Molecular Ecology and Diversity in Gut Microbial Ecosystems

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ABSTRACT

The microbial community inhabiting the gastrointestinal tract is represented by all major groups of microbes and is characterized by high population density, wide diversity and complexity of interactions. Resident microbial populations have been described as herbivores, omnivores, carnivores, and in a wide range of zoological classes where they contribute to the nutrition, physiology, immunology and protection of the host. Despite this vast amount of knowledge, the basic prerequisites for ecological studies, namely enumeration and identification of all community members have tremendous limitations. These limitations can be overcome using molecular ecology techniques based on sequence comparisons of nucleic acids (DNA and RNA) and can be used to provide a molecular characterization, while at the same time providing a classification scheme that predicts phylogenetic relationships. The use of nucleic acid-based techniques to detect, identify and quantify microbial populations in the gastrointestinal environment will be briefly reviewed. Some key discoveries revealed by application of the rRNA approach to characterization of morphologically conspicuous but as yet uncultured bacteria are described. The use of molecular ecology techniques will lead to major advances in our knowledge and provide the first complete description of gastrointestinal ecosystems.

Introduction

Animals of a wide range of orders or classes have a portion of their digestive system adapted to accommodate a fermentation which assists in digestion as well as providing a variety of other benefits (Table 1). Because of the refractory nature of the plant cell wall and the difficulty in digesting it, herbivores have anatomical and physiological adaptations of the digestive tract to allow assimilation of this material. Herbivorous reptiles, birds, and mammals usually have enlarged or elongated digestive tracts, often including fermentation chambers or sacs in the foregut or hindgut [19]. Cecum-colon (hindgut) fermenters represent an older differentiation than foregut fermenters which, in turn, are older than ruminants [17]. Advances in our understanding of fermentative digestion have tended to obscure the vital role that the gastrointestinal microbiota plays in the physiological, immunological and protective functions of the host animal. The association of microbes with tissues of the gastrointestinal tract of animals during evolution has resulted in a "balanced" relationship between resident microbes and the host. Numerous biochemical, physiological and immunological features that are considered intrinsic characteristics of animal species are actually responses by the animal to the physical presence and metabolic activities of the normal indigenous microbiota. This microbial challenge has modified the course of evolution in animals resulting in the selection of animal microbe relationships

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which are complex and which vary tremendously ranging from competition to cooperation [12].

Table 1. Orders of animals possessing regions of the digestive tract colonized by indigenous microbial populations which result in fermentative activity. Mammalian orders are classified as either hindgut or foregut fermenters.

	Order/Class	Family/Group (common name)
	Insecta	Isoptera (termites), Blattaria (cockroaches),
		Orthoptera (locusts, grasshoppers, beetles)
	Reptilia	Chelonia (turtles, tortoises)
		Sauria (iguana, agama lizard)
	Pisces	Perciformes (marine herbivovous fishes)
		Cyprinidae (carp)
	Aves	Ratites (emu, ostrich, rhea)
		Anseriformes (geese, ducks)
		Galliformes (grouse, ptarmigan)
		Cuculiformes (hoatzin)
Hindgut	Hominids	Troglodytes (apes, chimps, gorillas)
	Rodentia	Muridae (rats, mice, voles), Procavia (hyraxes)
		Castoridae (beaver), Hystricidae (porcupine)
		Caviidae (guinea pigs, cavy's)
	Lagomorpha	Leporidae (rabbitts, hares) Ochotonidae (pika)
	Marsupiala	Phascolarctidae (koala) Phalangeridae (gliders, possums)
	Other orders	Hyracoidea (hyraxes), Proboscidae (elephant)
		Sirenia (dugongs, manatees)
	Artiodactyla	Suidae (pigs, peccaries)
	Perissodactyla	Tapiridae (tapirs), Rhinocerotidae (rhinos)
		Equidae (horses, zebra, ass)
Foregut	Cetaceans	Cetacea (whales, dolphins)
	Marsupiala	Macropodidae (kangaroos, wallabies)
	Primates	Colobinae (langur, proboscis, colobus monkeys)
	Edentata	Bradypodidae (sloths)
	Artiodactyla	Hippopotamidae (hippo)
		Tylopoda/Camelidae (camel, llama)
		Tragulidae (chevrotain)
		Cervidae (deer)
		Giraffidae (giraffe, okapi)
		Antelocapridae (antelopes)
		Bovidae (sheep, goats, cattle, gazelles)

The gastrointestinal microbial community is characterized by its high population density, wide diversity and complexity of interactions. Our current knowledge of gut microbial diversity and ecology is largely based on classical anaerobic culture techniques, phenotypic characterization of culturable isolates as well as light and electron microscopic examination. These studies demonstrate that all major groups of microbes (bacteria, protozoa, fungi, yeasts and bacteriophage) are represented in the gastrointestinal tract. The human colon contains in excess of 10^{11} bacterial cells per gram of content which are reported to belong to as many as 400 different species [30]. The rumen, the most extensively studied gut ecosystem, contains large numbers of bacteria (up to 10^{11} viable cells per gram comprising 200 species), ciliate protozoa ($10^4 – 10^6$ per gram distributed over 25 genera), flagellate protozoa (lower numbers and less diverse than the ciliates), anaerobic chytridiomycete fungi (zoospore population densities of $10^3 – 10^5$ per gram divided into 5 genera) and bacteriophage particles (wide morphological diversity and genome size with density equivalent to $10^7 – 10^9$ particles per gram). Importantly, the mammalian gut ecosystem contains representatives of the three primary lines of descent Bacteria, Archaea and Eucarya articulated by Carl Woese and co-workers [40]. However, despite this vast amount of knowledge, microscopic and culture-based enumeration and classification schemes of microbial community members have tremendous limitations.

The two major problems faced by microbial ecologists studying the gut ecosystem are the inevitable bias introduced by culture-based enumeration and characterization techniques, and the lack of a phylogenetically based classification scheme. Modern molecular techniques based on sequence comparisons of nucleic acids can be used to provide molecular characterization while at the same time providing a classification scheme which predicts natural evolutionary relationships [27,37,40]. The group of Norman Pace [11,26] was among the first to recognize the power of combining Woese's new phylogeny with molecular biology and create what is now recognized as the field of **molecular microbial ecology**. This is defined as the application of molecular technology, usually based on comparative nucleic acid sequence information, to identify specific microorganisms in the environment, to assign functional roles to these specific microorganisms, and to assess their significance or contribution to specific environmental processes.

The Phylogenetic Basis of Molecular Microbial Ecology

In general, the principles (and techniques) outlined below can be applied to any gene but have been most commonly applied to ribosomal RNA (rRNA) molecules [30]. Due to the ubiquity of rRNA molecules (16S and 23S in Bacteria and Archaea, 18S and 28S in Eucarya, respectively) in all cellular life forms, comparative analysis of their sequences can be universally applied to infer relationships among organisms. The rRNA molecules comprise a mosaic of highly conserved sequence domains interspersed with more variable regions. Thus universal regions of sequence can be identified which can serve as primer binding sites for PCR amplification. In addition, it is possible to identify sequence motifs of increasing phylogenetic resolution. For example, signature sequences for Archaea, Bacteria and Eucarya, or Domain probes have been recognized as well as short stretches of more variable sequence characteristic of a number of the bacterial divisions such as the *Bacteroides-Prevotella-Porphyromonas* group [6], Enteric group [7], *Bifidobacterium* [7], *Lactobacillus* group [32], *C. leptum* subgroup [33], *Bacteroides fragilis* group [10] and *C. coccoides-E. rectale* group [10]. Species and even subspecies specific sequences have also

been identified such as for *B. distasonis* [10], *Fibrobacter succinogenes* and *F. intestinalis* [38, 18], *Lachnospira multiparus* [38], *Ruminococcus albus* and *R. flavefaciens* [25], and *Synergistes jonesii* [22]. Oligonucleotide probes targeting groups, families and species of methanogenic and sulfate-reducing bacteria are particularly well developed [30].

Some key discoveries revealed by application of the rRNA approach to studies of microbial diversity and ecology in gut ecosystems

Despite some limitations, molecular approaches have provided major advances in our understanding of microbial ecology and diversity. The following section describes advances in our understanding of the gut ecosystem that could not be achieved using conventional techniques alone. These examples cover large, unusual, morphologically conspicuous but as yet uncultured bacteria and symbioses.

Large ovals in the rumen

Several large, oval, morphologically conspicuous bacterial forms have been observed and reported since the earliest studies on rumen ecology. Quin's oval was briefly described by Woodcock and Lapage in 1913 [41] and reported by Quin [29] in large numbers in the rumen of sheep fed alfalfa diets, or supplemented with molasses, glucose or sucrose. This organism has a relatively large (4-8 μm) oblong-oval shape with tumbling motility and linear tufts of flagella on one side of the cell. It is gram-negative, non-spore-forming, occurs singly and in pairs, and reproduces by binary fission. This organism is easy to differentiate morphologically, even from *Magnovum eadii* (Eadie's ovals) which is much larger (12-20 μm), has gliding motility and a large number of peritrichous flagella. Quin's oval, considered an important member of the rumen microbiota of sheep, occurs in numbers from 2×10^5 to 3×10^8 per ml, but reaches numbers as high as 10^{11} per ml in sheep fed mainly molasses [39]. Further studies have been hampered by an inability to obtain pure cultures, and very slow and meticulous culture techniques required to determine carbohydrate fermentation and other characteristics.

Enriched suspensions (> 90% Quin's ovals based on cell count) were prepared by differential centrifugation and used for 16S rRNA sequence analysis. On the basis of previous phenotypic descriptions and phylogenetic assessment using 16S rRNA sequence analysis, a new genus and species (*Quinella ovalis*) was proposed [15]. Its closest relatives based on 16S rRNA sequences are the *Selenomonas-Megasphaera-Sporomusa* group in the low G + C gram-positive phylum. This information can be used to design selective enrichment techniques and to increase the possibility of obtaining pure cultures for definitive metabolic studies and to design specific oligonucleotide probes for ecological studies.

The largest known bacteria, Epulopiscium spp.

A further example of the power of the phylogenetic approach is provided by the following studies which describe the largest prokaryote known. The gut of a number of species of surgeonfish (Family: Acanthuridae) harbor symbiotic microorganisms (up to 80 x 600 μm) originally described as eukaryotic protists because of their large size [23]. However, their ultrastructure, determined by electron microscopy, is more characteristic of prokaryotes. Since these bacteria (named *Epulopiscium fishelsoni*) remain uncultured, taxonomic inferences based on biochemistry and physiology have been difficult to establish. Cells were purified by icromanipulation and PCR was used to obtain 16S rRNA

sequence information. Comparative sequence analysis demonstrated that *E. fishelsoni* purified from Australian surgeonfish formed a monophyletic group related to *Clostridium* in the low mol % G + C gram-positive bacteria. Furthermore, oligonucleotide probes designed to target 16S rRNA from *E. fishelsoni* hybridize with symbionts present in the gut of surgeonfish from the Red Sea, suggesting that these geographically isolated populations are related. Similar and apparently related "Epulos" representing at least 10 different morphotypes have subsequently been collected from surgeonfish in a variety of geographic locations (Hawaiian Islands, French Polynesia, Tuvalu, Guam, southern Japan, Papua New Guinea, the Great Barrier Reef, and South Africa [2]). So far, none of these organisms have been cultured. This suggests that geographically isolated populations of these symbionts have a similar role in digestion in marine herbivorous fish [5].

The guinea pig symbiont, Metabacterium polyspora

The gram-positive bacterium *Metabacterium polyspora* is an uncultivated symbiont of the guinea pig gastrointestinal tract [4,16]. Molecular analysis of 16S rRNA genes suggests an unusually close relationship between *Epulopiscium* spp and *M. polyspora* [1]. Generally, *M. polyspora* produces at least two endospores, and as many as 8 or 9, which is unusual among the known endospore forming species bacteria of the low G + C gram-positive bacteria. Endospore formation was shown to be coordinated with transit of the bacterium through the gastrointestinal tract of the guinea pig. For the majority of cells, sporulation is initiated in the ileum whereas later stages of development take place in the cecum. Fully fruited endospores, still contained within mother cells, are found in the animal's feces. Since guinea pigs are coprophagous, *M. polyspora* are reintroduced into the digestive tract. Mastication of feces and deterioration of the mother cell leads to release of mature endospores thus completing the cycle, suggesting a reliance on endospore formation for dispersal and reintroduction of the bacteria to its host.

Segmented filamentous bacteria

Although the phenomenon described above ("daughter cells" produced within bacterial cells) is rare, other organisms also release free-living cells. Notably, segmented filamentous bacteria (SFB) that live anchored to the ileal walls of rodents such as mice and rats produce live internal offspring and, at other times, dormant spores [3,13,14]. Filamentous segmented bacteria inhabit the lower small intestine of mice, rats and chickens as well as a wide range of other vertebrate and invertebrate hosts [14]. A phylogenetic analysis of 16S rDNA sequences revealed that SFB isolated from mice, rats and chickens form a natural group of different species which form a natural group related to the Group 1 *Clostridium* cluster [35,36].

The filaments range from 2 to 80 μm in length and 0.7 to 1.8 μm in diameter and have an intimate relationship with their host, being attached by one end to the epithelium. It is suggested that SFB's contribute to development and maintenance of host resistance to enteropathogens. Attempts to culture SFB's *in vitro* have been unsuccessful until now. However, as early as 1849, Leidy accurately described *Arthromitus* filaments in insects (Arthropods). Recent work led Margulis to hypothesize that, in nature, spore-forming aerobes in soil exist as one stage of the arthromitids. Complete sequencing of the 16S rRNA gene from four isolates confirms these as low G + C gram-positive *Bacillus cereus* [20]. They conclude that *B. cereus* and its close relatives that are easily isolated from soil

also enjoy filamentous growth in moist nutrient rich intestines of healthy arthropods and similar habitats.

Symbiotic relationships – anaerobic ciliate protozoa

A great majority of ciliate species are aerobes. However, an anaerobic lifestyle has evolved independently in many unrelated groups [9]. The ciliates of the rumen and cecum of herbivorous mammals are believed to have originated from free-living (anaerobic) ciliates that were introduced into digestive tracts that most likely already harbored anaerobic prokaryotes [9]. These free-living ancestors underwent rapid adaptive radiation resulting in a wide diversity of species. Anaerobic ciliates (as well as anaerobic chytrids) contain modified mitochondria or hydrogenosomes. These sub-cellular organelles function to ferment pyruvate produced by glycolysis into acetate and hydrogen and in this process couple energy conservation with ATP synthesis via substrate level phosphorylation [24,8,21]. These H_2-evolving fermentations in anaerobic unicellular prokaryotes have resulted in widespread symbiosis with methanogens. Most anaerobic protozoa have the other microbes living either inside (endosymbionts) or attached to their external surfaces (exosymbionts).

Recent research using a SSU rRNA probe targeting ruminal methanogens (Archaea) revealed a taxon-specific association between protozoal and methanogen populations in the rumen and a continuous culture fermentor system [34]. Methanobacteriaceae were the most abundant population in the rumen comprising 89.3% of total archeae and 99.2% in the protozoal fraction. This value decreased to 54% of archaeal signal after 48 hr of fermentor operation and was correlated with loss of protozoa from the system. In contrast, the Methanomicrobiales, the most abundant archaeal population, accounted for 12.1% of archaeal signal in rumen fluid and was not detected in the protozoal fraction, suggesting a free-living lifestyle. This group increased to 26.3% of archaeal signal in fermentor content without protozoa. These studies suggest the importance, and perhaps specificity, of Methanobacteriaceae as symbionts of rumen protozoa.

Conclusions and Future Directions

While the emphasis in this short review has been on the use of molecular techniques to generate advances in microbial ecology of the gastrointestinal tract that could not have been achieved using conventional techniques, the value and role of classical microbial ecology techniques should not be underestimated. Indeed molecular methods make use of information originating from organisms that have been cultured and deposited in various culture collections. It is generally agreed that these reference organisms represent a small fraction of the biodiversity present in nature. However, it is on the basis of nucleic acid sequences in these reference strains that “universal” primers for amplification are designed, without consideration of the fact that the particular “universe” used as a reference is very small [28]. The lack of resolving power limits the inferential value of rRNA sequence comparisons at the level of species, the most important unit for expressing biodiversity. Thus, Palleroni [28] concludes that molecular approaches should have an important role as *guides* for the isolation and characterization of prokaryotic diversity. “Isolation” does not necessarily imply the cultivation of pure cultures, but the creation of conditions which simulate those in the natural environment that allow microorganisms to express their biological and biochemical properties. Thus cultural based techniques should be used in combination with new molecular techniques to improve cultivation, speciation, and the

study of biodiversity. Studies that combine molecular measures of species composition and abundance with the measurement of biochemical activity are required to determine the precise role or function a specific organism plays in its natural environment and its quantitative contribution to the whole. Future emphasis should be on "microbial ecology" and not "molecular", since molecular techniques are tools that enable the study of microbial ecology in gut ecosystems to be a more vigorous, hypothesis driven, experimental science rather than a description of numbers and types of microorganisms.

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