Elucidating Syntrophic Butyrate-Degrading Populations in Anaerobic Digesters Using Stable-Isotope-Informed Genome-Resolved Metagenomics

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ABSTRACT  Linking the genomic content of uncultivated microbes to their metabolic functions remains a critical challenge in microbial ecology. Resolving this challenge has implications for improving our management of key microbial interactions in biotechnologies such as anaerobic digestion, which relies on slow-growing syntrophic and methanogenic communities to produce renewable methane from organic waste. In this study, we combined DNA stable-isotope probing (SIP) with genome-centric metagenomics to recover the genomes of populations enriched in $^{13}$C after growing on $^{[13C]}$butyrate. Differential abundance analysis of recovered genomic bins across the SIP metagenomes identified two metagenome-assembled genomes (MAGs) that were significantly enriched in heavy $^{[13C]}$DNA. Phylogenomic analysis assigned one MAG to the genus Syntrophomonas and the other MAG to the genus Methanothrix. Metabolic reconstruction of the annotated genomes showed that the Syntrophomonas genome encoded all the enzymes for beta-oxidizing butyrate, as well as several mechanisms for interspecies electron transfer via electron transfer flavoproteins, hydrogenases, and formate dehydrogenases. The Syntrophomonas genome shared low average nucleotide identity ($<95\%$) with any cultured representative species, indicating that it is a novel species that plays a significant role in syntrophic butyrate degradation within anaerobic digesters. The Methanothrix genome contained the complete pathway for acetoclastic methanogenesis, indicating that it was enriched in $^{13}$C from syntrophic acetate transfer. This study demonstrates the potential of stable-isotope-informed genome-resolved metagenomics to identify in situ interspecies metabolic cooperation within syntrophic consortia important to anaerobic waste treatment as well as global carbon cycling.

IMPORTANCE  Predicting the metabolic potential and ecophysiology of mixed microbial communities remains a major challenge, especially for slow-growing anaerobes that are difficult to isolate. Unraveling the in situ metabolic activities of uncultured species may enable a more descriptive framework to model substrate transformations by microbiomes, which has broad implications for advancing the fields of biotechnology, global biogeochemistry, and human health. Here, we investigated the in situ function of mixed microbiomes by combining stable-isotope probing with metagenomics to identify the genomes of active syntrophic populations converting butyrate, a C$\alpha$ fatty acid, into methane within anaerobic digesters. This approach thus moves beyond the mere presence of metabolic genes to resolve “who is doing what” by obtaining confirmatory assimilation of the labeled substrate into the DNA signature. Our findings provide a framework to further link the genomic identities of uncultured microbes with their ecological function within microbiomes driving many important biotechnological and global processes.

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Who is doing what within wastewater bioreactors? Ziels and colleagues use stable-isotope-informed metagenomics to identify interspecies cooperation in syntrophic butyrate-degrading populations within anaerobic digesters. @RyanZiels @SousaDZ

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Linking microbial genomic identity with ecological function is considered a “Holy Grail” in microbial ecology (1) and has broad implications for improving our ability to manage microbial communities in engineered biotechnologies. Anaerobic digestion is an example of a biotechnology that enables resource recovery from organic waste by generating methane gas as a renewable biofuel and thus plays a role in establishing a circular economy (2). The production of methane in anaerobic digestion is executed through a series of trophic interactions constituting a metabolic network of hydrolyzing and fermenting bacteria, syntrophic acetogens, and methanogenic archaea (3, 4). Metabolic reconstructions based on shotgun metagenomic sequencing data have highlighted potential partitioning of functional guilds within anaerobic digester micro-biomes (4). Yet, our understanding of the ecophysiology of the microorganisms present in anaerobic digesters is limited by the high community complexity and lack of cultured representatives (4). Elucidating the nature of interspecies interactions between different trophic groups in the anaerobic digester metabolic network may help to better understand and optimize the conversion of organic wastes into renewable methane.

The terminal steps in the anaerobic metabolic network, syntrophy and methanogenesis, are responsible for a considerable portion of carbon flux in methanogenic bioreactors, as fatty acids are often produced during fermentation of mixed organic substrates (5). The accumulation of fatty acids in anaerobic digesters is often responsible for a reduction in pH and process instability (3). In particular, syntrophic degradation of the 4-carbon fatty acid butyrate can be a bottleneck for anaerobic carbon conversion, as this metabolism occurs at the thermodynamic extreme. Butyrate degradation to acetate and hydrogen is thermodynamically unfavorable under standard conditions ($\Delta G^\circ = 53$ kJ/mol) and yields only $-21$ kJ/mol under environmental conditions typical of anaerobic bioreactors (pH 7, 1 mM butyrate and acetate, 1 Pa H₂) (see equation S1 in Table S2 in the supplemental material). Thus, cooperation between syntrophic bacteria and acetate- and hydrogen-scavenging methanogenic partners is necessary to maintain thermodynamic favorability. Cultured representative species carrying out syntrophic fatty acid oxidation are potentially underrepresented due to their slow growth and difficulty of isolation in the lab (6). So far, only two mesophilic (Syntrophomonas and Syntrophus) and two thermophilic (Syntrophothermus and Thermosyntropha) genera (12 bacterial species in total) have been shown to oxidize butyrate in syntrophic cooperation with methanogenic archaea, and they all belong to the families Syntrophomonadaceae and Syntrophaceae (6). Despite their major roles in processing carbon within anaerobic bioreactors, many syntrophic fatty acid-oxidizing bacteria have evaded detection with quantitative hybridization-based techniques (7), which is likely due to their low biomass yields (8) or our incomplete knowledge of active syntrophic populations within anaerobic digesters (9). Broad metagenomic surveys of anaerobic digester communities have similarly observed poor resolution of syntrophic populations, owing to their low abundance (4, 10). Thus, highly sensitive culture-independent approaches are needed to expand our understanding of the ecophysiology of syntrophic populations to better control and predict metabolic fluxes in anaerobic environments.

Recently, we demonstrated the potential of combining DNA-stable-isotope probing (DNA-SIP) with genome-resolved metagenomics to identify syntrophic populations degrading the long-chain fatty acid oleate (C₁₈:₁) within anaerobic digesters (11). Stable-isotope-informed metagenomic sequencing can enrich metagenomic libraries with genomic sequences of actively growing microbes that incorporate $^{13}$C into their biomass from an added labeled substrate (12) and thus allows for a “zoomed-in” genomic view of low-abundance populations, such as syntrophs. We also demonstrated that this approach was amenable for recovering high-quality microbial genomes using a differential coverage-based binning approach, as genomes from active microbes have
low abundance in heavy DNA from ¹²C controls but are enriched in heavy DNA from ¹³C-amended treatments (11). Here, we applied stable-isotope-informed metagenomics to resolve the genomic makeup of active syntrophic butyrate-degrading populations within anaerobic digesters treating manure and sodium oleate (13). These same anaerobic digesters were previously used for DNA-SIP with oleate (11) at a similar time point, thus allowing for genomic comparisons using a multisubstrate SIP data set. This approach identified potential metabolic flexibility in a syntrophic bacterium implicated in the degradation of multiple fatty acids within the study anaerobic digesters, and elucidated an *in situ* syntrophic partnership between the acetogenic bacterium and an acetoclastic methanogen via interspecies metabolite transfer during butyrate degradation.

**RESULTS AND DISCUSSION**

**DNA-SIP of methanogenic microcosms with [¹³C]butyrate.** Two laboratory-scale anaerobic digesters fed dairy manure were either pulse fed every 48 h or fed semicontinuously with sodium oleate (C₁₈:₁) for over 230 days (13). Quantitative PCR and 16S rRNA gene amplicon sequencing indicated that *Syntrophomonas* became enriched within the reactors from oleate feeding (13). DNA-SIP-informed metagenomics confirmed that a majority of oleate-degrading bacteria in the two digesters belonged to *Syntrophomonas* (11). Here, we investigated whether any of the populations implicated in oleate degradation were also involved in the degradation of the short-chain fatty acid butyrate (C₄). Digestate from the pulse-fed and continuously fed anaerobic digester were incubated in duplicate microcosms, which were spiked with either [¹²C]- or [¹³C]butyrate (40 mM) for approximately 50 h. The added butyrate was converted into methane at a >80% conversion efficiency based on chemical oxygen demand (COD) recovery (see Fig. S1 in the supplemental material). After the 50-h incubation, the contents of the microcosms were sacrificed for DNA extraction, density gradient centrifugation, and fractionation.

The abundance of 16S rRNA genes of the known butyrate-degrading genus *Syntrophomonas* was quantified across density gradient fractions using quantitative PCR (qPCR) to identify DNA fractions that were enriched in ¹³C. Density fractions with a buoyant density from 1.70 to 1.705 had 2.0 to 2.2 times more *Syntrophomonas* 16S rRNA genes (normalized to the maximum concentration) than the ¹²C controls (Fig. S2). Those DNA fractions were selected from each SIP microcosm for metagenomic sequencing, as well as for 16S rRNA gene amplicon sequencing.

The microbial communities in the heavy density gradient fractions were assessed through paired-end 16S rRNA gene amplicon sequencing for all ¹²C- and ¹³C-incubated duplicate microcosms (Fig. 1). Differential abundance analysis of operational taxonomic unit (OTU) read counts with DESeq2 (14) showed that approximately 50% (7 of 15) of the significantly enriched (P < 0.05) OTUs in heavy [¹³C]DNA samples relative to heavy [¹²C]DNA were taxonomically classified as *Syntrophomonas* for the pulse-fed digester (Fig. S3). For the continuously fed digester, approximately 40% of the ¹³C-enriched OTUs (7 of 17) were assigned to *Syntrophomonas* (Fig. S3). Additionally, two ¹³C-enriched OTUs in both digesters were assigned to *Methanothrix* (formerly *Methanoseta*), which likely scavenges the [¹³C]acetate generated by *Syntrophomonas* during [¹³C]butyrate degradation. While one previous study observed that *Syntrophaceae* was enriched predominantly in anaerobic digester granular sludge incubated with [¹³C]butyrate (9), various other studies also detected *Syntrophomonadaceae* populations (i) as active syntrophic butyrate degraders in anaerobic digester sludge using [¹⁴C]butyrate and microautoradiography–fluorescent in situ hybridization (MAR-FISH) (15), (ii) in anaerobic digest sludge by means of SIP using [¹³C]oleate (11), and (iii) in rice paddy soil with SIP using [¹³C]butyrate (16). In the last two studies, acetate-scavenging partners (*Methanothrix* and *Methanosarcinaceae*) were also enriched. Indeed, syntrophic interaction with acetoclastic methanogens is beneficial, as acetate accumulation can thermodynamically hinder butyrate oxidation (e.g., the ΔG exceeds the theoretical threshold for catabolism (~10 kJ/mol) when acetate accumulates be-
Beyond 10 mM (pH 7, 1 mM butyrate, and 1 Pa H₂) (assumptions appear in Table S2). Notably, H₂- and formate-consuming methanogens necessary for syntrophy were not detected during degradation of [13C]butyrate, likely because these archaea utilize CO₂ as a carbon source and no [13C]CO₂ is produced during butyrate oxidation. Our results also found 13C-enriched OTUs from lineages not known to degrade butyrate under methanogenic conditions: Treponema, Luteimonas, Thauera, Christensenellaceae (Firmicutes), and Anaerolineaceae (Chloroflexi) (Fig. S3). Other studies using [13C]butyrate also detected enrichment of populations likely unable to degrade butyrate, including Tepidanaerobacter and Clostridium, in a thermophilic anaerobic digester operated at 55°C (9) and Chloroflexi and Planctomycetes in rice paddy soil (16). Members of Tepidanaerobacter and Clostridium are known to syntrophically oxidize acetate under thermophilic conditions (17) and may have thus been enriched in [13C]RNA from [13C]acetate produced during the beta-oxidation of labeled butyrate in the study by Hatamoto et al. (9). Similarly, the Chloroflexi and Planctomycetes populations were hypothesized to have become enriched due to cross-feeding of intermediate metabolites, like acetate, in rice paddy soil (16). Thus, the “peripheral” populations detected in our study may grow on cellular-decay products, as genome-resolved metagenomics recently indicated that some uncultured Anaerolineaceae species are likely fermenters in anaerobic digesters (18). These results thus suggest that carbon cross-feeding may occur between multiple microbial trophic groups during the syntrophic degradation of butyrate in anaerobic digesters.

**Identifying active metagenome-assembled genomes (MAGs) in SIP metagenomes.** Metagenomic sequencing of heavy DNA from duplicate [13C]- and [12C]butyrate-amended microcosms yielded an average of 30 million paired reads per sample for both digesters (n = 8) (Table S1). The filtered reads from heavy [13C]DNA were coassembled, yielding a total assembly length of 516 Mb of contigs larger than 1 kb, with an average (N₅₀) contig length of 5 kb. The fraction of filtered short reads that mapped to the coassembly were 66% ± 3% (standard deviation) and 69% ± 1% for the [12C]- and [13C]-labeled metagenomes, respectively (n = 4 each) (Table S1). The coassembly generated from [13C] reads thus captured much of the genomic information present in the heavy-DNA fractions.

The assembled metagenomic contigs were organized into 160 genomic bins at various levels of completion and redundancy (Data Set S1). Differential abundance
analysis of the mapped read counts for the bins across the $^{13}$C- and $^{12}$C-labeled metagenomes with DESeq2 (14) identified two genomic bins that were significantly ($P < 0.05$) enriched in $^{13}$C-DNA (Table 1). These genomic bins were enriched in $^{13}$C-DNA in both the pulse-fed and continuously fed bioreactors. Based on suggested completion and redundancy metrics for MAGs (19), one genomic bin is classified as a high-quality MAG (completion, >90%; redundancy, <10%), while the other is a medium-quality MAG (completion, >50%; redundancy, <10%). Taxonomic classification with CheckM (20) assigned one of the MAGs to the genus Syntrophomonas and the other to Methanothrix (Table 1).

The phylogenomic placement of the $^{13}$C-enriched Syntrophomonas BUT1 MAG was consistent with its taxonomic assignment, as it was located in the Syntrophomonas genome cluster within the family Syntrophomonadaceae (Fig. 2). The closest relative to Syntrophomonas BUT1 based on single-copy marker genes was Syntrophomonas PF07, which is a genomic bin enriched in $^{13}$C from DNA-SIP with labeled oleate ($^{13}$C$_{18}$:1) using sludge from the same pulse-fed digester used in this study (11). A high average nucleotide identity (ANI) of 99% was observed between the Syntrophomonas BUT1 and Syntrophomonas PF07 genomes (Fig. S4), suggesting that these two organisms likely originated from the same sequence-discrete population (21). The next-closest relative of Syntrophomonas BUT1 based on the phylogenomic analysis was Syntrophomonas zehnderi OL-4 (Fig. 2), which was isolated from an oleate-fed anaerobic granular sludge bioreactor (22). However, the ANI between Syntrophomonas BUT1 and Syntrophomonas zehnderi OL-4 was below 95% (Fig. S4), suggesting that these two organisms are different species (23). Thus, the active butyrate-degrading bacterial MAG identified in this study is distinct from any species obtained by isolation at this time. The detection of the sequence-discrete population of Syntrophomonas BUT1 within heavy $^{13}$C-DNA from both experiments with universally labeled butyrate and oleate indicates that this syntrophic population may be metabolically flexible; that is, it may grow on fatty acids of various lengths and degrees of saturation. An alternative explanation may be that Syntrophomonas BUT1 was detected in the SIP experiment with universally labeled $^{13}$C-oleate due to its degradation of shorter fatty acids, such as butyrate, excreted during oleate degradation by other community members. These findings have implications for current frameworks for mathematical modeling of anaerobic digesters, which typically assume that long-chain fatty acid (LCFA)-degrading and butyrate-degrading populations are distinct and do not cross-feed (24). Thus, the incorporation of genomic and functional characterization, as obtained through DNA-SIP genome-resolved metagenomics, may help to improve our ability to accurately model anaerobic digestion processes by accounting for metabolic flexibility or cross-feeding within key functional guilds.

A phylogenomic analysis of the $^{13}$C-enriched Methanothrix BUT2 MAG based on archaeal single-copy marker genes placed the MAG within the genus Methanothrix, consistent with its taxonomic assignment with CheckM (Fig. 3). Methanothrix BUT2 was closely clustered with the genome of Methanothrix soehngenii GP6, along with four MAGs reported in the study of Parks et al. (25). Congruently with the phylogenomic analysis, Methanothrix BUT2 shared an ANI of over 98% with Methanothrix soehngenii GP6 and the same with four MAGs from the work of Parks et al. (25) (Methanothrix UBA243, Methanothrix UBA458, Methanothrix UBA70, Methanothrix UBA356), indicating that these genomes likely form a sequence-discrete population (Fig. S4). A second,

### TABLE 1 Genomic feature summary of the two metagenome-assembled genomes that were significantly enriched in $^{13}$C-DNA after the degradation of $^{13}$C-butyrate

| Name               | Bin ID | Taxonomy          | Size (Mb) | GC (%) | Completion (%) | Redundancy (%) |
|--------------------|--------|------------------|-----------|--------|----------------|----------------|
| Syntrophomonas BUT1| Bin 26_1 | Syntrophomonas    | 2.87      | 51.2   | 96.4           | 1.4            |
| Methanothrix BUT2  | Bin 26_2 | Methanothrix     | 1.44      | 53.6   | 74.7           | 3.1            |

*Based on phylogenetic placement of single marker genes with CheckM (20).

*Measured with anvi’o (71).
closely related population, including three MAGs from the work of Parks et al. (25) (Methanothrix UBA372, Methanothrix UBA332, Methanothrix UBA533) shared an ANI of 96% with the Methanothrix BUT2 population (Fig. S4).

DNA-SIP using [13C]oleate with the same anaerobic digester biomass as in this study did not identify any 13C-enriched methanogenic archaea in the genome-resolved metagenomic analysis (11). One possible explanation for the higher relative enrichment of methanogens on [13C]butyrate than on [13C]oleate may be the higher fraction of overall free energy partitioned toward methanogens during anaerobic butyrate degradation than during oleate degradation. For the overall conversion of 1 mol of butyrate to CO₂ and CH₄ under environmental conditions in anaerobic digesters, the thermodynamic yields would be −21.1, −9.4, and −58.9 kJ for the acetogenic bacteria, hydrogenotrophic methanogens, and acetoclastic methanogens, respectively (Table S2). For a similar conversion of 1 mol of oleate, the thermodynamic yields would be −219.9, −70.6, and −264.9 kJ, respectively (Table S2). Thus, the acetogen would gain a lower percentage of the overall free energy yield from the conversion of butyrate (24%) than from that of oleate (40%). As cell yield can depend on free energy (26), the

FIG 2  Phylogenomic tree showing the relationship of 13C-enriched Syntrophomonas BUT1 to other genomes available from the Syntrophomonadaceae family in the NCBI nr database (downloaded April 2018). The tree is based on a concatenated alignment of 139 bacterial single-copy marker genes (77) obtained using anvi’o (74). Open reading frames were predicted with Prodigal v.2.6.3 (70) and queried against sequences in a database of bacterial single-copy marker genes using HMMER v.2.3.2 (81). The tree was calculated using FastTree (82). The Clostridium ultunense genome was used as the outgroup.
lower yield of the butyrate degradation likely leaves a higher fraction of acetate for assimilation by an acetoclastic methanogen. The relative growth yields may also be particularly relevant due to the compositional nature of genome abundance data from the DNA-SIP metagenomes. As the stable-isotope-informed analysis utilized in this study depended on incorporation of the added $^{13}$C into biomass, it was not expected that autotrophic (i.e., hydrogenotrophic) methanogens would be highly enriched in the heavy $[^{13}C]$DNA because no CO$_2$ is produced during butyrate beta-oxidation and microcosms were preflushed with N$_2$-$^{12}$C$^{12}$CO$_2$ (Table S2). Comparing the enriched communities from DNA SIP with different fatty acids, along with bicarbonate, may highlight differences in energy partitioning between syntrophic bacteria and different archaeal partners.

**Metabolic potential of $^{13}$C-enriched MAGs.** Functional annotation and metabolic reconstruction of the $^{13}$C-enriched MAGs revealed their capacity to metabolize the $[^{13}C]$butyrate into methane through syntrophic cooperation.

A complete pathway for butyrate $\beta$-oxidation was annotated for *Syntrophomonas BUT1*, indicating that this MAG was capable of metabolizing the added $[^{13}C]$butyrate (Fig. 4). Notably, several homologues were detected for genes in the $\beta$-oxidation pathway. The *Syntrophomonas BUT1* genome encodes 6 acyl coenzyme A (acyl-CoA) transferases, 7 acyl-CoA dehydrogenases, 8 enoyl-CoA hydratases, 5 3-hydroxybutyryl-CoA dehydrogenases, and 10 acetyl-CoA acetyltransferases (Data Set S2). The presence of homologous $\beta$-oxidizing genes was also observed in the type strain *Syntrophomonas wolfei* subsp. *wolfei* Göttingen DSM 2245B (27). The large number of homologous $\beta$-oxidizing genes may afford *Syntrophomonas BUT1* flexibility to metabolize multiple
fatty acid substrates, as its genomic population was detected in heavy $^{13}$C DNA during SIP with both $^{13}$C butyrate (C4) and $^{13}$C oleate (C18) (11). The different homologous /H9252-oxidizing genes may also have different kinetics and/or affinities, which may allow Syntrophomonas BUT1 to adapt to various substrate concentrations. Fluctuating environments are thought to lead to robustness toward gene loss within metabolic networks through an increase in multifunctional enzymes (28). Thus, the presence of various homologous genes for /H9252-oxidation in Syntrophomonas BUT1 may have been selected for by fluctuating fatty acid concentrations, such as those imposed from pulse-feeding the anaerobic digester (13). It is also possible that the Syntrophomonas BUT1 population was enriched in $^{13}$C from labeled oleate due to cross-feeding of shorter-chain intermediates during /H9252-oxidation of the C18 LCFA, as other syntrophic bacteria were enriched to a high degree during growth on $^{13}$C oleate (11). Yet, the enrichment of Syntrophomonas BUT1 on $^{13}$C butyrate, along with the presence of the complete butyrate /H9252-oxidation pathway, strongly suggests that it is at least capable of $\beta$-oxidizing shorter-chain fatty acids (e.g., C4) produced in anaerobic environments.

**FIG 4** Cell diagram showing proposed metabolic pathways for anaerobic butyrate degradation in syntrophic cooperation between Syntrophomonas BUT1 and Methanothrix BUT2. The H2/formate-utilizing methanogenic partner is shown for conceptual purposes but was not identified with $^{13}$C DNA-SIP in this study due to its autotrophic growth in the microcosms. Dotted lines indicate the direction of electron flow. Details of predicted proteins are given in Data Sets S2 and S3. Enzyme abbreviations are as follows: Fd, ferredoxin; (Syntrophomonas BUT1) Acd, acyl-CoA dehydrogenase; Crt, enoyl-CoA hydratase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; AtoB, acetyl-CoA acetyltransferase; AckA, acetate kinase; Pta, phosphate acetyltransferase; Etf, electron transfer flavoprotein A; EtfB, electron transfer flavoprotein B; EtfD, EtfAB:quinone oxidoreductase; HydABC, bifurcating [Fe-Fe] hydrogenase; HyABC, [NiFe] hydrogenase; FdhA-HylBC, formate dehydrogenase (electron bifurcating); FdnGH, formate dehydrogenase (membrane bound, quinone reducing); FixX, electron transfer flavoprotein dehydrogenase; FixX, FixABC-associated ferredoxin; (Methanothrix BUT2) Acs, acetyl-coenzyme A synthetase; CooS, carbon monoxide dehydrogenase; CdhA, acetyl-CoA decarboxylase/synthase complex; Mtr, methyltetrahydrodihydro methanopterin:CO methyltransferase; McrABG, methyl-coenzyme M reductase; HdrED, coenzyme B-coenzyme M heterodisulfide reductase; FpoABDKLMMNO, F420H2 dehydrogenase.
Syntrophomonas BUT1 lacks genes for aerobic or anaerobic respiration, which is similar to genomes of *S. wolfei* and *Syntrophus aciditrophicus* that are capable of syntrophic butyrate degradation (27, 29). Electrons derived from butyrate oxidation (reduced electron-transferring flavoprotein [ETF] from butyryl-CoA oxidation and NADH from 3-hydroxybutyryl-CoA oxidation) must be disposed of through reduction of CO₂ to formate and H⁺ to H₂ via formate dehydrogenases and hydrogenases, respectively (30–33). In the Syntrophomonas BUT1 genome, we identified genes encoding butyryl-CoA dehydrogenase, ETF alpha and beta units (EtfAB), and two EtfAB:quinone oxidoreductases (Data Set S2), indicating that this organism may transfer electrons from butyryl-CoA oxidation into membrane electron carriers using ETF. The Syntrophomonas BUT1 genome contains five gene clusters encoding formate dehydrogenases and four gene clusters encoding hydrogenases (Data Set S2). These include a membrane-bound cytochrome b-dependent selenocysteine-containing formate dehydrogenase and [NiFe] hydrogenase, which may receive butyrate-derived electrons via menaquinol (30). The quinone binding site of the selenocysteine-containing formate dehydrogenase was on the cytoplasmic side, indicating that it likely utilizes proton motive force to drive unfavorable electron transfer to CO₂-reducing formate generation outside the cell. Energy investment via “reverse electron transport” is critical to drive the uphill electron transfer from the butyryl-CoA/crotonyl-CoA couple to CO₂/formate or H⁺/H₂ couples. In contrast, the quinone binding site of the [NiFe] hydrogenase was on the periplasmic side, indicating that it couples outward vectorial proton transport with H₂ generation.

Previous genomic and proteomic studies also highlight the importance of ETF-based electron transfer, membrane-bound formate dehydrogenases/hydrogenases, and reverse electron transport (5, 27, 33–36).

To complete syntrophic butyrate oxidation, NAD⁺ must also be regenerated through oxidation of NADH. However, NADH oxidation coupled with CO₂/H⁺-reducing formate/H₂ generation is thermodynamically unfavorable. To address this obstacle, anaerobic organisms are known to utilize electron bifurcation (or confurcation), which involves the coupling of endergonic and exergonic redox reactions to circumvent energetic barriers (37). For instance, *Thermotoga maritima* utilizes a trimeric hydrogenase to couple the endergonic production of H₂ from NADH with the exergonic production of H₂ from reduced ferredoxin (38). Two trimeric formate dehydrogenase- and two trimeric [FeFe] hydrogenase-encoding gene clusters in Syntrophomonas BUT1 appear linked to NADH, as they all contained an NADH:acceptor oxidoreductase subunit (Data Set S2). Yet, if the trimeric hydrogenases and formate dehydrogenases in Syntrophomonas BUT1 produce H₂/formate via electron bifurcation with NADH and ferredoxin, it remains unknown how Syntrophomonas BUT1 regenerates reduced ferredoxin, as the known butyrate β-oxidation pathway does not generate reduced ferredoxin (30). Moreover, the Syntrophomonas BUT1 genome does not encode an Rnf complex, which would be necessary to generate reduced ferredoxin from NADH. Recently, the Fix (homologous to ETF) system was shown to perform electron bifurcation to oxidize NADH coupled with the reduction of ferredoxin and ubiquinone during N₂ fixation by Azotobacter vinelandii (39). The Syntrophomonas BUT1 genome encoded a Fix-related ETF-dehydrogenase, FixC, as well as its associated ferredoxin, FixX (Data Set S2). A Fix system has also been detected in *S. wolfei* and was postulated to serve as a means of generating reduced ferredoxin for H₂ or formate production via the bifurcation mechanism (30). Yet, reduced ferredoxin production with the Fix system would be energetically costly, especially with regard to the low energy yields during syntrophic butyrate oxidation (40). Another mechanism was proposed for generating reduced ferredoxin in Rnf-lacking syntrophs that involves a heterodisulfide reductase complex (HdrABC) and an ion-translocating flavin oxidoreductase (Flx or Flox) (41). The *flxABCD-hdrABC* gene cluster was shown to be widespread among anaerobic bacteria, and the protein cluster (FlxABCD-HdrABC) is proposed to function similarly to the heterodisulfide reductase (HdrABC)-[NiFe]-hydrogenase (MvhADG) complex (HdrABC-MvhADG) involved in flavin-based electron bifurcation in hydrogenotrophic methanogenic archaea that couples the exergonic reduction of CoM-CoB heterodisulfide.
(CoM-S-S-CoB) with the endergonic reduction of ferredoxin with H₂ (42). A full floxABCD-hdrABC gene cluster was detected in the genome of *Syntrophomonas* BUT1 (Data Set S2). During the syntrophic growth of *Syntrophomonas* BUT1 on butyrate, the FlxABCD-HdrABC protein cluster may oxidize NADH with reduction of ferredoxin along with the reduction of a high-redox-potential disulfide acceptor (42). In *Desulfovibrio vulgaris*, it has been proposed that the DsrC protein serves as the high-redox thiol-disulfide electron carrier that is reduced by the FlxABCD-HdrABC complex during growth (43). The DsrC protein was also detected in the syntrophic benzoate-degrading *Syntrophorhabdus aromaticivorans* strain UI, along with an flxABCD-hdrABC gene cluster (41), suggesting that the reduction of a thiol-disulfide electron carrier may be a conserved mechanism for generating reduced ferredoxin in syntrophic bacteria. Yet, the *Syntrophomonas* BUT1 genome does not encode a DsrC protein, and thus an alternative and unknown thiol-disulfide electron carrier would be needed. Another possibility is that the trimeric hydrogenase can drive NADH-dependent H₂ generation, as shown in *S. wolfei* Goettingen (40). Nonetheless, this genomic analysis demonstrates that *Syntrophomonas* BUT1 has the potential capacity to overcome energetic barriers during syntrophic butyrate β-oxidation and contains multiple possible mechanisms for H₂ and formate production.

In addition to interspecies electron transfer via molecular hydrogen and formate, a potential mechanism has been proposed for direct interspecies electron transfer (DIET), in which electrons are shared via electrically conductive nanowires (44). DIET activity has been suggested in enrichment communities degrading propionate and butyrate, in which *Syntrophomonas* was detected (45, 46). However, DIET has not been demonstrated with pure cultures of *Syntrophomonas* to date. The direct transfer of electrons is thought to depend on electrically conductive type IV pili and external polyheme cytochromes (47, 48). The *Syntrophomonas* BUT1 genome encodes a type IV pilin assembly protein, PilC, but no genes that encoded the structural protein PilA, which is associated with DIET (48), were found. Moreover, the type IV pilin genes identified in the *Syntrophomonas* BUT1 genome were of the type Flp (fimbrial low molecular protein weight), which are smaller than the Pil-type pilin utilized for DIET in *Geobacter* (49, 50). A multiheme c-type cytochrome was detected in the *Syntrophomonas* BUT1 genome that had 59% amino acid identity (89% coverage) with the multiheme c-type cytochrome OmcS from *G. sulfurreducens*, which has been implicated in DIET (48) (Data Set S2). However, that gene also had higher homology (69% identity, 94% coverage) with the cytochrome c nitrite reductase from *S. wolfei* (GenBank accession no. WP_081424886). Therefore, the roles of DIET in the metabolism of *Syntrophomonas* BUT1 remain unclear but warrant further attention via expression-based profiling.

In addition to encoding potential genetic mechanisms for energy conservation during syntrophic growth, *Syntrophomonas* BUT1 encodes a capsule biosynthesis protein (CapA), which appears to be specific to syntrophic growth (51). The function of CapA in syntrophic growth is unclear but may be related to the production of exopolymeric substances that facilitate interactions with methanogenic partners (51). The *Syntrophomonas* BUT1 genome also contains the ftsW gene, which is related to shape determination and is also a postulated biomarker of a syntrophic lifestyle (51). Based on the presence of these “syntrophic biomarkers” along with genes for β-oxidation and H₂/formate production, the genomic repertoire of *Syntrophomonas* BUT1 aligns with that of a syntrophic butyrate degrader.

The genome of *Syntrophomonas* BUT1 was compared with published genomes of the *Syntrophomonas* genus (*S. wolfei* subsp. *wolfei*, *S. wolfei* subsp. *methylbutyratica*, and *S. zeihnderi*) to investigate whether metabolic genes for beta-oxidation and energy conservation were conserved (Data Set S4). Cutoffs of 42% amino acid similarity and 80% sequence overlap were employed based on the lowest first-quartile amino acid similarity that we observed for top BLAST hits (minimum of 20% amino acid similarity and 80% overlap) of *Syntrophomonas* BUT1 genes to each aforementioned *Syntrophomonas* genome (42.0%, 43.5%, and 43.5%, respectively). Based on these similarity thresholds, only 34% (1,050 out of 3,066) of protein-coding genes in the *Syntrophomo-
nas BUT1 genome have closely related homologs present in all of the other sequenced *Syntrophomonas* genomes. Notably, 40% of the *Syntrophomonas* BUT1 protein-coding genes have no homologs in other *Syntrophomonas* genomes that meet the similarity criteria described above. Reflecting this genomic diversity, *Syntrophomonas* BUT1 encodes several beta oxidation-related genes that have no homologs in the other *Syntrophomonas* genomes that meet the above criteria: one acetyl-CoA acetyltransferase, acetyl-CoA dehydrogenase, acryl-CoA reductase, and acyl-CoA thioesterase (Data Set S4). In addition, the *Syntrophomonas* BUT1 genome harbors putative isobutryl-CoA mutase genes (SYNBUT1_v1_1780025–27) highly similar to those of *Syntrophothermus lipocalidus* (65.0 to 83.4% amino acid similarity), suggesting that *Syntrophomonas* BUT1 may also be capable of syntrophic isobutyrate degradation. Hydrogenases, formate dehydrogenases, and energy conservation genes were generally conserved among *Syntrophomonas* BUT1 and the other *Syntrophomonas* genomes. Only the cytochrome b-dependent [NiFe] hydrogenase has no homologs in the *S. wofei* subsp. *wofei* genome. This implies that *Syntrophomonas* BUT1 may have distinct capabilities for fatty acid oxidation, but the levels of energy conservation necessary to drive syntrophic beta oxidation may not vary between *Syntrophomonas* species.

A genomic analysis of the *Methanothrix* BUT2 genome indicated that it contained the complete pathway for methane production from acetate (Fig. 4; Data Set S3). This observation agrees with the physiology of other *Methanothrix* species, which are known aceticlastic methanogens (52, 53). *Methanothrix* BUT2 also contained genes that likely are involved in energy conservation during aceticlastic methanogenesis. The genome of *Methanothrix* BUT2 harbored acetyl-CoA synthetase for acetate activation, bifunctional CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) to oxidatively split acetyl-CoA into CO₂ and CH₃H₂MPT, tetrahydromethanopterin S-methyltransferase, and methyl-CoM reductase for methyl-CoM reduction to CH₄ (Data Set S3). To couple acetyl-CoA oxidation and reductive CH₄ generation, BUT2 must transfer electrons from reduced ferredoxin to coenzyme M (CoM-SH) and coenzyme B (CoB-SH). We identified an FpO-lacking F₄₃₀H₂ dehydrogenase (FpO) complex and heterodisulfide reductase (HdrDE) that could facilitate this (Data Set S3) and also generate an ion motive force (S4). This energy conservation system is highly similar to *Methanothrix thermophila* acetate oxidation (S4). In previous studies, *Methanothrix* species have been observed to cooccur with *Syntrophomonas* in LCFA-degrading (13) and butyrate-degrading (55–57) anaerobic environments. In this study, the stable-isotope-informed metagenomic analysis strongly suggests that the labeling of *Methanothrix* BUT2 DNA was due to the incorporation of [¹³C]acetate produced during the degradation of [¹³C]butyrate by *Syntrophomonas* BUT1.

A nearly complete pathway for methane production from CO₂ was also observed in the *Methanothrix* BUT2 genome (Data Set S3). The only gene lacking in the CO₂-reducing pathway was an F₄₃₀-dependent N₉,N₁₀-methylene-tetrahydromethanopterin dehydrogenase (Mtd). While *Methanothrix* is thought to be an obligate aceticlastic methanogen (52, 53), the presence and expression of the CO₂-reducing pathway in *Methanothrix* were previously reported (58–60) and were hypothesized to be involved in methane formation via DIET. However, the mechanism through which *Methanothrix* directly accepts electrons from its syntrophic partner has not been identified (58, 59). The other known electron donors for methane production from CO₂ are hydrogen and formate. A membrane-bound hydrogenase (mbhAB) was observed in the *Methanothrix* BUT2 genome (Data Set S3). In other studies, negligible hydrogenase activity was observed with *Methanothrix* species (61). Two monomeric formate dehydrogenase enzymes (FdhA) were also encoded by *Methanothrix* BUT2 (Data Set S3). Experiments with thermophilic *Methanothrix* sp. strain CALS-1 and mesophilic *Methanothrix concilii* showed that they displayed formate dehydrogenase activity by splitting formate into hydrogen and CO₂; however, the produced CO₂ was not used for methane generation (61, 62). Yet, the mesophilic *M. soehngenii* did not show formate dehydrogenase activity (S3). Thus, the roles of the hydrogenases, formate dehydrogenases, and CO₂-reducing pathway for methane generation in *Methanothrix* BUT2 are not clear. Transcriptomic,
metabolomic, and/or proteomic approaches are needed to elucidate the activity of the CO₂-reducing methanogenesis production pathway during syntrophic growth on butyrate with *Syntrophomonas BUT1*.

**Conclusions.** In this study, stable-isotope-informed genome-resolved metagenomics was used to provide genomic insights into syntrophic metabolism during butyrate degradation in anaerobic digesters. The results obtained via genome binning and metabolic reconstruction showed that a ¹³C-enriched *Syntrophomonas* genome contained the genetic capacity to convert butyrate into precursor metabolites for methane formation: acetate, hydrogen, and formate. A ¹³C-enriched *Methanothrix* genome likely consumed the acetate produced during butyrate degradation, incorporating some ¹³C into biomass. The presence of a CO₂-reducing pathway, as well as formate dehydrogenase and hydrogenase genes, in the *Methanothrix* genome leaves open the possibility of flexible metabolism during methanogenesis. As syntrophic fatty acid-degrading populations are often slow-growing and thus difficult to isolate, this study demonstrates a new approach to link ecophysiology with genomic identity in these important populations involved in anaerobic biotechnologies as well as global carbon cycling. Advancing our understanding of in situ metabolic activities within anaerobic communities is paramount, as these microbiomes contain multiple interacting functional groups that, in cooperation, enable the processing of degradable organic carbon into methane gas. Coupling SIP-informed metagenomics with other activity-based techniques, such as metabolomics, transcriptomics, and proteomics, may further illuminate the structure of anaerobic metabolic networks as well as quantify metabolite fluxes, thus enabling newly informed process models to predict rates of anaerobic carbon transformation.

**MATERIALS AND METHODS**

**Batch incubations with [¹³C]butyrate.** Two 4-liter anaerobic digesters treating dairy manure and sodium oleate were operated for over 200 days at a solids retention time of 20 days and a temperature of 35°C, as described by Ziels et al. (13). The two digesters were operated with different feeding frequencies of sodium oleate. One digester received sodium oleate once every 48 h, while the other digester was fed semicontinuously every 6 h (13).

On day 228 of digester operation, 10-ml samples were collected from each digester and immediately transferred to 35-ml glass serum bottles that were prepurged with N₂-CO₂ (80:20) and capped with butyl rubber septa. At the time of biomass sampling, total effluent volatile fatty acids (VFA) and LCFA (liquid plus sorbed) levels were below 70 mg/liter. Duplicate microcosms were fed with a 1 M solution of either ¹²C sodium butyrate or ¹³C-labeled sodium butyrate (>98% atom purity; Cambridge Isotope Laboratories, Tewksbury, MA, USA) to reach an initial butyrate concentration of 40 mM. The ¹³C-labeled sodium butyrate was universally labeled at all 4 carbons. Triplicate blank controls were incubated in parallel to measure background methane production from the inoculum. Methane production was measured approximately every 4 h over the 50-h incubation time using a digital manometer (series 490A; Dwyer Instruments) and gas chromatograph-flame ionization detector (GC-FID) (item no. SRI 8610C), according to the methods of Ziels et al. (13). Cumulative methane production from butyrate degradation was determined by subtracting the cumulative methane production in unamended controls over time. A 50-h incubation time was used to limit cross-labeling of peripheral populations with by-products of endogenous decay (11, 12), while also providing sufficient time for nearly all of the substrate (>80%) to be converted.

**Stable-isotope probing.** DNA was extracted from the duplicate 10-ml microcosms after the 50-h incubation, separated via density gradient centrifugation, fractionated, precipitated, and recovered as previously described (11). DNA was measured in 24 density gradient fractions using Qubit (Invitrogen, MA, USA). *Syntrophomonas* 16S rRNA genes were quantified in gradient fractions as described by Ziels et al. (11), using previously developed primers and probes (63). Heavy-DNA fractions with buoyant densities between 1.70 and 1.705 g/ml (see Fig. S2 in the supplemental material) were selected for each microcosm sample and sent for metagenomic sequencing at MR DNA Laboratories (Shallowater, TX, USA), as well as for 16S rRNA gene iTag sequencing at the U.S. Department of Energy Joint Genome Institute (JGI), according to the method of Ziels et al. (11). Metagenome libraries were prepared using the Nextera DNA sample preparation kit (Illumina Inc., Hayward, CA, USA) by following the manufacturer’s instructions. The metagenome libraries were sequenced in 150-bp paired-end mode on a HiSeq 2500 sequencer (Illumina Inc., Hayward, CA, USA).

**16S rRNA gene amplicon sequence analysis.** Raw 16S rRNA gene amplicon reads were filtered by trimming the first 10 bp, truncating forward reads at 265 bp, truncating reverse reads at 180 bp, and filtering all reads based on a maximum expected error of 2 using DADA2 (64). The filtered and trimmed reads were then dereplicated and denoised into exact sequences using estimated error parameters with DADA2. Forward and reverse sequences were then merged with DADA2 using a minimum overlap of 20 bp and zero allowed mismatches. Merged and denoised sequences were then truncated to 390 bp
and clustered into OTUs with a 99.5% similarity cutoff after chimera removal with UPARSE v8.1 (65). Representative sequences of the 99.5% OTUs were classified against those in the SILVA SSU Ref nonredundant data set, v.123, using the RDP classifier (66).

**Metagenome binning, annotation, and statistical analysis.** All metagenomic reads were initially trimmed and quality filtered using illumina-utils (67) (available from https://github.com/merenlab/illumina-utils) according to the parameters of Minoche et al. (68). Metagenomic reads from all [13C]butyrate-fed microcosms were coassembled using MEGAHIT v1.1.1 (69). Open reading frames were called with Prodigal v2.6.3 (70) and were taxonomically classified with GhostKOALA (71). Short reads from the 13C and 12C metagenomes were mapped onto the contigs using Bowtie 2 (72) with default parameters and parsed with SAMTools v1.3.1 (73). Additionally, bulk community metagenomic reads from the total biomass collected from each digester within 2 days of the butyrate SIP incubations were mapped onto the assembled contigs for their inclusion in the subsequent differential-coverage binning.

The contigs were then binned according to the workflows of Eren et al. (74) and Lee et al. (75) using anvio v2.4.0 and CONCOCT v1.0.0 (76). Briefly, single-copy genes were searched using the “anvi-run-hmms” command. Single-copy genes were identified using hidden Markov models in anvio based on the Campbell et al. (77) and Rinke et al. (78) bacterial and archaeal gene data sets, respectively. The “anvi-profile” command was used to parse contig coverage across all samples from the BAM files with SAMTools (73). The “anvi-merge” command was used to compile the coverage information for contigs across all samples into a single anvio profile. The initial binning was conducted with the “anvi-cluster-with-concost,” which uses CONCOCT (76), by constraining the number of bins to 40 (“–num-clusters 40”) to minimize fragmentation error (i.e., splitting up a single bin into multiple smaller bins) (75). Bins that displayed “ contamination error” (i.e., a bin has multiple populations and/or contamination) (75) were interactively refined using the “anvi-refine” command based on completion and redundancy estimates from the presence of bacterial and archaeal single-copy genes, taxonomies of open reading frames (ORFs) from BlastKOALA, tetra-nucleotide frequency, and coverage patterns across multiple samples. After manual refinement of the bins using anvio, we obtained a set of 160 genomic bins that were assessed for completeness and contamination with CheckM (20) (Data Set S1). The differential abundance of each genomic bin in the [13C]- and [12C]butyrate metagenomes of each digester was determined using DESeq2 (14) using mapped read counts. A significant difference in abundance between 13C and 12C metagenomes was established by a P value of less than 0.05. The average nucleotide identity (ANI) between 13C-enriched genomic bins and publicly available genomes from closely related organisms was calculated with pyANI (available from https://github.com/widdowquinn/pyani). Open reading frames were annotated with the MicroScope platform (79), and metabolic reconstructions were performed with Pathway Tools (80). Potential type IV pilin genes were identified with the PilFind program (49).

**Data availability.** We have made publicly available the following: raw sequence reads and metagenome assemblies for the butyrate DNA-SIP metagenomes in NCBI’s Sequence Read Archive under BioProject no. PRJNAS24401, genomic FASTA files for each 13C-enriched genomic bin (https://doi.org/10.6084/m9.figshare.7761776), and the annotation data for the two 13C-enriched MAGs (https://doi.org/10.6084/m9.figshare.7761710). The bulk community raw metagenomic reads from the study by Ziels et al. (11) that were used in differential coverage binning are available via the U.S. Joint Genome Institute’s Genome Portal (https://genome.jgi.doe.gov/portal/) under the project identifiers 1105507 and 1105497. 16S rRNA gene amplicon sequences are available via the U.S. Joint Genome Institute’s Genome Portal under project no. 1105527, with sample identifiers 112232 to 112239.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00159-19.

**FIG S1** PDF file, 0.01 MB.
**FIG S2** PDF file, 0.03 MB.
**FIG S3** PDF file, 0.03 MB.
**FIG S4** PDF file, 0.1 MB.
**TABLE S1** DOCX file, 0.01 MB.
**TABLE S2** DOCX file, 0.01 MB.
**DATA SET S1** XLSX file, 0.1 MB.
**DATA SET S2** XLSX file, 0.02 MB.
**DATA SET S3** XLSX file, 0.02 MB.
**DATA SET S4** XLSX file, 0.2 MB.

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