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Use of a Fluorescent Analog of Glucose (2-NBDG) To Identify Uncultured Rumen Bacteria That Take Up Glucose

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ABSTRACT Few characteristics are more important to a bacterium than the substrates it consumes. It is hard to identify what substrates are consumed by bacteria in natural communities, however, because most bacteria have not been cultured. In this study, we developed a method that uses fluorescent substrate analogs, cell sorting, and DNA sequencing to identify substrates taken up by bacteria. We deployed this method using 2[*N*-(7-nitrobenz-2-oxa-1,2-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG), a fluorescent glucose analog, and bacteria of the bovine rumen. This method revealed over 40 different bacteria (amplicon sequence variants [ASVs]) from the rumen that take up glucose. Nearly half of these ASVs represent previously uncultured bacteria. We attempted to grow these ASVs on agar media, and we confirmed that nearly two-thirds resisted culture. In coculture experiments, the fluorescent label of 2-NBDG was not transferred to nontarget bacteria by cross-feeding. Because it is not affected by cross-feeding, our method has an advantage over stable isotope probing. Though we focus on glucose, many substrates can be labeled with the fluorophore NBD. Our method represents a new paradigm for identifying substrates used by uncultured bacteria. It will help delineate the niche of bacteria in their environment.

IMPORTANCE We introduce a method for identifying what substrates are consumed by bacteria in natural communities. Our method offers significant improvement over existing methods for studying this characteristic. Our method uses a fluorescently labeled substrate which clearly labels target bacteria (glucose consumers in our case). Previous methods use isotope-labeled substrates, which are notorious for off-target labeling (due to cross-feeding of labeled metabolites). Our method can be deployed with a variety of substrates and microbial communities. It represents a major advance in connecting bacteria to the substrates they take up.

KEYWORDS DNA sequencing, bacteria, flow cytometry, rumen, substrates

Few characteristics are as important to a bacterium as the substrates it consumes. A bacterium that consumes only glucose, for example, cannot grow in an environment devoid of this substrate. Accordingly, when a bacterium is cultured in the laboratory, one of the first characteristics determined is which substrates it can consume. Many species of bacteria are differentiated by this characteristic (1, 2).

Despite its importance, this characteristic cannot be studied for most bacteria in natural communities—most bacteria have not been cultured (3). Several culture-independent methods have been developed to try to overcome this roadblock. Most methods provide a microbial community with an isotopically labeled substrate and then pinpoint which bacteria took up labeled substrate using microautoradiography (4, 5), secondary ion mass spectroscopy (6), or Raman spectroscopy (7). The identity of the labeled bacteria is established with fluorescently labeled nucleotide probes. In stable

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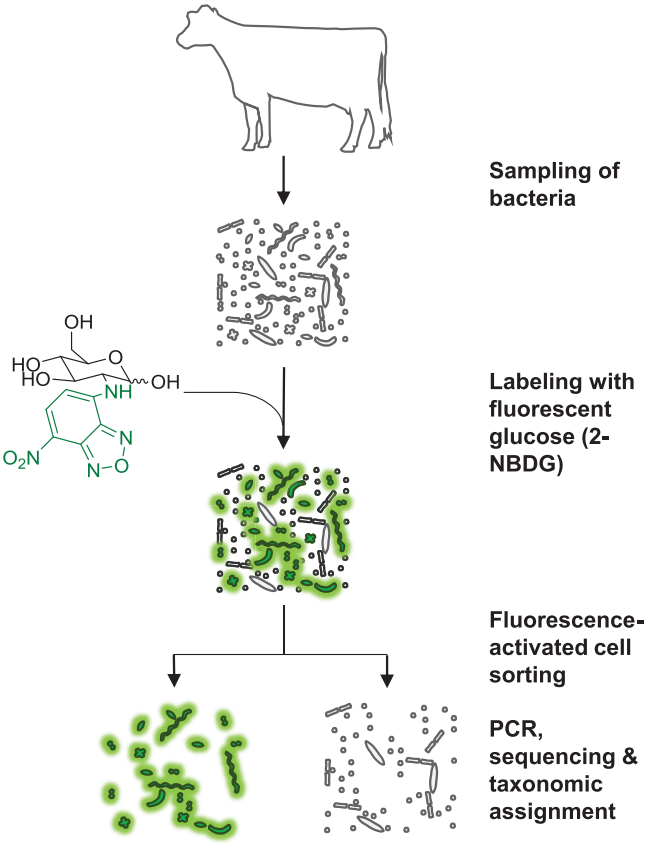

FIG 1 Outline of our method of identifying uncultured rumen bacteria that take up glucose with 2-NBDG.

isotope probing, bacteria assimilate label into nucleotides, and then nucleotides are separated and sequenced to establish identity (8).

A problem with isotope-based methods is that they can lead to off-target labeling of bacteria. Even if a bacterium did not take up a substrate directly, it can become labeled by taking up labeled metabolites released by another (target) bacterium. Because such cross-feeding is pervasive in microbial communities, these methods have low specificity and identify bacteria that are not true consumers of substrate (9, 10). This cross-feeding would be desirable only if the investigator wants to trace label through food chains (9, 10).

Other methods identify which bacteria take up a substrate by providing communities with a nonlabeled substrate and then detecting which cells become metabolically active. They may detect activity by (i) using probes for reductases (11) or (ii) tracking incorporation of labeled water (12) or amino acid analogs (13) into cell biomass. However, such activity is only a proxy for consuming substrate. Also, these methods have low specificity when bacteria become metabolically active by cross-feeding.

Genome sequencing is another culture-independent method. A bacterium's genome sequence can reveal which transporters and metabolic pathways exist for different substrates (14–16). This information can be used to infer which substrates are taken up. This method has pitfalls, however. Despite improved techniques (17, 18), genomes assembled from community DNA are often incomplete or contaminated or lack 16S rRNA genes (an important phylogenetic marker). Single-cell sequencing leads to genomes of low (~40%) completeness and is plagued by high (~65%) failure rates (19). Errors in annotation (20) further complicate use of genome sequencing.

Here we present an improved method for identifying substrates taken up by bacteria. This method uses fluorescently labeled analogs (Fig. 1). For proof of concept, we deploy the method with 2[*N*-(7-nitrobenz-2-oxa-1,2-diaxol-4-yl)amino]-2-deoxy-

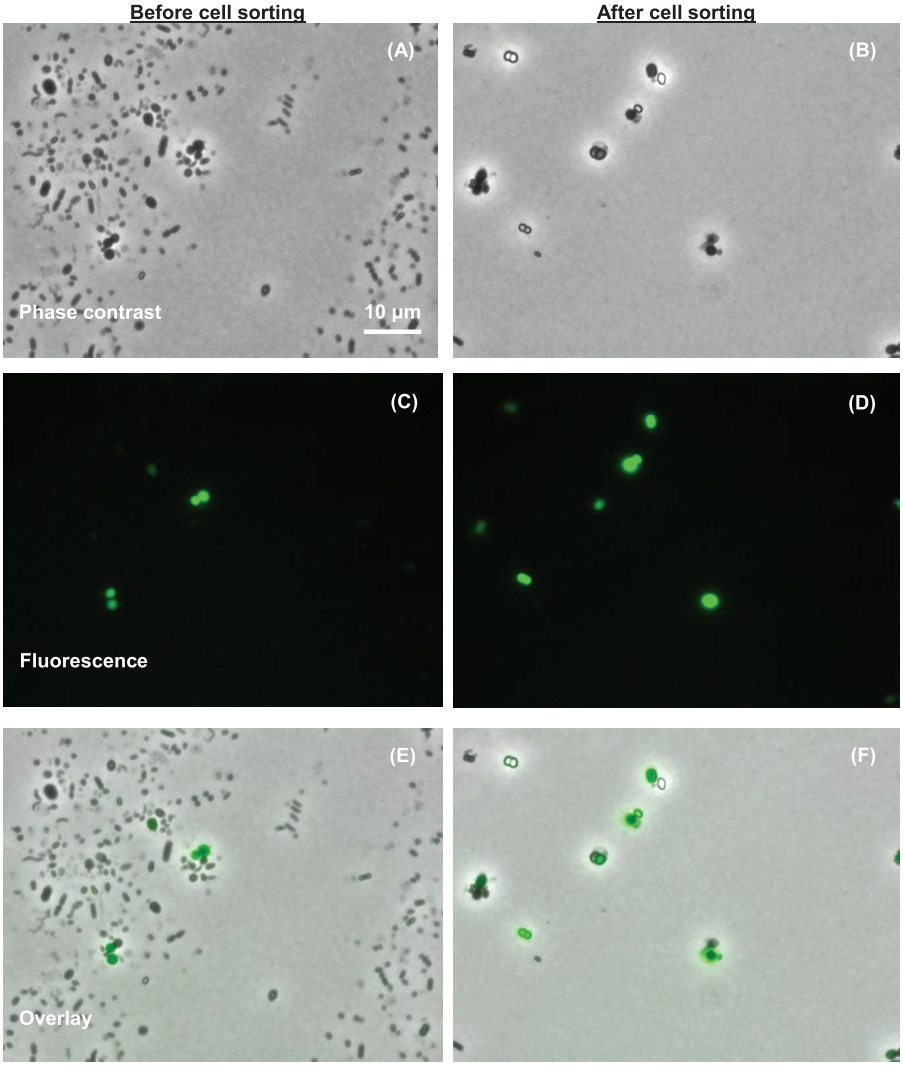

FIG 2 Microscopic image of mixed rumen bacteria incubated in 2-NBDG. Images show cells before cell sorting (A, C, and E) and after two sequential rounds of sorting (C, D, and F). Cells labeled with 2-NBDG are green. Images are from one of two independent experiments.

glucose (2-NBDG), a widely used analog of glucose (21, 22). Because 2-NBDG is degraded intracellularly to a nonfluorescent derivative (23), the method should not lead to the off-target labeling observed with isotope-based methods. With our method, we identified over 40 different bacteria from the bovine rumen that take up glucose. Several are from previously uncultured groups, including one (order RF39) that has been associated with efficiency of milk production.

RESULTS

Mixed rumen bacteria readily take up 2-NBDG. To determine if our method was feasible, we first determined if mixed rumen bacteria can take up 2-NBDG. We used both microscopy and flow cytometry for this purpose. Microscopy showed that a group of cells readily took up 2-NBDG and were labeled green (Fig. 2A, C, and E). Flow cytometry confirmed results with microscopy and showed that this group constituted ~10% of total cells (Fig. 3A). To verify that 2-NBDG was taken up by live cells only (not adsorbed onto dead cells), we examined uptake by cells fixed with formalin. No uptake by these cells was observed (see Fig. S1 in the supplemental material). The results showed that a live group of rumen bacteria can take up 2-NBDG.

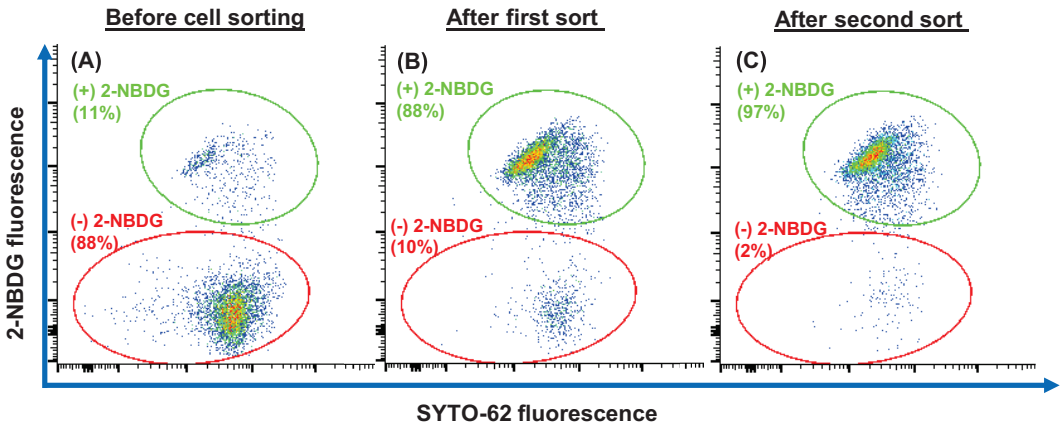

FIG 3 Flow cytometry histogram showing bacteria labeled with 2-NBDG and their separation by fluorescence activated cell sorting (FACS). Shown are cells before sorting (A), after one round of sorting (B), and after two rounds (C). Over 95% purity was achieved. Panels B and C present results of a postsort analysis. SYTO 62 was added to stain DNA. Histograms are from 1 of 15 independent experiments.

Sorting 2-NBDG-labeled cells by FACS achieves high purity. After we confirmed a group of rumen bacteria can be labeled with 2-NBDG, the next step of our method was to determine if cells labeled with 2-NBDG could be sorted out by fluorescence-activated cell sorting (FACS). We found that we could indeed separate cells this way, but postsort analysis showed that a single round of sorting achieved only 80% to 90% purity (Fig. 3B and Data Set S1).

We could achieve a much higher purity by subjecting cells from the first round of sorting to a second round (Fig. 3C and Data Set S1). Across experiments, postsort analysis showed purity of 94.9% (standard error of the mean [SEM], 0.3%) ($n = 30$) after this round. Microscopy confirmed that high purity was achieved (Fig. 2B, D, and F). In sum, after performing two rounds of cell sorting, 2-NBDG-labeled cells increased from 10% to 95%.

A unique group of bacteria was enriched during FACS. To determine if bacteria sorted out by FACS represented a unique group, we sequenced the 16S rRNA genes of sorted cells. Specifically, we sequenced of the V4 region and used DADA2 to identify amplicon sequence variants (ASVs). We also performed sequencing on the original (unsorted) samples. A total of 893 ASVs in sorted and 1,723 ASVs in unsorted samples were identified (Data Set S2). All ASVs, their sequences, and taxonomic assignments are reported in Data Set S3.

To determine if sorted and unsorted cells differed, we analyzed their diversity. When we measured β diversity using weighted UniFrac distances and visualized the data with principal-coordinate analysis (PCoA) plots, sorted and unsorted samples clustered apart ($P = 0.001$ [Fig. S2A]). When we measured α diversity using the phylogenetic diversity metric, we found that it was lower for sorted versus unsorted samples ($P < 0.001$ [Fig. S2B]). Together, these analyses reveal large differences between sorted and unsorted samples, indicating that a unique group of bacteria was enriched during FACS.

Several different bacteria can be identified as taking up 2-NBDG. In the last step of our method, we formally identified the ASVs that take up 2-NBDG. Though 893 ASVs were identified in FACS-sorted cells, many were at low abundance (Data Set S2). These ASVs may represent cells that do not take up 2-NBDG but nonetheless were detected in sorted cells because of <100% purity during sorting. To identify which bacteria truly took up 2-NBDG, we used a Wilcoxon signed-rank test to determine which had a relative abundance higher ($P < 0.05$) than 0. Importantly, we deployed this test in a way to account for <100% purity during sorting (see Materials and Methods).

Our method revealed 43 ASVs that took up 2-NBDG (Fig. S3). Among the most abundant ASVs were those assigned to *Pseudobutyrivibrio*, *Succinivibrio*, and *Streptococcus* (Fig. 4), which are well-known cultured groups in the rumen (24). Other

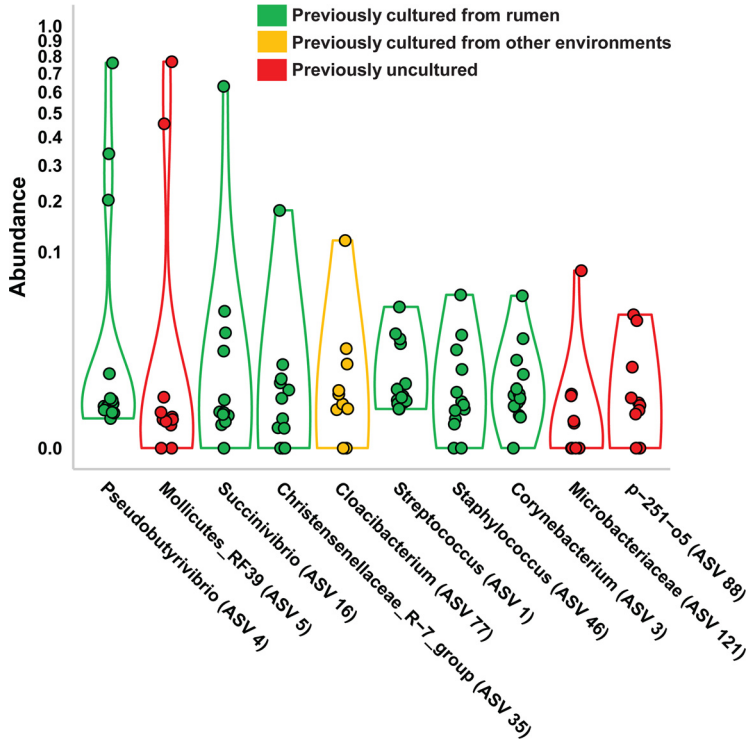

FIG 4 Violin plot of the 10 most abundant bacteria that take up 2-NBDG. A single-sided Wilcoxon signed-rank test was performed to test if abundance was >0 after accounting for impurity from unsorted samples. Each observation represents 1 of 15 independent experiments. See Fig. S3 for the full list of bacteria.

abundant ASVs were assigned to less well-known groups, such as RF39 and *Cloacibacterium*. Members of these less well-known groups might represent uncultured bacteria.

Our method identified some bacteria that resist culture. To determine if ASVs that took up 2-NBDG are related to cultured bacteria, we compared their sequences to those of cultured strains in the Hungate 1000 collection and SILVA database (Data Set S4). Out of all ASVs ($n = 43$), we found one-fifth ($n = 9$) were closely related to cultured rumen bacteria. They matched, at 100% sequence identity, strains from the Hungate 1000 collection. A match at 100% identity suggests that they belong to the same species as these cultured strains (25). An additional one-third of ASVs ($n = 15$) were closely related to bacteria from other environments; they exactly matched strains in the SILVA database. One half of ASVs ($n = 19$) were not closely related to bacteria from the rumen or from other environments. These may represent bacteria that are hard to culture.

To confirm that some ASVs resist culture, we performed our own culture experiments. We grew dense cultures of rumen bacteria on media containing glucose, rumen fluid, and agar (RGA) or rumen fluid and agar (RA) alone. After colonies grew, we harvested and sequenced them *en masse*. Our method revealed 385 ASVs that grew on RGA medium and 424 ASVs that grew on RA medium (Data Set S2). We compared these ASVs with the 43 that took up 2-NBDG.

Several ASVs that took up 2-NBDG did not match those that grew on our media, or they were found only at a low abundance. Nearly two-thirds that took up 2-NBDG ($n = 27$) had a mean abundance of <0.1% on both RGA and RA media (Fig. S3). Nearly one-third ($n = 14$) had <0.001% abundance (Fig. S3). This supports the assertion that our method had identified bacteria that resist culture, at least on the RGA and RA media used.

Our method identified other bacteria that are easily culturable. Though our method identified bacteria that resist culture, others could be cultured at high abun-

dance. Of the 10 most abundant ASVs that grew on RGA media, 7 were also identified as those taking up 2-NBDG in FACS-sorted cells (Fig. S4A). Similarly, 6 of the top 10 ASVs that grew RA medium also took up 2-NBDG (Fig. S4B). This supports the notion that our method with 2-NBDG can identify predominant, cultured bacteria.

Our intention in using RGA was to grow glucose-consuming bacteria, but ASVs that grew on RGA and RA were similar. This is shown by analysis of both α and β diversities (Fig. S2). Thus, our methods identify bacteria that are readily cultured, but they cannot identify glucose-consuming bacteria specifically.

2-NBDG is not subject to cross-feeding between rumen bacteria. Our method assumes that 2-NBDG labels only those cells that take up the compound. This assumption would be violated if the fluorescent group were transferred from one bacterium to another in a process known as cross-feeding. Cross-feeding occurs when a metabolite released by one bacterium is taken up by another. It is common in the rumen and other microbial communities (26–28).

To test if there is cross-feeding of 2-NBDG, we conducted coculture experiments with two bacteria which form a cross-feeding relationship. *Streptococcus equinus* JB1, a coccus, takes up glucose and releases lactate (29). *Anaerovibrio lipolyticus* 5S, a crescent, takes up lactate (but not glucose [29]). As expected, *Streptococcus bovis* JB1 took up 2-NBDG in cocultures, as shown by green labeling of cocci under the microscope (Fig. 5). Additionally, *A. lipolyticus* 5S remained unlabeled, as shown by the absence of green labeling of crescents. This result shows that there is no transfer of the fluorescent group in 2-NBDG by cross-feeding. Labeling by 2-NBDG should be specific to those bacteria that first took up the compound.

DISCUSSION

Using a fluorescent glucose analog (2-NBDG) and rumen bacteria, we demonstrated how fluorescently labeled analogs can identify the substrates taken up by bacteria in a natural community. With this novel method, we identified over 40 different bacteria (ASVs) from the rumen that take up glucose. Several ASVs belong to groups resistant to culture in our study and previous ones. Our method can be immediately deployed with other substrates and microbial communities, and thus, it represents a key advance in connecting bacteria to the substrates they take up.

Our method identifies substrates taken up by bacteria with high specificity. By using a fluorescently labeled substrate analog like 2-NBDG, our method can identify substrates used by bacteria with higher specificity than previous methods. Methods that use isotopically labeled substrates, such as stable isotope probing, are not specific to bacteria of interest when cross-feeding occurs (9, 10). One bacterium may become labeled by feeding off the labeled metabolites of another bacterium, leading to false identification. According to Dumont and Murrell (10), cross-feeding "will always remain a potential problem" for these methods.

The fluorescent analog 2-NBDG that we used is not subject to cross-feeding. In *Eshcerichia coli*, 2-NBDG is degraded to a nonfluorescent derivative intracellularly (23), which should make cross-feeding impossible. Our coculture experiments confirm that the analog specifically labels a glucose-consuming bacterium (*S. equinus* JB1), and it does not label a second bacterium (*A. lipolyticus* 5S) that feeds off fermentation products released by the first. These results, along with work identifying glucose transporters responsible for 2-NBDG uptake (22), support the notion that our method is specific for bacteria that take up glucose.

The price paid for high specificity is low sensitivity—2-NBDG is not taken up by all glucose-consuming bacteria. When applied to the rumen community, our method identified only 7 out of the 10 most abundant bacteria (ASVs) that grew on glucose-containing media. Further, only 10% of cells took up 2-NBDG, whereas previous studies have found that $>$40% of cells grew on glucose-containing media (30, 31).

Using more glucose analogs may overcome this low sensitivity. 2-NBDG is compatible with only certain glucose transporters of bacteria. Specifically, it is taken up only by transporters that tolerate modification (addition of the NBD fluorophore) to the C-2

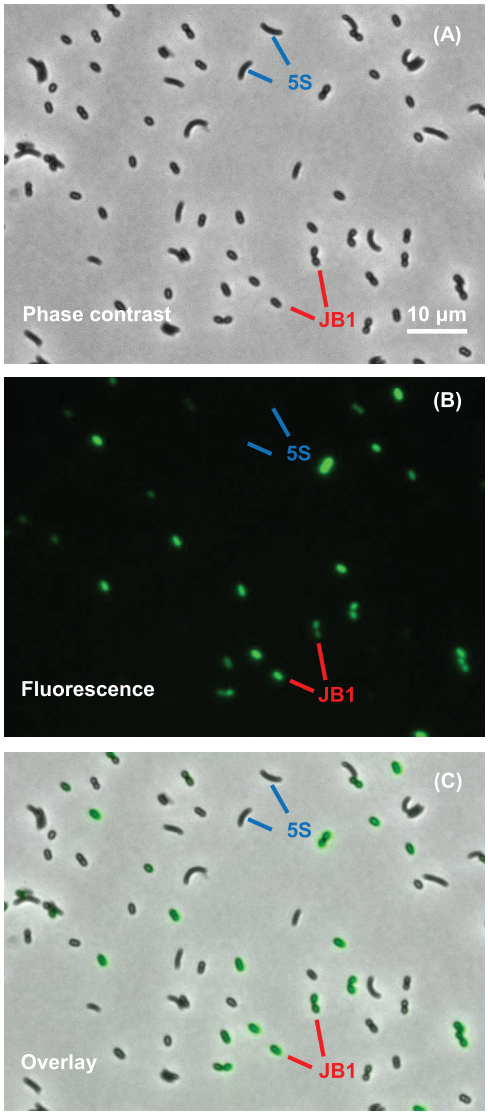

FIG 5 Microscope image that shows absence of cross-feeding of 2-NBDG between *Streptococcus equinus* JB1 (coccus) and *Anaerovibrio lipolyticus* 5S (crescent). Cocultures of *S. equinus* JB1 and *A. lipolyticus* 5S were given 2-NBDG (5 mM). *S. equinus* JB1 takes up 2-NBDG and is labeled green, whereas *A. lipolyticus* 5S remains unlabeled; the fluorescent group in 2-NBDG is not transferred from the former to the latter. Images are from one of two independent experiments.

position of glucose (22). However, other analogs are compatible with other transporters. 1-NBDG, an isomer of 2-NBDG, is compatible with transporters that tolerate modification to the C-1 position (32). Using isomers like 1-NBDG could increase the number of different bacteria our method identifies.

2-NBDG is highly specific in more than one way. It is specific not only for bacteria that take up glucose but also for the process of glucose transport itself. 2-NBDG is phosphorylated when transported into the cell (22, 23), and then it is degraded to a nonfluorescent derivative thereafter (23). As a result, its further metabolism cannot be traced. In other words, 2-NBDG is a useful tracer for glucose transport, not for glucose metabolism.

Our method identifies important bacteria resistant to culture. Of the 43 different bacteria (ASVs) that our method identified, 2 belonging to order RF39 may be especially important. No member of this order has been previously cultured from any environment, and the two bacteria each grew at $<$0.05% mean abundance on RGA and RA media in our own study. Despite their recalcitrance to culture, members of RF39 are

TABLE 1 Diet fed to rumen fluid donor cows

Parameter	Alfalfa hay	Concentrate supplement[a,b]
Amt fed (kg of dry matter/day)	8.3	5.8
Composition (g/100 g of dry matter)		
Organic matter	90.7	92.2
Neutral detergent fiber[c]	37.4	18.5
Acid detergent fiber	31.7	12.9
Crude protein	18.8	15.2
Starch	1.5	39.7
Ether extract	2.3	4.5

[a]Shiloh finisher, Corbett's feed.
[b]Ingredients included corn, molasses, soybean meal or cottonseed hulls, vitamins, minerals, and fat (inclusion rates of ingredients are proprietary).
[c]Analyzed with heat-stable amylase and expressed exclusive of residual ash.

likely important in the rumen and have been associated with efficiency of milk production by the cow (33, 34). Our method begins to unravel the role of this order in the rumen by showing that some members can take up glucose.

Fourteen other bacteria identified by our method resisted culture to an even greater extent, and they had a mean abundance of $<$0.001%. If this abundance were proportional to the number of colonies growing on plates, one would need to screen 10^5 colonies to obtain a single representative of these bacteria. The largest culture efforts for gut habitats have screened 10^3 to 10^5 colonies (35–37). It is not surprising, therefore, that none of these 14 bacteria matched the Hungate 1000, a collection of 501 sequenced rumen bacteria (38). This illustrates that our method can identify substrates taken up by bacteria that defy identification using culture.

Though several bacteria identified by our method resisted culture, others matched well-cultured groups. For example, our method identified bacteria belonging to *Pseudobutyrivibrio*, *Succinivibrio*, and *Streptococcus*. Species from these groups were well cultured in our study and previous ones, and they are well known in the rumen for consuming glucose and other sugars (24, 29, 39). Finding them helps validate our method.

Many substrates are compatible with our method. Our current work focused on a single glucose analog, 2-NBDG, for proof of concept, but our method can be deployed with other substrates analogs. The fluorescent group NBD has been used to label amino acids (40), lipids (41), and other sugars (42). Any substrate derivative with an amine can be labeled (40, 43). Additional work would need to verify that, like 2-NBDG, these substrates are specific to bacteria of interest and not subject to cross-feeding.

Conclusion. Our method was able to identify several uncultured bacteria from a natural community as capable of taking up glucose. Because it used a fluorescently labeled substrate, not an isotopically labeled one, our method was specific to bacteria of interest and not affected by cross-feeding. This gives the method considerable strength over stable isotope probing and other isotope-based methods. Because fluorescently labeled substrates are readily prepared, our method represents a crucial step in (i) linking uncultured bacteria with the substrates they take up and (ii) unraveling the role of such bacteria in the environment.

MATERIALS AND METHODS

Collection of rumen fluid. Rumen fluid was collected from three dry Holstein cows of approximately 750 kg (live weight). Animals were housed at the animal physiology unit at University of Florida in Gainesville, FL. The University of Florida Institutional Animal Care and Use Committee approved all animal procedures. Diet information is in Table 1.

Labeling of mixed rumen bacteria with 2-NBDG and SYTO 62. Mixed rumen bacteria were prepared from rumen fluid following our procedure of differential centrifugation (44). Briefly, rumen contents were strained through 4 layers of cheesecloth, protozoa and small feed particles were removed by centrifugation at 1,000 $\times$ *g* for 5 min at 4°C (F15-8x50cy rotor and Sorvall Legend XTR centrifuge; Thermo Fisher Scientific), and then bacteria were isolated by centrifugation at 10,000 $\times$ *g* for 10 min at 4°C. Bacteria were anaerobically resuspended and diluted to 1 g of protein liter^{-1} in N-free buffer (modified Simplex type [22]).

After isolation, mixed bacteria were incubated in 2-NBDG. A 1-ml aliquot of mixed bacteria was transferred anaerobically to a culture tube and prewarmed for 10 min at 39°C. After prewarming, 2-NBDG (Cayman Chemical) was added to a final concentration of 100 μM. Incubation continued at 39°C for 5 min. After incubation, cells were harvested anaerobically by centrifugation, washed, and resuspended in 1 ml of N-free buffer. The resuspended bacteria were filtered anaerobically through a nylon cloth (100-μm pore size) to remove any remaining cell clumps or feed particles. In control experiments, cells were killed (fixed with 10% formalin) before incubation in 2-NBDG.

To stain nucleic acids, SYTO 62 dye (Thermo Fisher Scientific) was added to mixed bacteria at a 5 μM final concentration. Incubation continued for at least 1 h on ice to maximize staining.

Flow cytometry and cell sorting. Mixed bacteria prepared above were sorted using a Sony SH800 cell sorter (Sony Biotechnology). Data analysis was carried out with FlowJo software (FlowJo LLC).

To increase purity, bacteria were subjected to two sequential rounds of sorting, as previously done with mammalian cells (45–47). Approximately 300,000 events were collected during the first round. Cells were sorted at a rate of 3,000 events/s. After cleaning the instrument with distilled and deionized H_2O for a 3-min wash cycle, cells from the first round were subjected to a second round of sorting. Approximately 70,000 events were collected during the second round at a rate of 200/s. Temperature was set at 5°C for both sample collection and the cell sorting chamber. Sorting was completed within 30 min.

The mode chosen for both rounds of sorting was semipurity. Preliminary experiments showed no improvement in purity when sorting under stricter modes (ultrapurity and single cell).

After sorting, bacteria were harvested by centrifugation (10,000 $\times$ *g* for 10 min at 4°C) and resuspended in irradiated ADS buffer. Unsorted cells were harvested the same way as a control. The ADS buffer (24, 48) contained 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of NaCl, 0.9 g of $(NH_4)_2SO_4$, 90 mg of $MgSO_4$, 0.119 g of $CaCl_2{\cdot}2H_2O$, 3 g of Na_2CO_3, and 0.5 g of cysteine hydrochloride per liter. To eliminate DNA contamination, buffer was irradiated in a multilinker (UVC-515UV; Ultra-Lum Inc.) for 80 min (49). Irradiation was done in Balch tubes (178 by 150 mm).

All procedures were done for 5 days on each of 3 cows ($n = 15$ independent experiments in total).

Flow cytometry under aerobic versus anaerobic conditions. We performed FACS under aerobic conditions, yet most rumen bacteria are anaerobic. In preliminary experiments, we determined if this was a problem by measuring the stability of 2-NBDG under aerobic versus anaerobic conditions. Using an Accuri C6 flow cytometer (Accuri Cytometers Inc.), we found that samples showed only a slight decrease (5%/h) in fluorescence after being exposed to air for 2 h (Fig. S5). Further, samples showed a similar decrease when kept under strictly anaerobic conditions (continuous gassing with CO_2) (Fig. S5). Because FACS was completed within 30 min, these experiments support the assertion that aerobic conditions were not deleterious to sorting.

We used an Accuri C6 for these experiments because the sample tube is left open to the environment, permitting a gassing needle to be inserted into it. These experiments were not possible with the Sony SH800 because the sample is kept behind a plastic shield.

Culture of mixed rumen bacteria on RGA and RA media. Rumen bacteria were cultured using media containing rumen fluid, glucose, and agar (RGA) or rumen fluid and agar alone (RA) (31). RGA medium contained 400 ml of clarified rumen fluid (see preparation below), 1.5 g of glucose, 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of NaCl, 0.9 g of $(NH_4)_2SO_4$, 90 mg of $MgSO_4$, 0.119 mg of $CaCl_2{\cdot}2H_2O$, 3 g of Na_2CO_3, and 0.5 g of cysteine hydrochloride per liter.

Rumen fluid was collected from cows, and a 10^{-3} dilution in irradiated ADS buffer was prepared. An aliquot of the dilution (0.1 ml) was ejected onto anaerobic bottle plates (50) containing 9 ml of medium. Rumen fluid was spread using 3-mm glass beads autoclaved with the plates. After incubation at 39°C for 7 days, colonies were harvested *en masse* with a cell scraper (51) into 4 ml of irradiated ADS buffer.

Clarified rumen fluid was supernatant from centrifuging rumen fluid at 1,000 $\times$ *g* for 10 min. It was incubated anaerobically at 39°C for 7 days to exhaust carbon and energy sources present in the rumen fluid (31).

All procedures were done on 4 days for each of 3 cows ($n = 12$ independent experiments in total). On each day, 4 bottle plates were prepared for each medium. Due to the intensity of the procedures, days for anaerobic culture were different from those for cell sorting.

Extraction and amplification of DNA. Bacterial cells from cell sorting or culture were subjected to DNA extraction and amplification with the REPLI-g single cell kit (Qiagen). The kit was used according to the manufacturer's instructions. Briefly, 10^2 to 10^5 cells in a 2-μl volume were lysed by incubation in alkaline buffer, and then DNA was then amplified with Φ29 polymerase. Amplification was performed isothermally in a thermal cycler (CFX96; Bio-Rad) for 8 h. To monitor the amplification, 0.5$\times$ SYBR green (Fisher Scientific) was also included in the reaction mixture. All procedures before quantitative PCR (qPCR) were done in a biological safety cabinet to minimize environmental contamination. Whole-genome amplified DNA was stored at -20°C.

For some samples, DNA extraction was also performed with the DNeasy PowerSoil kit (Qiagen). A comparison between PowerSoil and REPLI-g kits is shown in Fig. S6. The PowerSoil kit was used for this preliminary comparison only because it was impractical to use with sorted cells. The kit required 10^7 cells for PCR amplification of the V4 region (data not shown), and 10 h of sorting would be needed to collect this number of cells.

Sequencing of 16S rRNA genes and bioinformatic analysis. DNA prepared as described above was PCR amplified and then sequenced on the Illumina MiSeq platform. All procedures were performed by Molecular Research LP.

The V4 region of 16S rRNA gene was PCR amplified using the forward primer 515FB (GTGYCAGCM GCCGCGGTAA) and reverse primer 806RB (GGACTACNVGGGTWTCTAAT) (52). The forward primer included an 8-bp barcode for identification. A 30-cycle PCR was performed using the HotStarTaq Plus master mix kit (Qiagen). Amplification included the initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. Multiple samples were pooled in equal proportions based on the molecular weight and DNA concentration. Pooled samples were purified with calibrated AMPure XP bead kit (Beckman Coulter). Amplicons were sequenced with the Illumina MiSeq v.3 platform, generating 250-bp paired-end reads. Details on samples are found in Data Set S2.

After demultiplexing, sequence reads were clustered into amplicon sequence variants (ASVs) with the DADA2 package (version 1.8.0) (53). The steps of the DADA2 pipeline include error filtering, trimming, learning of error rates, denoising, merging of paired reads, and removal of chimeras. An average of 10.2% demultiplexed reads were removed during error filtering and other steps. During trimming, the forward and reverse reads were truncated at positions 220 and 180 to remove low-quality tails. During learning of error rates, the nbases parameter was set to 2e+08 to include more data and improve fit of the error model.

Taxonomic assignment of ASVs was done to the genus level using a naive Bayesian classifier (54) implemented in DADA2 with SILVA reference database (version 132) (55).

The ASV table generated by DADA2 was imported into QIIME pipeline for α and β diversity analyses (56). Phylogenetic trees were generated with aligned sequencing by using the qiime phylogeny fasttree and qiime phylogeny midpoint-root script. α and β diversity analyses were performed by using the qiime diversity core-metrics-phylogenetic script.

Matches between sequences of ASVs and previously cultured bacterial strains were determined using VSEARCH (57). The Hungate 1000 collection (38) was the source of sequences for cultured bacteria from the rumen. SILVA (55) was the source for cultured bacteria from all environments. Sequences from SILVA were selected by searching for "[T]" and "s[C]" in the organism field of the online database. The percent identity between ASVs and cultured bacteria was determined with the usearch_global command of VSEARCH (57).

Preparation of cocultures of rumen bacteria. Additional experiments were performed with a coculture of the rumen bacteria *Streptococcus equinus* JB1 and *Anaerovibrio lipolyticus* 5S. Strains were obtained from the ATCC.

Strains were grown anaerobically under O_2-free CO_2, in Balch tubes with butyl rubber stoppers at 39°C. *S. equinus* JB1 was grown with PC-VFA medium as described by Tao et al. (22).

A. lipolyticus 5S was grown with Prins semidefined medium. This medium contained 2 ml of glycerol, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 1 g of NaCl, 50 mg of $MgSO_4$, 100 mg of $CaCl_2{\cdot}2H_2O$, 3.3 g of $NaHCO_3$, 10 g of Trypticase peptone (product number 211921; BD), 10 g of Bacto yeast extract (product number 212750; BD), and 0.5 g of cysteine hydrochloride per liter (58).

Cultures were grown in at least quadruplicate to mid-exponential phase and then pooled. Cells were harvested the same way as for mixed rumen bacteria, except they were washed with N-free buffer twice. They were then filtered and incubated in 2-NBDG as for mixed rumen bacteria.

Microscopy and image analysis. Mixed rumen bacteria and pure cultures of rumen bacteria were imaged by epifluorescence microscopy using an Axio Scope.A1 microscope (Zeiss, Munich, Germany), 10× (A-Plan; numerical aperture, 0.25) or 100× oil (A-Plan; numerical aperture,1.25) objectives, 470 ± 40/525 ± 50nm band-pass green fluorescent protein filter, 605 ± 50/670 ± 50 nm band-pass Alexa Fluor 633 filter, and an AxioCam CM1 camera. Samples were prepared as wet mounts on a thin layer of 2% Noble agar. ZEISS ZEN was used for image acquisition. ImageJ (NIH) and Photoshop (Adobe) were used to linearly adjust brightness and contrast as well as to perform channel overlay. The adjustment was applied to whole images.

Statistical analysis. For Fig. 4 and S4, we determined which bacteria took up 2-NBDG by using a Wilcoxon signed-rank test. Specifically, we used this test to determine which bacteria had relative a abundance of >0 after accounting for the <100% purity achieved during cell sorting. The null (H0) hypothesis was

$$H0 = \frac{\text{relative abundance of ASVs in unsorted cells}}{100 - \text{percent positive before sorting}} \times \left(100 - \text{percent positive after 2nd round of sorting}\right)$$

The right-hand side of the equation is the relative abundance of an ASV expected when it belongs to the group negative for 2-NBDG fluorescence. It is 0 when purity (percent positive after 2nd round of sorting) is 100% and >0 when purity is <100%. Values for relative abundance of ASV sorted and unsorted cells are found in Data Set S2. Values for percent positive before sorting and percent after 2nd round of sorting are found in Data Set S1. The test was paired because we compared relative abundance for samples collected on the same date.

For Fig. S2 and S6, we determined if the phylogenetic diversity metric differed across treatments using a pairwise Kruskal-Wallis test at the highest rarefaction depth. Additionally, we determined if the within-treatment UniFrac distances and between-treatment UniFrac distances differ with a pairwise permutational multivariate analysis of variance (PERMANOVA; no Bonferroni correction applied).

Data availability. Sequence data are available at the NCBI database under BioProject accession number PRJNA484386.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03018-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.6 MB.

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