Diet Induced Changes in the Colonic Environment and Colorectal Cancer

Molecular Ecological Analysis of the Gastrointestinal Microbiota: A Review[1]

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ABSTRACT The gastrointestinal (GI) microbiota of mammals is characterized by its high population density, wide diversity and complexity of interactions. While all major groups of microbes are represented, bacteria predominate. Importantly, bacterial cells outnumber animal (host) cells by a factor of ten and have a profound influence on nutritional, physiological and immunological processes in the host animal. Our knowledge of the molecular and cellular bases of host-microbe interactions is limited, though critically needed to determine if and how the GI microbiota contributes to various enteric disorders in humans and animals. Traditionally, GI bacteria have been studied via cultivation-based techniques, which are labor intensive and require previous knowledge of individual nutritional and growth requirements. Recently, findings from culture-based methods have been supplemented with molecular ecology techniques that are based on the 16S rRNA gene. These techniques enable characterization and quantification of the microbiota, while also providing a classification scheme to predict phylogenetic relationships. The choice of a particular molecular-based approach depends on the questions being addressed. Clone libraries can be sequenced to identify the composition of the microbiota, often to the species level. Microbial community structure can be analyzed via fingerprinting techniques, while dot blot hybridization or fluorescent in situ hybridization can measure abundance of particular taxa. Emerging approaches, such as those based on functional genes and their expression and the combined use of stable isotopes and biomarkers, are being developed and optimized to study metabolic activities of groups or individual organisms in situ. Here, a critical summary is provided of current molecular ecological approaches for studying the GI microbiota. J. Nutr. 134: 465–472, 2004.

KEY WORDS: • *gastrointestinal microbiota* • *microbial ecology* • *16S rDNA* • *molecular techniques*

Bacterial populations as high as 10^{10} to 10^{11}/g contents belonging to as many as 400 different species are found in the hindgut of mammalian species including humans (1,2). Bacterial cells, predominantly anaerobes, outnumber animal cells by a factor of 10 and have a profound influence on immunological, nutritional, and physiological processes in the host (1).

The study of gastrointestinal (GI)[3] microbial ecology (3) involves investigation of the organisms present (abundance and diversity), their activity (usually determined in vitro, but ideally in vivo activity should be measured), and their relationship with each other and the host animal (synergistic and competitive interactions). The technological impetus for major advances in our knowledge of GI microbial ecology during the last 40 y has been derived from three major sources: 1) the development of anaerobic culture techniques; 2) the use of experimental rodent models to define relationships between intestinal bacteria and the host; and 3) the development of gnotobiotic technology by which germfree or defined-microbiota animal models could be derived and maintained (4).

While the estimate of cultivability of GI bacteria is relatively high (10–50%) compared to most microbial ecosystems (5,6), the culturable fraction is still a minority. The reasons for this cultivation anomaly include the unknown growth requirements of the bacteria, the selectivity of the media that are used, the stress imposed by the cultivation procedures, the

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[3] Abbreviations used: BAC, bacterial artificial chromosome; cfu, colony forming units; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent in situ hybridization; GI, gastrointestinal; IVET, in vivo expression technology; MPN, most probable number; OTU, operational taxonomic unit; rRNA, ribosomal RNA; SSCP, single strand conformation polymorphism; SSU, small subunit; TGGE; temperature gradient gel electrophoresis; T-RFLP, terminal-restriction fragment length polymorphism; TTGE, temporal temperature gradient gel electrophoresis.

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necessity of strictly anoxic conditions, and difficulties with simulating the interactions of bacteria with other microbes and host cells. The circumvention of these limitations requires culture-independent methods **(Fig. 1)**. A dramatic increase in the application of approaches based on the sequence diversity of the 16S ribosomal RNA (rRNA) gene have been made during the past decade to explore the diversity of bacterial communities in a variety of ecosystems, including the mammalian GI tract (6). Sequence comparisons of nucleic acids isolated from complex microbial ecosystems can be used to provide molecular characterization, while at the same time providing a classification system, which predicts natural evolutionary relationships (8,9). As such, the field of molecular microbial ecology is defined as the application of molecular technology, typically based on comparative nucleic acid sequence information, to identify specific microorganisms in a particular environment, to assign functional roles to these microorganisms, and to assess their significance or contribution to environmental processes. The current review provides a critical summary of the application of molecular techniques for studying the GI microbiota and discusses future directions.

Transition from cultivation to molecular analysis

To obtain an estimate of cultivability, microbial ecologists generally compare microscopic counts with total viable counts. The total viable count made on a nonselective agar-based medium estimates the number of colony forming units (cfu) per gram of sample. The finding that total viable counts are typically lower than total microscopic counts was thought to be due to the number of dead cells. Indeed, dead bacteria in feces may constitute up to one third of the total bacterial community (10). However, recent nucleic acid based studies indicated that a majority of bacteria in a variety of ecosystems are different from those described in culture. Therefore, it is reasonable to assume that the differences between microscopic and total viable counts are also due to an inability to culture the majority of the bacteria. This has led to an extensive development and application of culture-independent approaches to study complex microbial ecosystems.

Sequencing of SSU rDNA clone libraries

The construction of small subunit (SSU) rRNA libraries is required to inventory bacteria and archaea present in a given environment. In fact, sequencing of SSU rRNA genes has become a standard procedure in the identification of isolates, and it is now impossible to adequately describe microbial communities without SSU rRNA sequence data. Currently, >79,000 16S rRNA sequences are available in DNA databases, which is far greater than for any other gene (http://rdp.cme.msu.edu/html). Ribosomal RNA sequences can be obtained either directly from SSU rRNA or from their encoding genes (SSU rDNA) by reverse transcription (RT)- or regular PCR. In practice, SSU rDNA sequences are determined by creating rDNA clone libraries rather than cDNA libraries from rRNA. After library construction, the sequences of the cloned amplicons are determined and compared to sequences deposited in SSU rDNA databases (http://www.ncbi. nlm.nih.gov/BLAST/ and http://rdp.cme.msu.edu/html), and followed by phylogenetic analysis (11–13). Sequencing of SSU rDNA clone libraries from human feces (14–16), colonic and ileal samples (17,18) and the oral cavity (19,20) have confirmed that a significant fraction of resident bacteria have not been described previously. Similar results have been reported for several GI tract segements in a variety of animal species (21–26). Interestingly, most of the novel sequences from GI tract studies grouped in the low G+C Gram positive phylum, indicating that this group is particularly underrepresented by cultivation procedures. It is also important to estimate how much of the actual diversity is represented in SSU rDNA clone libraries. This diversity estimate is dependent on how the operational taxonomic units (OTUs) are defined. Unfortunately, OTU determination is not standardized with thresholds for sequence differences within OTUs varying from 1 to 5%, which makes statistical comparisons between clone libraries difficult (27).

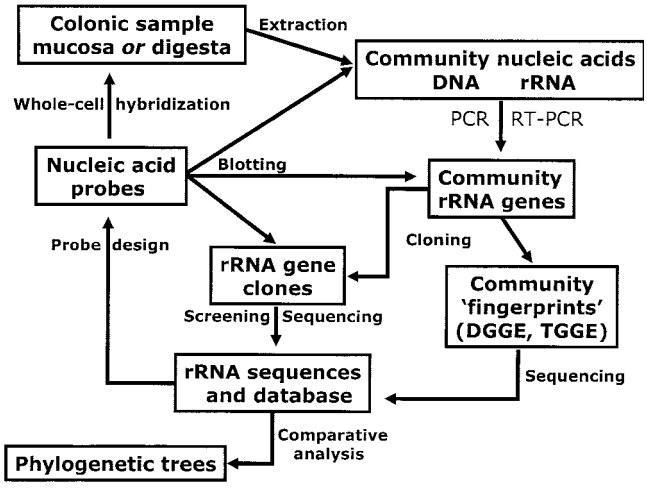

FIGURE 1 Current molecular methods used singularly or in combination to analyze complex microbial ecosystems. Modified from (5,7).

Traditionally, bacteria have been classified on the basis of phenotypic properties and only after the availability of nucleic acid based technology have SSU rDNA sequences been recognized as a standard phylogenetic classification tool in the description of bacterial strains (28). While large numbers of cloned SSU rDNA sequences from the GI tract of a variety of animal species (14–26) have been deposited in DNA databases, few examples exist of direct comparisons between SSU rDNA sequences retrieved from cloned amplicons and colony forming units from culturable GI bacteria. Also, SSU rDNA sequences from only a small fraction of all bacterial isolates have been deposited in DNA databases. In addition, novel cultivation strategies focusing for example on butyrate-producing or cellobiose-degrading bacteria have demonstrated recently that previously unknown bacteria can be isolated (29–31). An accurate estimate of the cultivability of GI bacteria will only be forthcoming after these discrepancies are resolved.

Although sequencing of cloned SSU rDNA amplicons gives significant information about the identity of uncultured bacteria, the data are not quantitative and PCR and cloning steps are not without biases (**Table 1**; 25, 32). Comparative analysis of SSU rDNA clone libraries from several GI tract studies indicated that the number of PCR cycles should be minimized, based on the estimated diversity represented by the libraries (14,21,33), and several steps to minimize PCR bias have been suggested (32,34). Nonetheless, the SSU rDNA clone libraries sequenced to date clearly demonstrate their utility in the quest to better understand the diversity of the GI microbiota.

SSU rDNA fingerprinting

Labor and expense make cloning and sequencing of SSU rDNA sequences unsuitable for monitoring communities in a

TABLE 1

A summary of current techniques used to study complex microbial ecosystems

Methods	Uses	Limitations
Cultivation	Isolation; "the ideal"	Not representative; slow & laborious
16S rDNA sequencing	Phylogenetic Identification	Laborious; subject to PCR biases
DGGE/TGGE/TTGE	Monitoring of community/population shifts; rapid comparative analysis	Subject to PCR biases; Semi-quantitative; identification requires clone library
T-RFLP	Monitoring of community shifts; rapid comparative analysis; very sensitive; potential for high throughput	Subject to PCR biases; semi-quantitative; identification requires clone library
SSCP	Monitoring of community/population shifts; rapid comparative analysis	Subject to PCR biases; semi-quantitative; identification requires clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires sequence information; laborious at species level
Dot-blot hybridization	Detection; estimates relative abundance	Requires sequence information; laborious at species level
Quantitative PCR	Detection; estimates relative abundance	Laborious
Diversity microarrays	Detection; estimates relative abundance	In early stages of development; expensive
Non-16S rRNA profiling	Monitoring of community shifts; rapid comparative analysis	Identification requires additional 16S rRNA-based approaches

culture-independent manner. However, several so-called fingerprinting techniques have been used to study bacterial communities and appear to be ideal for monitoring community shifts and comparing communities between GI sites and among animals. Denaturing gradient gel electrophoresis (DGGE) was first applied in microbial ecology to study bacterial diversity in a marine ecosystem (35). Since this pioneering study, a variety of microbial ecosystems have been analyzed using DGGE or similar techniques, including temperature gradient gel electrophoresis (TGGE), and only occasionally temporal temperature gradient gel electrophoresis (TTGE). Two additional microbial community fingerprinting techniques are single strand conformation polymorphism (SSCP) and terminal-restriction fragment length polymorphism (T-RFLP) analyses. Although the principles and technical procedures vary, all microbial community fingerprinting techniques are PCR-based and generate profiles representing the sequence diversity within the selected ecosystem. DGGE, TGGE, and TTGE are based on sequence-specific melting behavior of amplicons, SSCP on the secondary structure of single stranded DNA, and T-RFLP on specific target sites for restriction enzymes. Interestingly, with the exception of T-RFLP, all other techniques have been used successfully in mutation detection in clinical research before being applied to microbial ecology, which demonstrates their discriminative power. With improvements of statistical software, similarity indices can be calculated and cluster analysis of SSU rDNA profiles can be performed. Thus, fingerprinting techniques are very useful for analyzing and monitoring microbial communities over time or in response to dietary treatments. Several recent reviews provide more detailed explanation of the principles of fingerprinting techniques (6,7,36).

DGGE, TGGE, and TTGE analyses of SSU rDNA have been used successfully to characterize and monitor GI bacterial communities in humans (15,37–40), pigs (41–43), cattle (44), dogs (45), rodents (46,47) and chickens (48,49). It has been reported that DGGE or TGGE are sensitive enough to detect bacteria that constitute up to 1% of the total bacterial community (15,35). This means that only the most dominant bacteria will be represented. Although less frequently used, T-RFLP has also proven to be a useful fingerprinting technique to monitor the GI microbiota (50–52). These recent studies have contributed substantial knowledge concerning factors that affect microbial community structure such as environmental perturbations, physiological conditions, GI tract location, and the genetic background of the host as discussed below.

The stability of a bacterial ecosystem is directly related to its diversity index, with a decrease in diversity resulting in a less stable ecosystem (53). The first fluorescent in situ hybridization (FISH) and TGGE experiments describing the community structure of predominant bacteria in feces from healthy human adults revealed that the composition remains relatively stable over time (15,54). Whether this compositional stability also indicates a functional stability remains to be investigated. Previous cultivation-based studies of the fecal microbiota revealed that community shifts do occur, especially in newborn babies and elderly people (55–57). More recent culture-independent studies have confirmed these findings (58,59), while also revealing the utility of molecular-approaches for further investigating the relationship between microbial stability and enteric disease. For example, the fecal microbial community in individuals suffering from Crohn's disease was found to be unstable in structure (40), indicating the importance of determining whether the instability might be a cause or consequence of disease. Similarly, a recent investigation of the ileal microbiota in neonatal piglets nourished either enterally or parenterally revealed an inverse relationship between bacterial diversity and susceptibililty to colonization by the opportunistic pathogen *Clostridium perfringens* (60). The ileal microbiota of parenterally-nourished animals was less diverse and harbored a greater density of *C. perfringens* than that of animals fed enterally.

The first comparison of TGGE fingerprints from fecal samples of adult humans demonstrated that the composition of the predominant bacterial community was host-specific (15). Apparent host-specificity has also been observed in other human fecal samples (38,39), as well as in other animals, including pigs, dogs, chickens, and mice (41–49,61,62). This indicates that host-specificity of bacterial communities in the GI tract is a general phenomenon and not restricted to one animal species. This argues for a strong influence of the host genotype on the bacterial community, which was observed previously for the presence of methanogens in the GI tract of several vertebrate and invertebrate animals (63,64). For humans, this hypothesis has been tested recently by comparing DGGE profiles from human adults of varying genetic relatedness from unrelated persons to monozygotic twins (37). The similarity be-

tween DGGE profiles of monozygotic twins was significantly higher than that for unrelated individuals, indicating that one's genetic background indeed influences the composition of the predominant microbiota. The exact nature of the host influence remains to be determined, but it is most likely to be found in specific host-microbe interactions (65). These very important findings indicate that host-specific effects on GI tract communities cannot be neglected, and that nutrition as well as disease studies have to be designed in such a way that host- versus diet- effects can be distinguished.

Gastrointestinal microbial ecology is complicated by the fact that community structure varies between GI regions in most animals, including humans. The first fingerprinting data to reveal these differences were observed in pigs (41). For humans, it was found that the mucosa-associated bacterial community was uniformly distributed along the colon, but significantly different from feces (38). Although the number of comparative analyses at different locations in the GI tract is limited, available observations indicate that fecal samples do not necessarily reflect other parts of the GI tract, including the colon.

Quantification of SSU rDNA and SSU rRNA

Although PCR is the most sensitive technique to detect sequences that are present in very low concentrations in the environment, many factors can influence the amplification reaction and the fingerprinting techniques alone do not provide quantitative data (32). However, it is possible to determine quantitatively SSU rDNA or rRNA concentrations using PCR. Competitive (RT-)PCR is one approach to quantify the target, as was used initially to quantify messenger RNA (mRNA) from human cells (66). With this method, a specific standard of known concentration is added in different concentrations to the target followed by PCR amplification. The differences in size between the target and the standard allows discrimination and subsequent quantification on an agarose gel. Using competitive PCR, SSU rDNA of a variety of bacterial species could be quantified in rumen samples (67–69). A similar approach was used to quantify targets corresponding to single amplicons in TGGE profiles (70). The benefit of this approach is that the target and standard have similar sizes and can be discriminated by melting behavior. Similar observations were made when constant-denaturant capillary electrophoresis and quantitative PCR were combined (71).

Most probable number (MPN) PCR is another method to quantify bacterial SSU rDNA in environmental samples and has been used successfully to analyze fecal samples (72). The principle is similar to MPN-counting of bacteria. Target DNA is diluted until extinction and used as template for PCR using species or group specific primers. The method is relatively rapid and suitable to quantify major groups of bacteria, but is less useful for analysis of complex communities at the species level.

Another more recently applied quantitative PCR method is the real-time PCR approach, which has been applied successfully to characterize GI samples from the human and newborn pig as well as the rumen (60,73–75). Although real-time PCR still needs to be proven suitable for analyzing complex bacterial communities, this application looks promising because bacterial targets in very low concentration can be quantified, which is difficult using other approaches.

Dot blot hybridization has been used to measure the quantity of a specific 16S rRNA in a mixture relative to the total concentration of rRNA. In brief, total RNA is isolated from the sample, bound to a filter using a dot or slot blot manifold device and hybridized with labeled oligonucleotide probes. The relative concentration of rRNA may be estimated by dividing the concentration of specific probe by the concentration of labeled universal probe hybridized, after normalization of the signals with rRNA from control strains. This approach has been used to quantify rRNA from samples of the human, pig, and horse GI tract, and the rumen (40,76–80). Because PCR or other amplification procedures are not involved, the quantification is very accurate, and this procedure is well validated and widely used (81). Recently ProbeBase, a database containing >700 published oligonucleotide probes, has become available online (www.probebase.net; 82), which will facilitate the search for rRNA probes targeting numerous families, genera, and species of microorganisms.

Studies in which quantitative PCR or dot blot hybridization have been used often highlight the quantitative power of these approaches; however, the quantification is only relative, as has been reported recently (83). The concentration of 16S rRNA and the number of ribosomes per cell varies among bacterial species and with growth phase and activity of the cell. This makes extrapolation of dot blot hybridization data to bacterial numbers, especially the uncultured ones, invalid. Similarly, genome size and 16S rRNA gene copy numbers differ among bacterial genomes, which hampers the extrapolation of the data to cell number.

Fluorescent in situ hybridization

A frequently applied culture-independent approach to quantify bacterial cells in environmental samples is FISH using SSU rRNA-targeted oligonucleotide probes (5). This method combines the power of SSU rRNA probe hybridization with epiflourescent light microscopy, confocal laser microscopy, or flow cytometry for direct quantification of individual bacteria. This approach can be used to determine the relative importance of specific groups or genera of bacteria. FISH is being increasingly used to study the bacterial composition of the GI tract, and probes have been developed to quantify bacteria belonging to various genera including *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, *Collinsella*, *Eubacterium*, *Fusobacterium*, *Clostridium*, *Veillonella*, *Fibrobacter*, and *Ruminococcus* (84–89). To facilitate enumeration, FISH has been automated and combined with computerized image analysis (90). Ultimately, enumeration of GI bacteria is best addressed using this approach. However, with the exception of the murine cecum (84), FISH has been used mainly to quantify major groups of bacteria in human feces. FISH enables five ecological issues to be addressed simultaneously: 1) to identify subpopulations in natural ecosystems and to locate their habitat; 2) to obtain information on community structure by using nested sets of probes; 3) to circumvent cultivation problems; 4) to determine in situ cellular rRNA content; and 5) to accurately enumerate defined cell populations (6). Presently, the lowest level of detection is 10^6 cells per g of feces. Most counts have been performed using microscopy, but recently the application of flow cytometry to quantify fecal bacteria has also been demonstrated (83,88,89). Statistical analysis indicated that counts retrieved by microscopy and flow cytometry were similar (88). The combination of FISH and flow cytometry is a very promising approach for the near future since it is possible to sort uncultured bacteria (91) and, although these sorted bacteria are not viable, they can be used for molecular genetic studies. Disadvantages of FISH are that it is dependent on SSU rDNA sequences available in the databases, and that only a few probes can be used per analysis. In addition, FISH is dependent on the permeability of the bacterial cell, the

accessibility of the target, and the number of ribosomes per cell.

Diversity microarrays

A new and popular method to detect bacteria in environmental samples is the use of DNA microarrays (also called biochips, gene chips, or DNA chips). DNA microarrays are typically glass surfaces spotted with arrays of numerous covalently linked DNA fragments that are available for hybridization. Current applications include monitoring RNA expression of the arrayed genes in growing cells (transcriptional profiling) or detecting DNA sequence polymorphisms or mutations in genomic DNA. DNA microarray technology is also being optimized to study bacterial diversity in a variety of ecosystems (92,93). Two of the main problems regarding DNA microarray analysis are the hybridization specificity and quantification of the signals. El Fantroussi et al. (93) demonstrated that specific and nonspecific hybridization can be discriminated by determining the thermal dissociation curve for each probe-target duplex. Quantification of hybridization signals seems to be a difficult task at present since it has been shown that the signal intensities may vary significantly between targets even when perfectly matched to the probe sequence. The first attempts to generate DNA microarrays for application to gut ecosystems have been performed and look promising (94,95). Without question, DNA microarray technology will be expanded and further extended in the near future to study the ecology of the GI tract.

Non-SSU rRNA-based profiling

Several non-SSU rRNA-based profiling approaches, such as those based on cellular fatty acid composition (61,62) or G+C content of DNA (96–98) have also been used successfully to monitor shifts in the structure of GI bacterial communities. However, in contrast to SSU rRNA-based approaches, these methods are disadvantaged by the lack of phylogenetic databases.

Conclusions on GI tract communities from culture-independent data

The application of SSU rRNA based approaches has provided novel insights into the composition and structure of microbial communities in the GI tract. The number of SSU rDNA sequences directly retrieved from several GI tract locations in a variety of animals is large and still growing. Despite a lack of consistency between the molecular procedures and the definition of OTUs, one of the main conclusions, which can be drawn from these data are that the majority of GI bacteria have yet to be obtained in culture, leaving their description and possible contributions to GI health or disease unknown.

From structure to function

Characterizing the community structure of the GI microbiota is the first important step in studying this ecosystem, although such data provide limited information on microbe-microbe and host-microbe interactions. Based on the complexity of the GI microbiota and the limited culturability of many of its members, it is clear that determining the function of all contributing microbes is a very difficult task. However, some technological progress has been made in analyzing in situ activities of GI bacteria, and has great promise for future research **(Table 2)**.

Measuring the expression of functional genes is one approach to determine the in situ activity of bacteria in an ecosystem. Deplancke et al. (46) were able to specifically detect adenosine-5′-phosphosulfate reductase mRNA expression at different locations in the mouse GI tract by using an RT-PCR approach. Also, RT-PCR was used to monitor the expression of four *Helicobacter pylori* genes during its infection of human and mouse gastric mucosae (99). So-called in vivo expression technology (IVET) is another approach for measuring gene expression in situ. The IVET strategy allows the identification of promoters that are specifically induced when bacteria are exposed to certain environmental conditions (100). The approach has been used mainly to study gene expression of pathogens, but was also used recently to identify gene promoters, which were specifically induced in *Lactobacillus reuteri* during colonization of the mouse GI tract (101). Remarkably, in this instance the expression of only three genes could be linked to *Lactobacillus* colonization.

Despite the value of complete genome sequences and subsequent comparative genomics and the application of DNA microarrays to study transcriptional responses of microbes, these approaches are in an early state of development and are expensive, and thus prohibitive for many investigators. In addition, those genome sequences are retrieved from culturable, well-studied bacteria. New opportunities to search for functional genes, including those from uncultured microbes, are the use of bacterial artificial chromosome (BAC) vectors and subtractive hybridization. BAC vectors support the cloning of large DNA inserts (>100 kb) and libraries have been constructed from soil (102,103) and marine environmental DNA (104). These have been used to assess the diversity and metabolic potential within these complex ecosystems. In this way, it is possible to link SSU rRNA genes to functional genes in a culture-independent matter.

The use of isotopically labeled substrates is another way to gain insight into functional aspects of specific microbes within

TABLE 2

Molecular approaches for studying metabolic activities and gene expression in gastrointestinal microbes

Approach	Target molecule	Is cultivation required?	Is the identity of the target gene required?	Can microbes be identified directly?	Main purpose
BAC vector cloning	Genomic DNA	No	No	No	Identify functional genes
DNA microarray	mRNA	No	Yes	No	Obtain transcriptional fingerprints
In situ isotope tracking	Labeled biomarkers	Yes	No	Yes	Identify active microbes
IVET	Promoter regions	Yes	Yes	Yes	Detect induced promoters
RT-PCR	mRNA	No	Yes	No	Detect/measure gene expression
Subtractive hybridization	Genomic DNA	No	No	No	Recovery of unique genes

a complex community. Environmental samples are incubated with isotopically (stable or radioactive isotopes) labeled substrates and these isotopes can then be traced back by extracting biomarkers, such as DNA, rRNA, or lipids. This approach has been applied successfully to aquatic sediments and the microbes involved in methane oxidation and acetate-coupled sulfate reduction, respectively, could be identified (105). Recently, developments have been made in combining isotope tracking with DGGE of SSU rRNA RT-PCR products or high-throughput DNA microarray analysis (106,107). Another successful approach to link phylogenetic information to processes is the combination of microautoradiography and FISH (108,109). These approaches look very promising, although one should keep in mind that some microbes might be able to discriminate different isotopes (110). Nonetheless, it is evident that the use and development of novel approaches, such as those described above, should continue and be applied more frequently to better define microbe-microbe and host-microbe interactions as they pertain to GI health and disease.

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