J. Appl. Microbiol. 98* (2), 261−71.

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185

(34) Brejning, J., Jespersen, L., and Arneborg, N. (2003) Genome-wide transcriptional changes during the lag phase of Saccharomyces cerevisiae. *Arch. Microbiol. 179* (4), 278−94.
(35) Rolfe, M. D., Rice, C. J., Lucchini, S., Pin, C., Thompson, A., Cameron, A. D., Alston, M., Stringer, M. F., Betts, R. P., Baranyi, J., Peck, M. W., and Hinton, J. C. (2012) Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. *Journal of bacteriology 194* (3), 686−701.
(36) Ali, S. R., Anwar, Z., Irshad, M., Mukhtar, S., and Warraich, N. T. (2016) Bio-synthesis of citric acid from single and co-culture-based fermentation technology using agro-wastes. *J. Radiat. Res. Appl. Sci. 9* (1), 57−62.
(37) Haitjema, C. H., Solomon, K. V., Henske, J. K., Theodorou, M. K., and O'Malley, M. A. (2014) Anaerobic gut fungi: Advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. *Biotechnol. Bioeng. 111* (8), 1471−82.
(38) Godsy, E. M. (1980) Isolation of Methanobacterium bryantii from a Deep Aquifer by Using a Novel Broth-Antibiotic Disk Method. *Appl. Environ. Microbiol. 39* (5), 1074−1075.
(39) Belanche, A., de la Fuente, G., and Newbold, C. J. (2014) Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiol. Ecol. 90* (3), 663−77.
(40) Shou, W., Ram, S., and Vilar, J. M. (2007) Synthetic cooperation in engineered yeast populations. *Proc. Natl. Acad. Sci. U. S. A. 104* (6), 1877−82.
(41) Zhou, K., Qiao, K., Edgar, S., and Stephanopoulos, G. (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nat. Biotechnol. 33* (4), 377−83.
(42) Marchand, N., and Collins, C. H. (2016) Synthetic Quorum Sensing and Cell-Cell Communication in Gram-Positive Bacillus megaterium. *ACS Synth. Biol. 5* (7), 597−606.
(43) Fredrickson, A. G., and Stephanopoulos, G. (1981) Microbial competition. *Science (Washington, DC, U. S.) 213* (4511), 972−9.
(44) De Vrieze, J., Gildemyn, S., Vilchez-Vargas, R., Jauregui, R., Pieper, D. H., Verstraete, W., and Boon, N. (2015) Inoculum selection is crucial to ensure operational stability in anaerobic digestion. *Appl. Microbiol. Biotechnol. 99* (1), 189−99.
(45) Wright, A. D., and Pimm, C. (2003) Improved strategy for presumptive identification of methanogens using 16S riboprinting. *J. Microbiol. Methods 55* (2), 337−49.
(46) Tuckwell, D. S., Nicholson, M. J., McSweeney, C. S., Theodorou, M. K., and Brookman, J. L. (2005) The rapid assignment of ruminal fungi to presumptive genera using ITS1 and ITS2 RNA secondary structures to produce group-specific fingerprints. *Microbiology (London, U. K.) 151* (5), 1557−1567.
(47) Theodorou, M. K., Williams, B. A., Dhanoa, M. S., McAllan, A. B., and France, J. (1994) A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Technol. 48* (3), 185−197.
(48) Wood, I. P., Elliston, A., Ryden, P., Bancroft, I., Roberts, I. N., and Waldron, K. W. (2012) Rapid quantification of reducing sugars in biomass hydrolysates: Improving the speed and precision of the dinitrosalicylic acid assay. *Biomass Bioenergy 44*, 117−121.
(49) Anderson, I. J., Sieprawska-Lupa, M., Goltsman, E., Lapidus, A., Copeland, A., Glavina Del Rio, T., Tice, H., Dalin, E., Barry, K., Pitluck, S., Hauser, L., Land, M., Lucas, S., Richardson, P., Whitman, W. B., and Kyrpides, N. C. (2009) Complete genome sequence of Methanocorpusculum labreanum type strain Z. *Stand. Genomic Sci. 1* (2), 197−203.
(50) Li, D., Luo, R., Liu, C. M., Leung, C. M., Ting, H. F., Sadakane, K., Yamashita, H., and Lam, T. W. (2016) MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods (Amsterdam, Neth.) 102*, 3−11.
(51) Kang, D. D., Froula, J., Egan, R., and Wang, Z. (2015) MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ 3*, No. e1165.
(52) Alneberg, J., Bjarnason, B. S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U. Z., Lahti, L., Loman, N. J., Andersson, A. F., and Quince, C. (2014) Binning metagenomic contigs by coverage and composition. *Nat. Methods 11* (11), 1144−6.
(53) Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., Olson, R., Overbeek, R., Parrello, B., Pusch, G. D., Shukla, M., Thomason, J. A., 3rd, Stevens, R., Vonstein, V., Wattam, A. R., and Xia, F. (2015) RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep. 5*, 8365.
(54) Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., and Tanabe, M. (2012) KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res. 40*, D109.
(55) Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res. 25* (7), 1043−55.
(56) Langmead, B., and Salzberg, S. L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods 9* (4), 357−9.
(57) Saier, M. H., Jr., Reddy, V. S., Tsu, B. V., Ahmed, M. S., Li, C., and Moreno-Hagelsieb, G. (2016) The Transporter Classification Database (TCDB): recent advances. *Nucleic Acids Res. 44* (D1), D372−9.
(58) Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P. K., Xu, Y., and Yin, Y. (2018) dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res. 46* (W1), W95−w101.
(59) Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., and Tiedje, J. M. (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res. 42*, D633.
(60) Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods 7* (5), 335−6.
(61) Edgar, R. C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics 26* (19), 2460−1.
(62) DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G. L. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology 72* (7), 5069−72.
(63) Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Duenas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., Larsson, E., Lindahl, B. D., Lucking, R., Martin, M. P., Matheny, P. B., Nguyen, N. H., Niskanen, T., Oja, J., Peay, K. G., Peintner, U., Peterson, M., Poldmaa, K., Saag, L., Saar, I., Schussler, A., Scott, J. A., Senes, C., Smith, M. E., Suija, A., Taylor, D. L., Telleria, M. T., Weiss, M., and Larsson, K. H. (2013) Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol. 22* (21), 5271−7.
(64) Gilmore, S. P. (2018) *Engineering Synthetic Systems Inspired by Anaerobic Fungi*. University of California, Santa Barbara, ProQuest Dissertations & Theses A&I.

DOI: 10.1021/acssynbio.9b00271
ACS Synth. Biol. 2019, 8, 2174−2185