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Function and Phylogeny of Bacterial Butyryl Coenzyme A:Acetate Transferases and Their Diversity in the Proximal Colon of Swine

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ABSTRACT

Studying the host-associated butyrate-producing bacterial community is important, because butyrate is essential for colonic homeostasis and gut health. Previous research has identified the butyryl coenzyme A (CoA):acetate-CoA transferase (EC 2.3.8.3) as a gene of primary importance for butyrate production in intestinal ecosystems; however, this gene family (*but*) remains poorly defined. We developed tools for the analysis of butyrate-producing bacteria based on 12 putative *but* genes identified in the genomes of nine butyrate-producing bacteria obtained from the swine intestinal tract. Functional analyses revealed that eight of these genes had strong But enzyme activity. When *but* paralogues were found within a genome, only one gene per genome encoded strong activity, with the exception of one strain in which no gene encoded strong But activity. Degenerate primers were designed to amplify the functional *but* genes and were tested by amplifying environmental *but* sequences from DNA and RNA extracted from swine colonic contents. The results show diverse *but* sequences from swine-associated butyrate-producing bacteria, most of which clustered near functionally confirmed sequences. Here, we describe tools and a framework that allow the bacterial butyrate-producing community to be profiled in the context of animal health and disease.

IMPORTANCE

Butyrate is a compound produced by the microbiota in the intestinal tracts of animals. This compound is of critical importance for intestinal health, and yet studying its production by diverse intestinal bacteria is technically challenging. Here, we present an additional way to study the butyrate-producing community of bacteria using one degenerate primer set that selectively targets genes experimentally demonstrated to encode butyrate production. This work will enable researchers to more easily study this very important bacterial function that has implications for host health and resistance to disease.

Short-chain fatty acids (SCFAs) play a central role in the maintenance of colonic homeostasis, which is the delicate balance between the host, its immune system, and the gastrointestinal microbial partners (1). Butyrate in particular has potent effects on host tissues. As with other SCFAs, butyrate is consumed by the host as an energy source; however, unlike the other common SCFAs, such as propionate and acetate, butyrate is the preferred energy source for colonocytes (2) and is rapidly absorbed and used by the colonic epithelium. This rapid oxidation of butyrate reduces local oxygen concentrations, causing the epithelia to become hypoxic and thus limiting the growth of facultative aerobic pathogens, such as *Salmonella* species (3, 4). In addition, butyrate alters host gene expression to promote immune tolerance to the colonic microbiota and to improve the barrier function of the colonic epithelium. For example, butyrate has been shown to increase the secretion of antimicrobial peptides and mucus as well as the expression of tight junction proteins, thickening and strengthening the barrier while making it less hospitable to invasive microbes (5–7). Most of butyrate's immunomodulatory activities result in anti-inflammatory effects, including the production of extrathymic T-regulatory (T-reg) cells (8), the limitation of proinflammatory $CD4^+$ T cell activity (9), the stimulation of epithelial cells to produce retinoic acid (10), and the desensitization of colonic epithelial cells to gamma interferon (IFN-γ) (11). Although the maintenance of immune tolerance is complex and requires a balance among many regulatory factors, butyrate is a major signal for the host immune system leading to the inhibition of proinflammatory responses and to toleration of microbes that are present (12).

Because of butyrate's importance in maintaining colonic homeostasis and host health, characterizing and manipulating the bacterial populations responsible for its production are of great interest. Butyrate-producing bacteria do not form a monophyletic group, and at least four different fermentation pathways lead to butyrate production (13). The most common pathway for butyrate production in colonic environments entails the condensation of two molecules of acetyl coenzyme A (acetyl-CoA), followed by reduction to butyryl CoA. After butyryl CoA has been generated, two different enzymes are responsible for the final conversion to butyrate: butyrate kinase (Buk) and butyryl-CoA:acetate-CoA

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transferase (But) (14), with the But protein being the most common in the colonic environment (13). This enzyme takes the CoA group from butyryl-CoA and transfers it to acetate, yielding acetyl-CoA, thus regenerating a substrate of the main butyrate production pathway. This transferase is thought to be especially advantageous in the colonic ecosystem due to the high levels of acetate, allowing butyrate producers to take up and use a waste product of other microbes (15). Many studies have suggested that the majority of butyrate production in hindgut fermenters is the product of But enzyme activity, including in swine (13, 14, 16–18). It should be noted that previous work has suggested that not all enzymes capable of But activity are homologous. Previous work demonstrated that some bacteria from clostridial cluster XVI isolated from a chicken cecum had But activity despite lacking genes with significant homology to the *but* gene family (19). The authors identified genes similar to those encoding other known propionyl-CoA transferases in these genomes and suggested that these genes were responsible for the observed But activity. This work focuses only on genes encoding But enzymes commonly found in the *Ruminococcaceae* and *Lachnospiraceae* families (previously known as clostridial clusters IV and XIVa, respectively) and is not applicable to But-active enzymes with different evolutionary origins.

The sequence variation for the *but* gene family is poorly defined and currently includes closely related transferases that have differing substrate preferences (16, 17, 20). The FunGene But protein database is a large repository of But-like protein sequences and is an excellent resource; however, it contains But proteins and similar transferases that have distinct substrate specificities. Furthermore, few But proteins in this database have been functionally confirmed. Here, we have analyzed the *but* gene from previously identified butyrate-producing bacteria from swine (18), defined the functional diversity of the *but* sequences, developed degenerate *but* primers for PCR, and investigated the butyrate-producing bacterial community in the swine colonic environment. The results show that the degenerate *but* primers preferentially amplify genes encoding functional But enzymes over their paralogues, and that diverse *but* genes are transcribed in the swine colon.

MATERIALS AND METHODS

Identifying potential *but*-encoding sequences. Previous work identified nine strains of swine-associated intestinal bacteria as butyrate producers, as determined by gas chromatography. Additionally, these strains were also found to exhibit But activity, although the active genes could not be identified in all cases (18). These strains were subjected to shotgun genomic sequencing to identify the genes encoding their But activity. Genomic DNAs were isolated using a previously described protocol (21). Sequencing was performed using a HiSeq 2500 sequencer (2 × 150 bp, rapid mode; Illumina, San Diego, CA) or a MiSeq (2 × 300 bp) at the Iowa State University Office of Biotechnology (DNA facility, Ames, IA), a Pacific Biosciences sequencer (P6-C4 chemistry; PacBio, Menlo Park, CA) at the Yale Center for Genome Analysis (New Haven, CT), and Roche FLX-Titanium chemistry (Roche Diagnostics, Branford, CT, USA). Libraries were prepared according to the manufacturer's directions. The resulting data included some combination of PacBio reads, Roche FLX 2.3-kb mate-pair library reads, and Illumina 7.9-kb mate-pair library reads. These were assembled using the MIRA assembler in a *de novo* hybrid assembly (22). Potential *but* genes were identified in the genomes by performing a BLAST search with the amino acid sequence of the butyryl-CoA:acetate-CoA transferase gene from *Roseburia intestinalis* L1-82 (GenBank accession no. EEV00989).

Testing for butyrate transferase activity. Candidate genes were cloned into the pET-TOPO-101 vector (Invitrogen, Carlsbad, CA) and transformed into TOP10 *Escherichia coli* chemically competent cells, according to the manufacturer's instructions (primers used for cloning are listed in Table S1 in the supplemental material). Plasmid DNAs were isolated using the MinElute miniprep kit (Qiagen, Valencia, CA), and positive clones were confirmed to have full-length gene inserts by sequencing on an Applied Biosystems 3730xl DNA analyzer. Cloned DNAs were additionally transformed into *E. coli* BL21 Star competent cells for protein expression, in accordance with the kit protocol. Cultures (100 ml) were grown for 12 h in LB containing 50 μg/ml carbenicillin. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After an additional 6 h of growth, cultures were harvested by centrifugation, washed, and resuspended in 10 ml of sterile phosphate-buffered saline (PBS). Cells were lysed by two passages through a French press (Aminco, Silver Spring, MD). Lysates were centrifuged at 19,000 × *g* for 10 min to remove remaining unlysed cells. Protein expression was confirmed, and the amount of recombinant protein in each lysate was estimated by running 15 μg of total protein (determined by the Bradford assay [23]) in a 15% SDS-PAGE gel, staining with Coomassie blue, and comparing the 49-kDa band to all bands in the sample using a densitometry analysis in the ImageJ software package (24). Activities were normalized to the amount of protein present in the 49-kDa band.

Butyryl-CoA transferase (EC 2.8.3.8) activity was tested using the citrate synthase assay, as described previously (16), and activity was measured with acetate and butyryl-CoA as substrates (Sigma). The acetyl-CoA generated by butyrate transferase is condensed with oxaloacetate, liberating CoA, which reacts with 5,5′-dithio-bis-(2-nitrobenzoate) to form a yellow thiophenolate anion. The reaction rates were measured by monitoring the absorbance at 412 nm at 39°C on a Beckman (Indianapolis, IN) DU-650 spectrophotometer. Crude cell lysates were diluted with sterile water as necessary to achieve the linear range for the rate of the reaction. The reaction was repeated in the absence of acetate to confirm that the measured rate was not due to CoA-hydrolase activity.

Designing and validating conserved primers to *but*. All full-length functionally validated *but*-like genes were aligned using CLC Genomics Workbench (Aarhus, Denmark), and conserved regions were identified. Degenerate primers (funbut-FWD, 5′-CARYTIGGIATYGGIGGIAT SCC; funbut-REV, 5′-TGTCCGCCIGYICCRSWRAT) were designed to preferentially amplify those *but* genes with confirmed activity.

Full-length genes were downloaded from the FunGene *but* database on 21 March 2016, including only those sequences with a score of 275 or higher (25), resulting in 1,144 full-length sequences after removing redundant entries. The number of mismatches between the funbut primers and each gene in this data set was calculated with a Python script utilizing the Biopython libraries (26) (see Table S1 in the supplemental material). Previously published primer sets from Vital et al. (17) and Louis and Flint (20) were also analyzed for comparison. This script yielded a table listing the number of mismatches to each primer set for each gene entry. FastTree (27) was used to generate a phylogenetic tree from full-length amino acid sequences, and the R packages APE (28) and ggtree (29) were used to generate primer coverage figures. Sequences considered likely to amplify were those with two or fewer total mismatches to the primers.

To investigate potential amplification biases, the funbut primers were used in qPCR assays to determine which genes are preferentially amplified. Full-length gene amplicons were generated for each gene included in this study (see Table 1 for primer sequences). The amplicons were evaluated via NanoDrop (30) and diluted in 2 μg/ml sheared salmon sperm DNA to 10^7 copies/μl. The quantitative PCRs (qPCRs) were conducted with the Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) containing each of the funbut primers at 500 nM and 1 μl of *but* gene amplicon DNA in 20 μl on a Stratagene 3005P thermocycler (San Diego, CA). The cycling conditions were as follows: 95°C for 30 s, 53°C for 30 s, and 72° for 30 s, for 40 cycles in total. Inferences about amplification

TABLE 1 Primers used in cloning each *but*-like gene as well as for generating full-length gene amplicons for use in qPCR amplification preference assay[a]

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing temp (°C)	PCR product length (bp)
Butyricicoccus #1	CACCATGAGTATTTTTACAGAATACAGAAGCAAACTGCG	CAATATCGTTTGTTAGAGCGCCGC	53	1,338
Butyricicoccus #2	CACCATGGATTATCAGGCGCTCTATCAG	TTATCTGCGGTTGGAGGTTCTCCA	55	1,344
Megasphaera #1	CACCATGTACAAACAGAAACTGATGA	CCGTTTATTGCTATATCTCCAGATTTTCAT	50	1,323
Megasphaera #2	CACCATGTACAAACTTTCACAAATTCC	TTACATGGAAATCTTGCTGGTCTT	50	1,347
499	CACCATGGATTTTAATCAGGAATACCAGC	TTATTTATTGCTTCTTCTCCAAATATGCAT	53	1,341
35-6-1	CACCATGGATTATTCTAAAGAATATCAA	TTATCTCTTATTAGTTCTTCTCCAAATATTC	48	1,344
494a	CACCATGTCATTTACTCAAGAATATCAGA	TTATTTGTTAGATCTTCTCCAGATGTTAGC	53	1,341
831b	CACCATGGATTTTTCAACTGAATACAAAC	TTATTTGTTGCTTCTTCTCCAGATGTGCAT	53	1,341
992a	CACCATGAGTTACGCAAACGAATATCAA	TTACTTATTGCTGCGTCTCCAGATATGAG	54	1,341
27-5-10	CACCATGGAGCAAACAGAACTTTACCGG	TTACTTTCGGTTGCTTCTGCGCC	55	1,344
68-3-10 #1	CACCATGAGTAAGGATTTTGGATTGT	TTATTTCATCACCTTGCTGCTCG	50	1,383
68-3-10 #2	CACCATGTACAAGGTTTCGACTTTAG	CTAGAACGATGTTTTACTGGTGTATTTCC	50	1,359

[a] See Fig. 3.

preference were made by comparing threshold cycle (C_T) values for each gene. As each reaction mixture contained the exact same primer concentrations and numbers of target molecules, any difference in the C_T values among the different targets is due to amplification preferences.

MiSeq *but* amplicon library prep. Swine proximal colon contents (10 cm distal from the cecum) were immediately placed in RNA*later* and quickly homogenized to preserve the integrity of nucleic acids. Samples were subsequently frozen at −80°C until extraction (within 1 month). DNAs and RNAs were extracted using the PowerClean DNA and RNA extraction kits (MO BIO Laboratories, Carlsbad, CA) from proximal colon contents from six pigs fed a standard diet and associated with a different study (51). The iScript Select kit (Bio-Rad, Hercules, CA) was used to generate cDNA from the RNA using random hexamer primers. Amplicon sequencing libraries were prepared according to Illumina's 16S metagenomic sequencing library preparation (part 15044223 revision B), substituting the funbut primers for the 16S primers. This protocol uses a 2-step PCR procedure: the first step generates the amplicons from environmental samples, and the second step adds the indices and the sequencing adapters. In the first step, the funbut primers were used to amplify a 359-bp fragment using the AccuPrime *Taq* high-fidelity PCR system (Invitrogen). Due to the inclusion of multiple inosine bases, we were unable to produce a PCR product using a proofreading polymerase alone (31, 32), necessitating the use of a procedure that included *Taq* as well. The first-step PCR mixtures contained each primer at 500 nM and 100 ng of template and used an annealing temperature of 53°C for 35 cycles. The second PCR step was performed in accordance with the protocol using Kapa HiFi polymerase (Kapa Biosystems, Wilmington, MA) and the Nextera XT version 2 indices (Illumina). This library was sequenced on a MiSeq using a 2 × 300 version 3 reagent kit to generate 300-bp paired-end reads.

Sequence analysis. Sequences were processed using mothur (33) according to a modified version of the MiSeq standard operating procedure (SOP) (http://www.mothur.org/wiki/MiSeq_SOP). Paired-end reads were joined, quality screened, and aligned to full-length high-quality *but* genes downloaded from FunGene, as previously mentioned. Sequences passing quality filters were clustered at a 97% similarity cutoff, and representative sequences were obtained for each operational taxonomic unit (OTU). These representatives were used in a BLASTn search against a database comprising full-length sequences from the FunGene *but* gene data set plus genes from the current work to determine the closest matching published sequence (% identity). Communities were subsampled to 1,556 sequences per sample prior to further analysis. Sequences used as references in the phylogenetic trees have had their activities confirmed either in this work or in previously published work. Genes from human strains have been cloned and their activities confirmed in previous works, such as those of Charrier et al. (16) and Louis et al. (34). Other work has tested crude cell lysates for But enzyme activity, such as the study by Duncan et al. (15), and inferred *but* gene presence by measuring butyrate production, acetate consumption, and the presence of a gene homologous to confirmed *but* gene sequences. Additionally, reviews, such as that by Louis et al. (35), identified isolates with confirmed But enzyme activity. These reference sequences were trimmed to the length of the representative sequences for the OTUs and used to generate a maximum likelihood tree using RAxML (36).

Accession number(s). Bacterial genomes, butyrate transferase sequences, and amplicon sequencing data were deposited in GenBank under BioProject PRJNA341691. The code and data used to generate the figures in this paper are available at https://github.com/Jtrachsel/AEM-funbuts.

RESULTS

Activity encoded by the *but* gene is associated with an amino acid sequence motif. Putative But-encoding genes were identified in the genomic sequence data from the butyrate-producing bacteria isolated from swine. All of the nine genomes analyzed yielded at least one potential *but* gene, and three genomes were predicted to contain two (those of *Megasphaera*, *Butyricicoccus*, and *Eubacterium* spp.), resulting in a total of 12 putative But-encoding genes. Functional analyses revealed that eight genomes had strong But activity ranging from 7,004 $\mu M \cdot mg^{-1} \cdot min^{-1}$ (strain 27-5-10) to 27,819 $\mu M \cdot mg^{-1} \cdot min^{-1}$ (strain 831b; Table 2). Only one *but* gene per genome showed appreciable activity, with the exception of the *Eubacterium* (strain 68-5-10), in which neither putative But protein was highly active (Table 2). This lack of appreciable But activity in strain 68-5-10 is consistent with previous work with this strain using native whole-cell lysate in the same assay (18). All genes that showed strong activity also exhibited similar activity when propionyl-CoA was used as a substrate (Table 2). These results are in agreement with previous characterizations of this gene family (16) and demonstrate that these sequences encode But activity.

To determine sequence motifs associated with active But proteins, an amino acid alignment of all 12 putative *but* genes was generated. The alignment yielded several differences that demarked those with activity from those without. These differences occurred in a conserved region containing the amino acid motif LQLGIGG (Fig. 1). This motif was identical in all highly active But proteins; however, the proteins with low But activity contained at least one substitution in this motif. These data suggested that

TABLE 2 But enzyme kinetics and amino acid analyses[a]

Strain of origin	Closest related species (% 16S BLAST identity)	Gene name	Length (aa)[b]	Crude activity (μM/min · mg)		Similarity to *Roseburia* query sequence (aa)[c]	
				Butyryl-CoA	Propionyl-CoA	% ID	% POS
68-3-10	*Eubacterium nodatum* (93)	68-3-10 #1	460	77.3	406.4	47	66
68-3-10	*E. nodatum* (93)	68-3-10 #2	452	218.1	335.4	48	69
1161	*Megasphaera elsdenii* LC-1 (99)	*Megasphaera* #1	441	655.5	637.2	55	69
1161	***M. elsdenii* LC-1 (99)**	***Megasphaera* #2**	**448**	**23,515.6**	**20,747.3**	**49**	**69**
BB10	*Butyricicoccus pullicaecorum* (94)	*Butyricicoccus* #1	445	231.2	785.4	48	67
BB10	***B. pullicaecorum* (94)**	***Butyricicoccus* #2**	**447**	**13,367.9**	**12,665.3**	**73**	**85**
35-6-1	***Peptoniphilus grossensis* (97)**	**36-5-1**	**447**	**18,343.5**	**13,495.2**	**62**	**76**
27-5-10	***Intestinimonas butyriciproducens* (99)**	**27-5-10**	**447**	**7,004.6**	**5,725.3**	**72**	**85**
494a	***Anaerostipes butyraticus* (96)**	**494a**	**446**	**13,692.3**	**12,767.3**	**74**	**85**
992a	***Anaerostipes hadrus* (95)**	**992a**	**446**	**16,152.8**	**12,193.9**	**77**	**85**
499	***Roseburia hominis* (96)**	**499**	**446**	**8,276.8**	**7,946.9**	**83**	**91**
831b	***R. hominis* (97)**	**831b**	**446**	**27,819.8**	**27,892.9**	**86**	**91**

[a] Sequences with strong activity are shown in bold.
[b] aa, amino acids.
[c] ID, identity; POS, positive.

primers annealing to this site could be designed to preferentially amplify genes similar to those with high But activity, thus distinguishing genes with potential But activity from nonfunctional paralogues. The reverse primer-binding site was nondiscriminating, since we designed it to a gene region where But and But-like proteins shared similar amino acid residues. Unfortunately, no suitable alternative reverse primer-binding site would preferentially amplify all functional genes and still allow a primer of reasonable degeneracy for amplification.

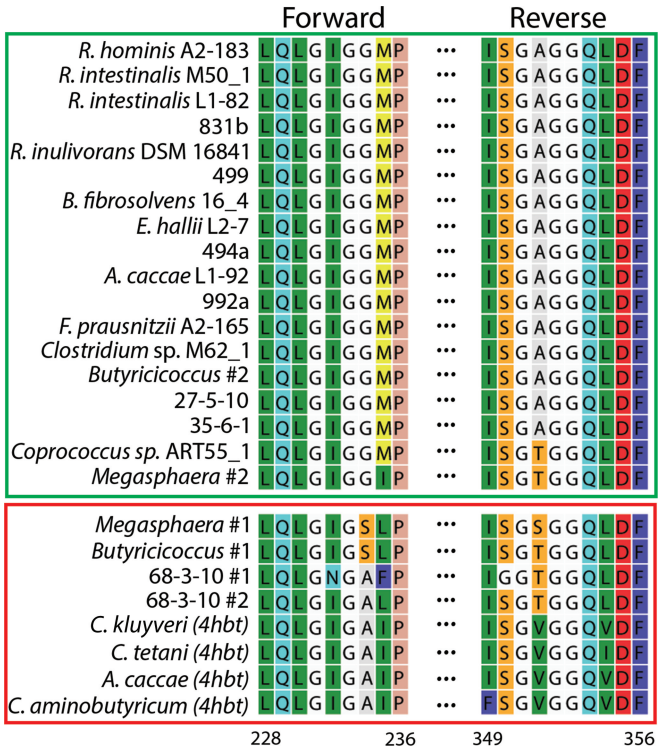

FIG 1 An amino acid alignment of the primer-binding regions. Residue numbering is based on the full-length But protein sequence from *Roseburia intestinalis* L1-82 GenBank accession no. EEV00989. Functionally confirmed sequences occupy the top green-bordered box. Sequences with little activity are bordered by the red box. The glycine at position 234 of this alignment is conserved in all highly active sequences. Amino acid residues are colored according to the RasMol convention. *4hbt*, 4-hydroxybutyryl-CoA:acetate-CoA transferase (outgroup).

Degenerate primers preferentially target function-associated But protein-coding sequences and amplify diverse swine-associated *but* genes. An *in silico* analysis compared the primer coverage of currently available *but*-targeting primer sets to the funbut primer pair and revealed that the funbut primers preferentially cover the clade containing all functionally confirmed sequences while having little coverage outside this clade (Fig. 2). The funbut primers are likely to amplify (two or fewer mismatches) 194 sequences, with 95% of these (184 sequences) in the main functional clade of interest. In contrast, the primers published by Vital et al. (17) are likely to amplify 517 sequences, 228 of these being in the clade of interest (44%). Finally, the primers published by Louis and Flint (20) are likely to amplify 5 sequences, all of which are in the main functional clade. It should be noted that the estimates for the number of sequences likely to be amplified are based only on the number of mismatches; amplification conditions also play a large role. Each of these primer sets could amplify more or less diversity than our estimate suggests depending on the exact conditions of the PCR. These results show that the funbut primers are more specific to diverse genes encoding functional But enzymes than are previously published *but* primers.

The *in silico* validation of the funbut primers suggests that they will preferentially amplify functional *but* genes over their paralogues, and to verify these findings, we investigated the amplification preference of our primers for the functionally validated But-encoding genes in this study. The funbut primers preferentially amplified sequences associated with But enzyme function over sequences associated with little or no But enzyme activity (Fig. 3). However, some *but* genes were amplified in fewer qPCR cycles and therefore more readily than others, revealing amplification biases even among the functionally confirmed genes (Fig. 3).

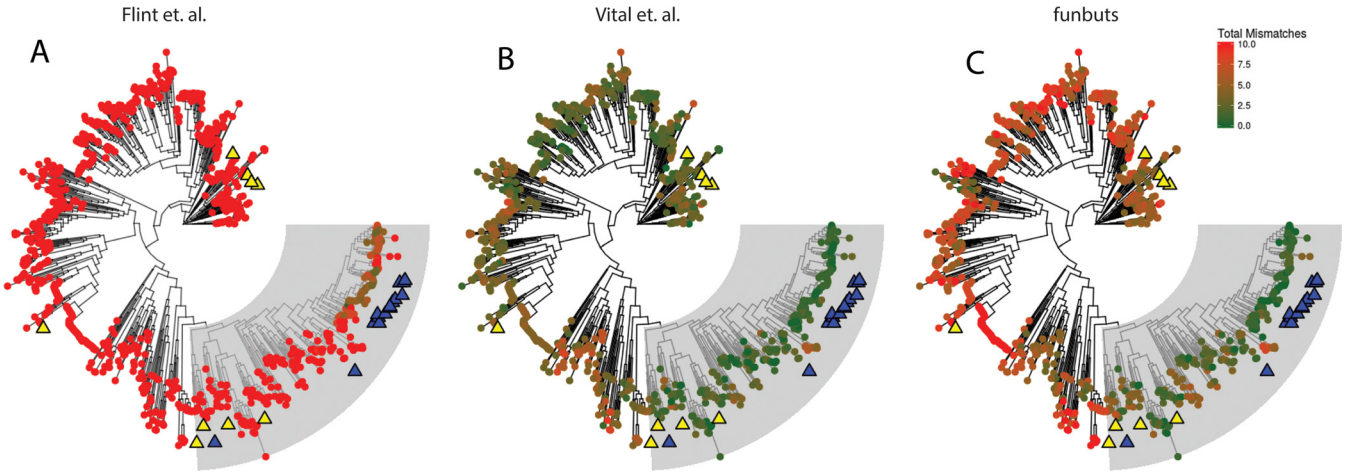

FIG 2 Primer coverage for the three available *but*-targeting primer sets. (A) Flint et al. (2). (B) Vital et al. (3). (C) funbut primers. Maximum likelihood trees of full-length protein coding sequences from the FunGene *but* database, with the tips of each branch colored to reflect the total number of mismatches each primer set has to that particular sequence (red to green). The clade containing all verified *but* genes is shaded gray. Sequences with confirmed activity are marked with a blue triangle, and *but* paralogues are marked with yellow triangles.

A large unexplored diversity of *but* genes exists in the swine hindgut. The funbut primers were applied to nucleic acids from a gut microbial community to evaluate *but* gene detection in this ecosystem. Diverse *but* genes were amplified from total DNA and RNA from swine proximal colonic contents. The colonic contents of six pigs yielded 90 OTUs from total DNA and 86 OTUs from total RNA (92 total unique OTUs, 97% similarity). Several OTUs were present in all animals, but these OTUs differed depending on which nucleic acid was used to profile the community. Fourteen OTUs were detected in RNA libraries from every pig, four OTUs were detected in all DNA libraries, and three OTUs were detected in every library regardless of nucleic acid type (OTU4, OTU14, and OTU23). The RNA-based communities harbored a greater diversity of *but* sequences, and these communities were more even than the DNA-based communities from the same sample (Fig. 4; Shannon diversity index with Wilcoxon paired test, $P = 0.03$; Shannon evenness with Wilcoxon paired test, $P = 0.03$). Similarly, community membership tended to differ between the RNA- and DNA-based communities (Fig. 5), suggesting that the most active butyrate producers may not be the most abundant.

Representative sequences of many of the OTUs differed greatly from previously known cultured butyrate producers, and some showed more similarity to *but* genes from organisms detected only in metagenomes (Fig. 6). In total, 33 out of 92 OTUs were represented by sequences that most closely matched organisms detected only in metagenomic data sets. Similarly, many OTUs were represented by sequences with relatively low identity to any previously detected *but* gene. The maximum identity detected was 100%, and the minimum identity was 71.2% (OTU4). Representative sequences from 82 OTUs showed <90% identity, and 23 showed <80% identity to previously detected genes in the reference databases, whether they were of metagenomic origin or not. These findings suggest that many as-yet-uncultured butyrate producers exist in the swine gut and that this community is underrepresented in databases. Furthermore, the abundances of many

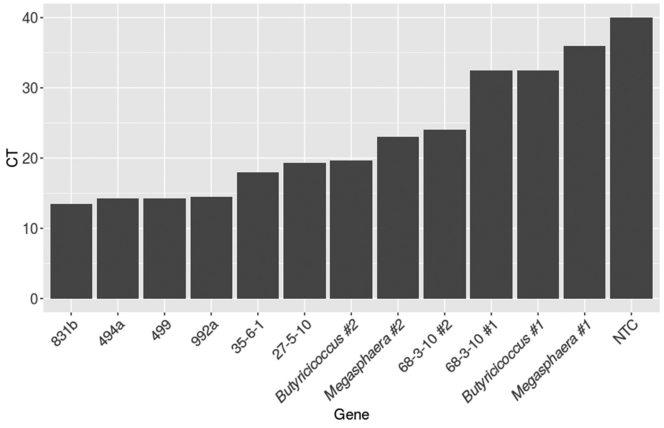

FIG 3 qPCR C_T values using the funbut primers against each gene identified in this study at 10^7 target molecules per reaction. Genes with lower C_T values are preferentially amplified over those with higher C_T values. NTC, no-template control.

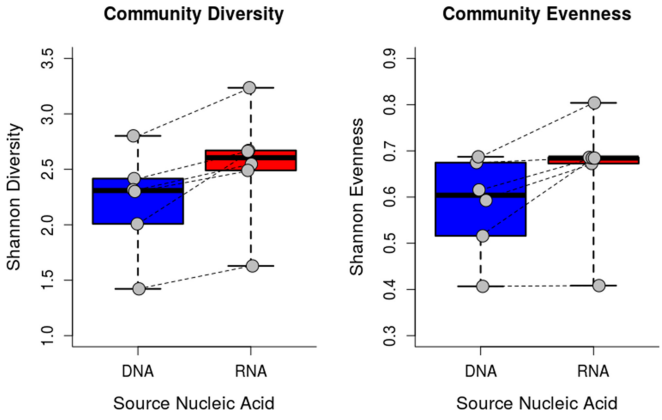

FIG 4 Shannon diversity and evenness indices of DNA and RNA *but* gene sequences, with communities from the same pig joined by a dotted line. The horizontal line within the box indicates the median, the boundaries of the box indicate the 25th and 75th percentiles, the whiskers extend to the minimum and maximum values, and individual data points are represented by gray circles.

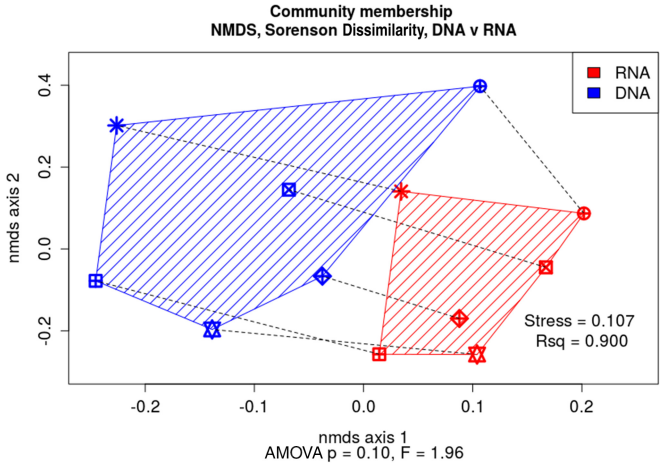

FIG 5 Nonmetric multidimensional scaling plot of Sorenson dissimilarity distances (membership) of the *but* gene sequence communities from six swine colons. Communities from the same animal are joined with a dotted line. AMOVA, analysis of molecular variance; nmds, nonmetric multidimensional scaling; Rsq, R^2.

OTUs from the same animal differed greatly depending on whether gene copy abundance (DNA) or transcript copy abundance (RNA) was considered (Fig. 6), supporting the idea that the transcriptionally active population is distinct from the most abundant.

Predicting function from phylogenetic analysis of But protein sequences. The genes encoding highly functional But proteins are phylogenetically separated from potential paralogues, but this separation is not perfect (Fig. 7), with some verified But enzymes and potential paralogues occupying the same clades on the tree. An example of this is the *Megasphaera* #2 gene, which encoded stronger But enzyme activity than the paralogue *Megasphaera* #1 gene from the same genome, but it was more divergent from the large functional clade than other sequences with confirmed activity. Phylogenetic placement was used to determine whether the OTUs detected by the funbut primer set encoded highly functional But enzymes or paralogues with lower activity. The representative DNA sequence from each OTU was aligned with confirmed *but* DNA sequences from the literature and this work (trimmed to the amplicon length), and a maximum likelihood phylogenetic tree was constructed from this alignment (Fig. 8). Similar to the tree constructed with full-length sequences, most of the confirmed reference sequences grouped together in one main clade apart from potential *but* paralogues, with the *Megasphaera* #2 gene being the exception. The vast majority of the OTUs detected by the funbut primers clustered more closely with *but* sequences encoding highly active enzymes than with sequences encoding low activity. Out of 18,672 total sequences, 18,559 sequences (99.4%) were contained within OTUs in the main functional clade, while only 113 sequences (0.6%) were outside this clade near potential *but* paralogues. The results suggest that phylogenetic relatedness is predictive of function for the majority of *but* gene sequences and that the funbut primers preferentially amplify functional *but* genes. However, function is more difficult to predict for distantly related deeply branching sequences within the *but* gene family.

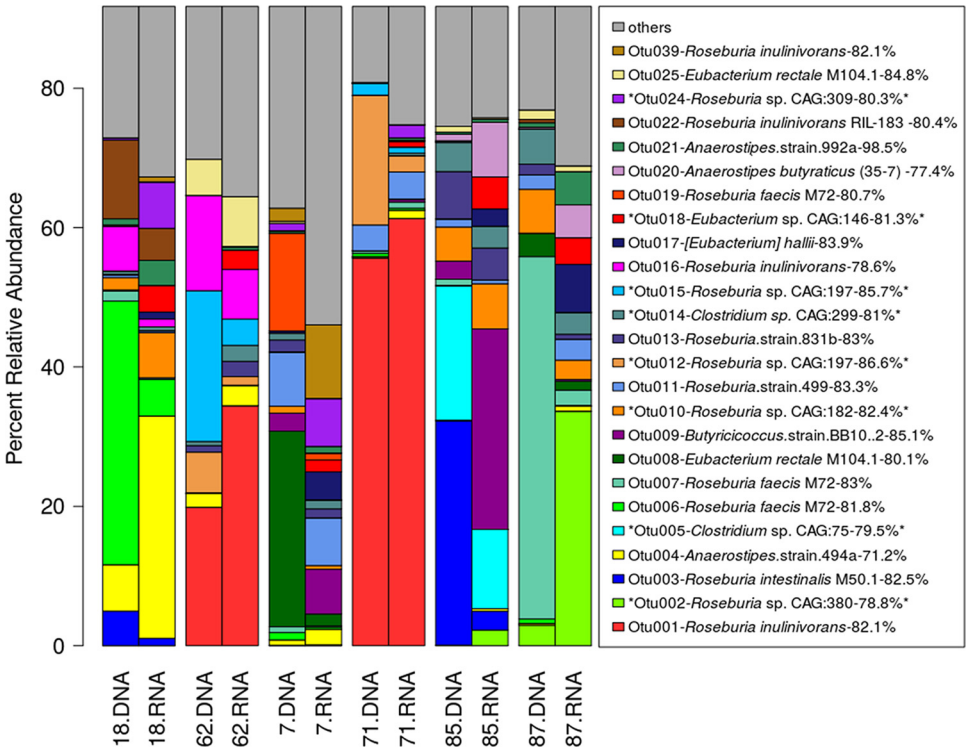

FIG 6 Twenty-five most abundant OTUs, clustered at 97% similarity at the DNA level. Listed are the names of the genomes containing the top homologues of representative sequences for each cluster, followed by percent identity (top BLAST hit). Asterisks denote genomes assembled from fecal metagenomes. Target sequences for this BLAST search were from the FunGene *but* gene database as well as the genes identified in this work.

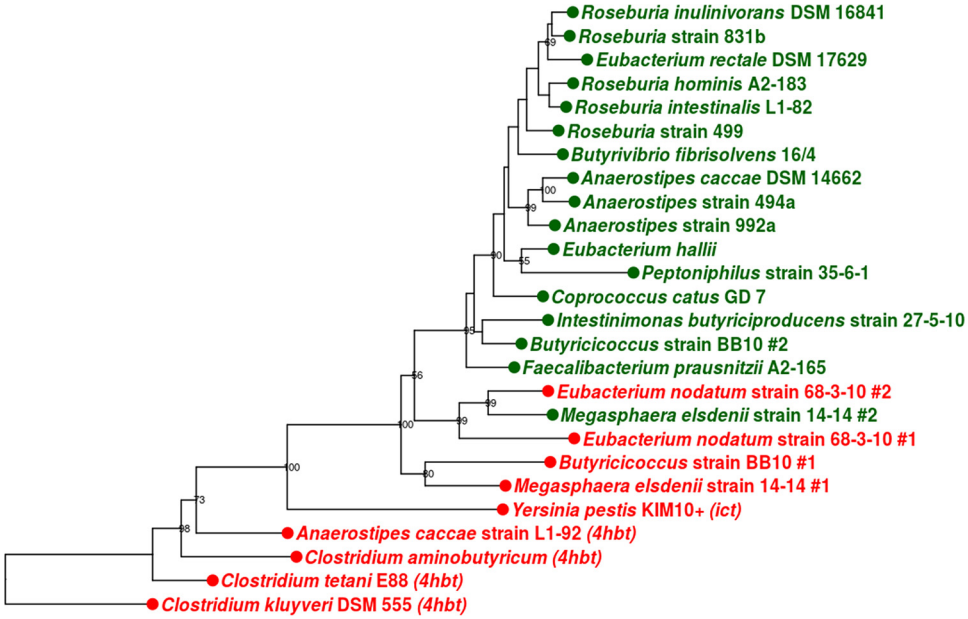

FIG 7 A phylogenetic tree (maximum likelihood) of full-length protein coding *but* sequences. Sequences with confirmed function are shown in green, and potential paralogues are shown in red. Note how functional But-encoding sequences are interspersed with paralogues toward the root of the tree. *4hbt*, 4-hydroxybutyryl-CoA:acetate-CoA transferase; *ict*, itaconate-CoA transferase.

DISCUSSION

Butyrate is centrally important to colonic homeostasis and is present in every vertebrate gut system studied to date (37). The butyrate-producing microbiota has been implicated in host health in many different disease models (35, 38–47), resulting in increased interest in studying this community. It is therefore important to have a reliable tool to identify butyrate-producing microbes and to detect their activity. The *but* gene is an excellent candidate for this probe due to its ubiquity in colonic environments, and we show the rational design of a primer set that detects *but* genes associated with butyrate transferase function. However, any analysis of the butyrate-producing community that examines only the *but* gene cannot be considered exhaustive. The bacterial butyrate synthesis pathway can be completed by other proteins, such as Buk and Ato, as well as nonhomologous enzymes capable of But activity (13, 19, 48). Characterizing a wide variety of functional genes is a critical step in designing targeted primers and probes. These data are additionally valuable when conducting comparative analyses of amplicon and metagenomic data sets from gut bacterial communities under different conditions.

Comparison of currently available *but* primer sets. Several primer sets targeting the *but* gene are currently available. The first was described by Louis and Flint in 2007 (20). This primer set was designed to be used in qPCR assays to estimate the total number of *but* gene copies in complex environments, such as feces. Much care was taken to avoid any amplification of nontarget, closely related transferases, resulting in a conservative primer set. These primers are unlikely to amplify paralogues of the *but* gene but also miss much of the full diversity of functionally verified gene products. Due to the specificity of these primers, very little spurious amplification is observed when used on complex samples, such as feces.

Conversely, Vital et al. recently described a more promiscuous set of *but* primers (17). These primers were designed to amplify a wide range of *but*-like sequences, acknowledging that they would amplify closely related non-*but* genes. The primers were used to elucidate the diversity of *but* gene sequences in humans and many other vertebrate species via Roche's 454 pyrosequencing (17, 37). The *in silico* processing pipeline they describe attempts to remove some of the non-*but* sequences by eliminating those that closely match reference sequences that reside outside the phylogenetic cluster formed by functionally confirmed But-encoding genes. This method, while useful, is imperfect; the functional validation of *but* gene family members presented here has revealed that the phylogenetic separation of verified But-encoding genes and their paralogues is not absolute (Fig. 7 and 8). Additionally, because of the degeneracy of this primer set, spurious off-target amplification and incorrectly sized PCR products regularly occur with these primers, necessitating the inclusion of a gel extraction step in sequencing library prep (17, 37).

The development of the funbut primer set built upon these two approaches by amplifying functionally verified yet diverse *but* gene sequences. The funbut primer set amplifies a greater diversity of *but* genes than the primers described by Louis and Flint (20) and fewer non-*but* paralogues than the primers described by Vital et al. (17). However, as is the case with most primer sets, these will likely misrepresent or underrepresent some important groups. For example, although we detected some *but* sequences similar to those of *Faecalibacterium* spp., these appeared at a much lower abundance than would be expected from 16S rRNA gene sequence-based studies of the swine gut. Importantly, due to the increased specificity of the funbut primers compared to the primers in the study by Vital et al. (17), no incorrectly sized PCR product has been observed when amplifying from complex fecal or mucosal samples, even at annealing temperatures as low as 45°C. This allows for the omission of the gel extraction step when preparing sequencing libraries and for the possibility of using these primers in SYBR-based qPCR assays.

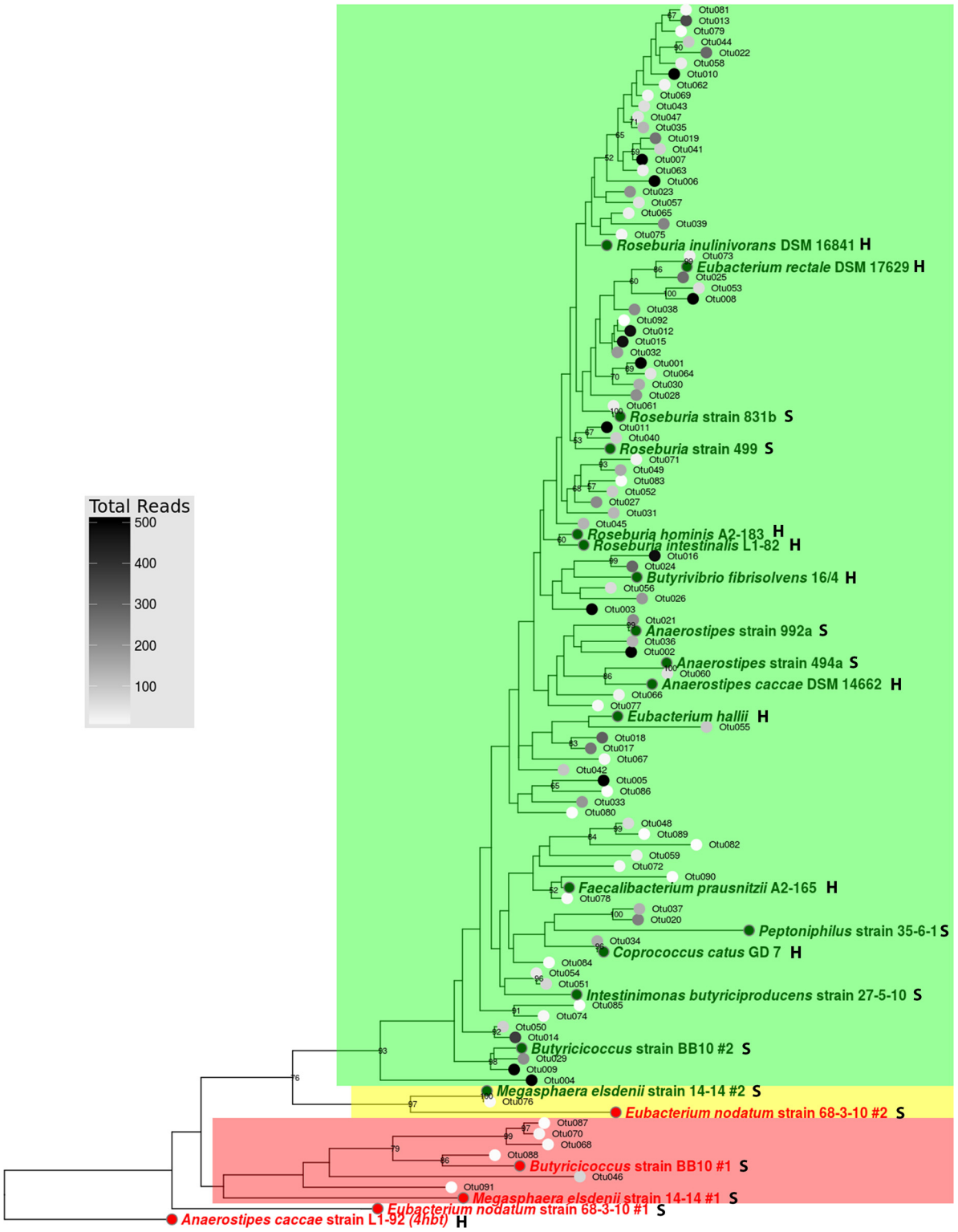

FIG 8 A phylogenetic tree (maximum likelihood) of representative sequences from OTUs from this study, and previously studied But-like sequences identified in Vital et al. (1). Sequences were generated with the funbut primers and clustered at 97% similarity. Sequences with confirmed But enzyme activity are shown in green, and sequences that have failed to demonstrate activity in functional assays are shown in red. Numbers at the nodes are bootstrap values after 1,000 resamplings. The main clade containing all functionally confirmed reference sequences is shaded green, the clade containing both confirmed But-encoding genes and potential paralogues is shaded yellow, and the clade containing only potential paralogues with low activity is shaded red. Reference sequences from human-associated bacterial isolates are marked with an H, and those from swine-associated bacterial isolates are marked with an S.

Active members of the butyrate-producing community differ from the abundant members. The funbut *but* primers detected striking differences in community composition from the same starting material (proximal colon contents) depending on whether DNA or RNA was used as the template. Sequences detected in DNA-derived libraries are not necessarily being transcribed and translated into proteins and may be representative of microbes simply passing through the intestinal tract, producing butyrate through alternative pathways, or utilizing metabolisms not involving butyrate production. At best, DNA-based *but* libraries represent the functional potential in the ecosystem. In contrast, sequences detected in RNA-based libraries represent microbes that are actively transcribing *but* genes. These active microbes represent a subset of the total *but*-containing community; however, we detected a greater diversity of *but* genes in the RNA-based libraries than in the DNA-based libraries. Other studies have identified similar differences between the metagenome and metatranscriptome, such as the observation that functional genes for methanogenesis in the human gut were far more abundant in the metatranscriptome than in the metagenome (49). This reinforces the idea that the gut ecosystem contains microbes that may be in low abundance but are highly active, and that DNA-based studies may overlook their importance. When profiling the butyrate-producing community, RNA may be a more appropriate source molecule than DNA.

Phylogenetic relatedness of But protein sequences informs potential function. Within the currently defined But protein family, there are genes encoding functional But enzymes and very similar paralogues. These two groups are more similar to each other than to the next most similar gene family, the 4-hydroxybutyrate transferases. One large clade harbored the vast majority of functionally confirmed *but* genes as well as the majority of all *but*-related OTUs detected, supporting the use of phylogenetic analyses to predict function for this family of But proteins. Outside this clade, functionally confirmed and unconfirmed But enzymes were interspersed (Fig. 7 and 8). However, this work does not rule out the possibility that paralogues also exist in the main functional clade as well. Because But enzyme activity is advantageous in colonic ecosystems, it is possible that enzymes specializing in this function have evolved multiple times from different ancestor proteins. Work by Eeckhaut et al. identified But enzyme activity from bacteria that lacked genes similar to *but* genes or their paralogues (19). They proposed that genes most similar to propionyl-CoA transferases were responsible for But enzyme activity in these organisms. Additional discovery and analysis of butyrate-producing organisms are required to delineate the full functional sequence diversity of deeply branching But protein sequences and to identify other protein families capable of But enzyme activity.

Further emphasizing the need to more fully characterize this family, and in agreement with previous gene-targeted studies (17, 37), we detected many OTUs with low identity to both confirmed sequences and cultured organisms. This work reveals many gaps in our knowledge of the *but* gene family. Due to the importance of this bacterial function in nearly all colonic ecosystems, better characterization of this community is necessary. It follows that culturing novel butyrate producers and identifying their functional genes remain important steps to improve *but* data sets.

Potential identities of some *but* gene paralogues. Many similar fatty acid-CoA transferases are easily confused for *but* genes. The 4-hydroxybutyryl-CoA:acetate-CoA transferases (4-*hbt*) are known to be similar; however, several researchers have been investigating genes that are more similar to *but* genes than 4-*hbt* genes and are required for full pathogenicity in *Yersinia pestis* and *Salmonella* species (50). These genes have been proposed to be itaconate-CoA transferases. They transfer a CoA group from succinyl-CoA onto itaconate, thus activating it and enabling its degradation into acetyl-CoA and pyruvate. Indeed, many entries in the FunGene database for *but*-like genes are from *Salmonella* and *Yersinia* genomes. These genes cluster more closely with functionally confirmed *but* genes than to the 4-*hbt* genes. It is likely that many other genes that closely resemble *but* genes act to move CoA moieties among various fatty acids.

Analyses of butyryl-CoA transferases in the animal intestinal ecosystem enable the study of a functional aspect of the gut microbiota and how it relates to health and disease. This research provides a tool to investigate functional butyrate transferases in the swine gut microbiota and could also be applied to other animals or other environmental samples, or it could be used to generate *but* amplicon data sets from metagenomic samples. This advances the analyses of the host-associated butyrate-producing community for enhancing swine health and improving food safety.

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