Screening of Intestinal Microflora for Effective Probiotic Bacteria

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Increasing consumer awareness of health-promoting intestinal bacteria has fueled the addition of viable probiotic bacteria as functional ingredients in certain foods. However, to effectively market the enhanced attributes of these foods, the added probiotic bacteria need to have scientific credibility. The scientific rationale for using many of the strains of probiotic bacteria currently on the market is weak. Furthering the current understanding of what features a bacterium needs to have for effective probiotic functionality will enable the selection of strains with a more credible scientific rationale. To screen for effective strains, one must understand the microbial diversity in the intestines of healthy individuals. The advent of molecular tools has greatly enhanced our ability to accomplish this. These tools comprise genetic fingerprinting, specific probes, molecular speciation, and techniques for the in situ analysis of specific microbial groups in the intestine. This review will detail these scientific approaches and how their impact will improve criteria for selection of probiotic bacteria.

Keywords: *Probiotic bacteria; intestinal microflora; Bifidobacterium; Lactobacillus*

INTRODUCTION

The concept of ingesting live microorganisms for the purpose of improving one's intestinal health and general well-being can be traced back to the beginning of the 20th century (*1*, *2*). This practice is now referred to as "probiotics" and is the subject of intense scientific research directed toward obtaining effective probiotic bacteria and establishing their health benefits. Although numerous genera of bacteria (and yeasts) are currently being marketed as probiotic cultures throughout the world, the two most commonly used genera are *Lactobacillus* and *Bifidobacterium*. The market for probiotic cultures is very significant in Asia, particularly Japan, and has attained significance in Europe during the past decade. The probiotic market is lower in the United States, but it is growing and has tremendous growth potential if strains can be obtained with the required scientific evidence that allows the U.S. Food and Drug Administration (FDA) to permit specific health claims. In recent years, there has been a growing body of scientific evidence bolstering specific health claims of certain probiotic strains. The health claims are quite diverse, ranging from diarrhea prevention to cholesterol reduction, highlighting the tremendous potential for the probiotic field. Although this is encouraging, the evidence is not yet consistent enough to convince all scientists or the FDA. It is pertinent, however, to review the current evidence supporting specific health claims.

Prevention and Treatment of Diarrhea. It is widely accepted that the normal gastrointestinal microflora exert a protective role against attack by enteric pathogens (*3*−*5*). Tissier (*2*) first advocated the ingestion of live bifidobacteria as a remedy against diarrhea in children. Although the use of probiotic bacteria for prophylactic and clinical uses against gastrointestinal infections is a valid concept, proper strains are not yet available for effective use among a wide range of individuals. However, promising studies have been carried out in this regard. Bifidobacteria have been used to successfully treat intestinal disorders (*6*) and in the prevention of rotaviral diarrhea in children (*7*). Some studies also support the use of a *Lactobacillus* strain, specifically *L. rhamnosus* GG, for the prevention (*8*) and treatment (*9*, *10*) of diarrhea in children.

Antibiotic-associated gastrointestinal disturbances are a well-recognized problem, and Black et al. (*11*) observed that *Bifidobacterium longum*, delivered with *Lactobacillus acidophilus*, decreased the incidence of ampicillin-associated diarrhea and the time required for recolonization. Furthermore, Colombel et al. (*12*) have shown an alleviation of the adverse effects, particularly diarrhea, of erythromycin treatment by administration of yogurt containing *B. longum*. *L. rhamnosus* GG was also found to be an effective prophylactic against antibiotic associated diarrhea (*13*, *14*).

Establishment of a Healthy Flora in Premature Babies. Premature infants generally take longer to establish a characteristic intestinal flora, which can render them more susceptible to certain intestinal infections, such as ulceronecrotic colitis (*15*). Akiyama et al. (*16*, *17*) administered lyophilized strains of *Bifidobacterium breve* or *B. longum* to premature infants and found that either culture resulted in a bifidobacteria population becoming established more quickly in their intestines compared to a control group. The *B. breve* strain, however, appeared to be more suited to the ecological conditions of the intestines of these infants as even after 6 weeks it was the only species detected. In the case of *B. longum*, other resident bifidobacteria gradually displaced this culture, suggesting that the strain used was not ideally suited to these intestinal environments. These studies do indicate that feeding of any bifidobacteria may be beneficial to premature infants to help establish a bifidobacteria flora. However, feeding of lactobacilli, including *L. rhamnosus* GG, was

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not found to prevent the establishment of deleterious flora in premature infants (*18*, *19*).

Alleviation of Constipation. Constipated bowel movement is a significant problem for many people, but especially the elderly. The lower intestinal bifidobacteria population in the elderly may be a contributing factor to constipation. Kleessen et al. (*20*) recently showed that feeding inulin (a prebiotic that enhances bifidobacteria in the large intestine) to constipated elderly individuals significantly increased the bifidobacteria population in their intestines and had a significant laxative effect. A study in Japan also reported that feeding a *Bifidobacterium* cultured milk to elderly people significantly improved stool frequency (reviewed in ref *21*). Furthermore, in feeding studies conducted in this laboratory, a significant effect in stool consistency was observed when large numbers of bifidobacteria were ingested (unpublished observations). It is possible that a laxative effect of bifidobacteria will only require high numbers of transient bifidobacteria through the intestine. Therefore, this effect may not be strain/subpopulation specific, which would greatly simplify the selection of strains for this effect. Further studies into this possible laxative effect of bifidobacteria will be needed to establish any prophylactic or pharmaceutical roles.

Alleviation of the Symptoms of Lactose Maldigestion. Dairy foods are a very important part of a healthy diet, but numerous individuals suffer from some symptoms of lactose maldigestion. Fermented dairy products appear to be more tolerable to lactose maldigesters than nonfermented foods, because of a decrease in lactose concentration of the food, as a result of microbial metabolism (*22*), and perhaps because of the delivery of β-galactosidase (*23*). Several studies have examined the contribution of both lactobacilli and bifidobacteria for the improvement of lactose tolerance. Two different strains of bifidobacteria in unfermented milk were found to reduce the breath hydrogen response (a measure of colonic carbohydrate metabolism) and symptoms of lactose malabsorption in a study of 15 subjects (*24*). When feeding yogurt fermented with bifidobacteria to lactose malabsorbers, Martini et al. (*25*) observed an improvement in lactose digestion by subjects. A *Lactobacillus acidophilus* strain with good bile and acid tolerance was found to reduce symptoms associated with ingesting lactose (*26*). However, other studies did not observe a significant improvement in the absorption of lactose in lactose malabsorbers after the delivery of other bifidobacteria cultures (*27*, *28*) or other strains of *L. acidophilus* (*29*). These apparent discrepancies are likely due to differences in the strains used and in the intestinal ecological conditions of the individuals in the feeding studies.

Enhancement of Immune Function. Recent studies support the assumption that the interaction of certain strains of lactobacilli and bifidobacteria with specific loci in the human intestine can stimulate both general immunity (increased levels of cytokines and IgA) and also specific antibodies to certain pathogens. Although the majority of studies investigating this clinical aspect rely on mice models, some human studies are now also supporting them. Ingesting certain strains of lactobacilli has been shown to increase secretory IgA levels (*30*) and rotavirus specific antibodies (*31*, *32*). A recent, doubld-blind, placebo-controlled study of 12 human subjects found that ingesting a strain of *Bifidobacterium lactis* enhanced general immunity (*33*). Further substantiation of these types of studies will greatly strengthen this very marketable health benefit.

Suppression of Tumorigenesis. Some strains of bifidobacteria have been suggested to be associated with anticarcinogenic, antimutagenic, and antitumorigenic activities. The suggested mechanisms for these health claims are plausible, but there is no real direct human evidence to support the claims. However, some animal studies are supportive of a role for bifidobacteria and some lactobacilli in the suppression of tumorigenesis. In a rat feeding study, cultures of *B. longum* reduced carcinogenesis by a food mutagen, 2-amino-3-methylimidazo[4,5-*f*]quinoline (*34*). In another study, a strain of *B. longum* was found to suppress azomethane-induced colon carcinogenesis in rats (*35*). Certain strains of lactobacilli have also been shown to significantly suppress intestinal tumors in rats by chemical mutagens (*36*). The anticarcinogenic, antitumorigenic, and antimutagenic activities of these probiotic bacteria have been proposed to occur via some modification of mutagens in vitro (*37*), reduction in carcinogen-generating fecal enzymes in vivo (*38*−*40*), and suppression of precancerous lesion or tumor formation (*34*, *40*−*42*). Further research is essential to comprehend these effects in humans and elucidate roles for specific strains of probiotic bacteria and their metabolic and enzymatic products.

Cholesterol Reduction. The claim that ingestion of certain probiotic bacteria can lower serum cholesterol levels is perhaps the least scientifically validated health claim of probiotics. However, there are some human feeding studies with certain strains of lactobacilli that suggest a possible effect (*43*, *44*). Although these and other studies do postulate a cholesterol-lowering effect for some lactobacilli strains, they are not very convincing because of the lack of a convincing basal line for cholesterol measurements prior to and after the feeding studies.

There are some cultures currently available with studies suggesting a role in some of the specific health claims outlined above, but the evidence is not substantial enough to impact a stringent scientific regulatory committee, such as used by the FDA. A major reason for the lack of effective probiotic cultures is an incomplete knowledge of how to select proper strains. Recent advances in understanding the physiology of these bacteria have led to a better selection process. However, there are still many gaps in our knowledge of how these bacteria interact successfully, in their intestinal habitat, for maximum health benefits. Improving this knowledge void will greatly increase the ability to obtain probiotic bacteria with the proper characteristics to elicit specific health effects. Modern molecular tools are providing the means for studying the microecology of these bacteria. The following sections will overview the current molecular tools available for studying the gastrointestinal (GI) tract microflora and also the current knowledge of selection criteria for effective probiotic bacteria.

MOLECULAR TOOLS FOR STUDYING THE GI TRACT MICROFLORA

The available molecular tools for probiotic bacterial research can be grouped into tracking tools, speciation tools, and in situ tools.

Tracking Tools. The ability to accurately track a bacterial strain through the GI tract is essential for answering many scientific questions about potential

probiotic bacteria. Classical approaches cannot differentiate to the strain level, so specific questions regarding a particular feeding strain would remain unanswered. Currently there are a number of different DNA fingerprinting methodologies available, most of which have already been used in probiotic research. All of these methodologies have unique advantages and limitations, which need to be considered before one is chosen for a particular study. This section will overview some of the more pertinent tracking tools for probiotic research.

Colony Hybridization with Nucleic Acid Probes. Genus and species specific probes for certain bacteria have been obtained and are useful for monitoring the types of organisms in an individual's fecal or intestinal matter. Although strain-specific probes are much harder to come by, the future availability of complete genome sequences should facilitate their design. Probes are chosen such that they are unique to the particular genus, species, or strain. Technically, the procedure is rapid as colonies can be directly probed, by lysing the colony to expose the nucleic acid content and allowing access for the probe. The label on the probe can be either radioactive, enzymatic, or fluorescent, which can be readily detected. The selection of probes, however, is the key to success with this approach, as any cross-reactivity can give ambiguous results. Probes can be obtained using either a shotgun or a directed approach. The shotgun approach is to randomly isolate DNA fragments and test them for probe reactivity against a bank of isolated strains. This approach is laborious and requires extensive testing for specificity. A directed approach to probe selection leaves less to chance, as it relies on probes directed at target sequences that are thought to be unique to the particular organism or group of organisms under study. One strategy is to identify enzymes unique to a group of organisms and direct probes at targets within the enzyme gene sequence. A potential example would be the bifidobacteria enzyme fructose-6-phosphate phosphoketolase (F6PPK), which is used by members of this genus to metabolize carbohydrates via a unique pathway often called the fructose-6-phosphate shunt. As this enzyme is unique to this genus (with the possible exception of *Gardnerella*), it potentially is a good source of probes for this genus and should afford an effective means for tracking these microorganisms in the gastrointestinal tract. However, the sequence of this gene is not yet available.

Generating short (~20 bases) oligonucleotide probes directed at regions of the ribosomal RNA (rRNA) is the most common means of obtaining genus- and species-specific probes. This approach is possible as there are some short variable sequence regions within this molecule that can distinguish to the genus or species level. During the probe design process, probes can be tested against the extensive database of rRNA sequences using computer models. With the correct design procedure, the resulting probes should have a low cross-reactivity. Using this strategy, genus-specific probes have been designed and evaluated for the detection of *Bacteroides* (*45*) and *Bifidobacterium* (*46*) from human fecal samples. Species-specific probes for bifidobacteria (*47*), *Bacteroides* (*48*), and *Lactobacillus* (reviewed in ref *49*) have also been developed.

Pulse Field Gel Electrophoresis (PFGE). PFGE can be applied to fingerprinting a bacterial isolate by digesting its genome into a relatively few (5−50) large segments and separating them by specific orientated electric pulses. The fragments are obtained by digesting the genome with rare cutting restriction enzymes, which generally have an 8 bp recognition site or a 6 bp recognition site, which may be statistically rare for the particular genome. Because the DNA fragments are large, they cannot be manipulated in aqueous solutions or they would be sheared mechanically. Therefore, all manipulations, including DNA isolation and restriction, are carried out on cells embedded in agarose plugs. The restricted fragments in the agarose plug are inserted then into a well in an agarose gel and separated by PFGE based on fragment size. The resulting pattern of DNA fragments is referred to as a restriction fragment length polymorphism (RFLP) and is highly characteristic of the particular organism. It should be noted that this fingerprint represents the complete genome and has the added advantage of detecting specific changes (DNA deletion, insertion, or rearrangements) within a particular strain over time. This feature also makes this fingerprinting technique one of the most discriminatory (if not the most) techniques available. It is also very reproducible. Disadvantages of this technique are that colonies need to be cultured to obtain enough cells and that it is technically challenging, as well as labor intensive. The usefulness of the technique has been demonstrated by McCartney et al. (*50*) and Kimura et al. (*51*) to monitor the prevalence of lactobacilli and bifidobacteria in human fecal samples over a period of time.

Ribotyping. A ribotype is essentially an RFLP consisting of the restriction fragments from a particular genome that contain rRNA genes. To obtain a ribotype for an organism, it must first be cultured to obtain enough cells for the procedure. Total DNA is then isolated and is totally restricted into multiple fragments of sizes ranging from <1 kb to >20 kb, using a restriction enzyme with a frequently occurring recognition sequence, generally a 6 bp recognizing enzyme. The restricted fragments are then separated by agarose gel electrophoresis and subsequently hybridized with a probe targeted to either the 16S, 23S, or 5S rRNA genes. In practice, probes to the 16S rRNA are the most commonly used. Following probe detection, restriction bands containing copies of the rRNA genes are visualized and the pattern of the band sizes represents a characteristic fingerprint. The basis of the technique is that many bacteria generally contain multiple copies (up to eight or more) of the rRNA genes throughout their genome, thus enabling the RFLP to be obtained. However, some bacteria contain as few as one copy of rRNA genes, thus limiting the effectiveness of ribotyping for fingerprinting these bacteria. Bacteria with a single copy of the rRNA operon are usually slow-growing bacteria. Examples are *Coxiella burnetti*, *Bradyrhizobium japonicum*, and *Mycobacterium paratuberculosis*. An advantage of ribotyping is that a single rRNA probe can be used to type all bacteria. It is also very reproducible, and its effectiveness for the analysis of human intestinal microflora has been demonstrated (*50*−*52*). Limitations of this fingerprinting technique are that it is not as discriminative as PFGE, requires culturing of bacteria, and is labor intensive. However, the development of an automated ribotyping instrument (DuPont, Wilmington, DE) has increased the usefulness of this technique for the analysis of large numbers of isolates.

A related fingerpinting methodology is the use of a probe targeted to specific regions within a genome, such as a virulence gene or other unique characteristic of a particular organism. As this targets only a single genetic locus, its discriminatory power is low, but it is highly effective for analyzing a population of organisms for specific traits. Probes can also be targeted at other sequences that may exist in multiple copies in a genome, such as insertion sequences (IS elements), thus enabling a characteristic RFLP to be generated. This technique has been used to fingerprint bacteria (*53*) but has not yet been used in the study of the human intestinal ecosystem. However, given the propensity of IS elements in many lactic acid bacteria (*54*), this may be an applicable technique for this purpose.

Multiplex Polymerase Chain Reaction (PCR). The PCR, which was developed by 1993 Nobel prize recipient Kary Mullis (reviewed in ref *55*), is one of the most useful molecular tools of modern times. In its simplest form, PCR is used to amplify a specific DNA sequence over a billionfold from a single copy, using a thermostable DNA polymerase (usually *Taq* DNA polymerase), deoxynucleotides (dNTP), and two primers, having sequences complementary to either end of the targeted sequence. This is achieved using multiple cycles of the PCR, generally 30−40. During each cycle of the PCR, the reaction tube is first heated to ∼94 °C, which denatures the double-stranded template DNA. The temperature is then dropped to <55 °C (typically), which allows the primers to anneal to their target sequences, and then to 72 °C to enable the *Taq* polymerase to extend from both primers, thus creating a duplicate copy of the DNA region between the two primers. This duplicated region is generally <5 kb, although it is now possible to amplify much larger fragments. As each duplicated copy becomes template for the next cycle of PCR, the amplification is exponential, whereby a single copy is potentially amplified to 2^n (n = number of cycles). Therefore, in a typical PCR of 35 cycles, $\sim 3.4 \times 10^{10}$ copies can potentially be generated.

In a multiplex PCR, more than one set of primers is included to enable the simultaneous amplification of a number of target DNA regions. The more target regions amplified, the more reliable the technique. A disadvantage of the technique is that prior sequence knowledge is required, and it is technically challenging to design optimal reaction conditions.

RFLP of the 16S rRNA Gene. This is a rapid technique involving amplification of the 16S rRNA gene using the PCR with primers targeted at universally conserved regions within this gene. The resulting amplicon is then restricted with an appropriate restriction enzyme, and the resulting restriction fragments are size separated by agarose gel electrophoresis, forming a characteristic RFLP. The choice of restriction enzyme depends on the particular genus and must be experimentally determined. Kullen et al. (*56*) used this fingerprinting technique to differentiate an ingested bifidobacteria isolate from the indigenous bifidobacteria in human subjects. In this study, the restriction enzyme *Hae*III, which recognizes the sequence GGCC, was found to generate a characteristic RFLP for this genus. As this is a PCR-based technique, it can be carried out on very few cells, thus eliminating the need to culture colonies. This is a major advantage of all PCR-based fingerprinting techniques. The discriminatory power of this technique is generally low because of the conserved nature of this gene. However, it has probably the highest reproducibility of all the PCR-based fingerprinting techniques.

Arbitrary Primed (AP) PCR. AP-PCR differs from conventional PCR in that only a single short primer (usually 10−12 bases), the sequence of which is arbitrarily chosen, is used. To enable the primer to anneal to the template DNA, the stringency of the reactions is reduced, allowing the primer to bind to regions where it exhibits nearest homology. When these primer binding sites are within a few thousand bases and are on opposite strands, the DNA region between can be amplified. The more products that are amplified, the more discriminatory the technique. This fingerprinting technique was first described in 1990 and was termed AP-PCR (*57*) or RAPD (*58*). As this rapid technique is very discriminative and can be applied to organisms for which no sequence information is known, numerous protocols have been developed for many bacterial genera. The major disadvantage of the procedure is that subtle changes in reaction conditions can change a banding pattern, thus compromising the reproducibility of the technique.

Triplet Arbitrary Primed (TAP) PCR. The low reproducibility of arbitrary priming results from unintended changes in reaction conditions. By purposely introducing specific changes to the reactions in three otherwise identical reactions, the amplicons, which are susceptible to changes in the reactions, can be identified. The triplet reaction is conducted in parallel at three different annealing temperatures (38, 40, and 42 °C) and, following gel electrophoresis of each reaction, the banding patterns are compared. Bands that are present in at least two lanes are considered to be resilient to small changes in reaction conditions and are therefore considered in the fingerprint analysis. The technique can be discriminative to the species and strain levels and has been demonstrated to reliably fingerprint bifidobacteria and lactic acid bacteria (*59*).

Speciation Tools. Accurate and unambiguous identification of probiotic bacteria is essential to conduct credible scientific studies. A recent study of commercially available lactobacilli and bifidobacteria showed that many were misclassified at the species level (*60*). Identification and characterization of the isolates by classical methods have many shortcomings, in particular, a lack of accuracy and its labor-intensiveness. The advent of molecular tools has greatly expanded the ability to reliably identify isolates and also to calculate the evolutionary relatedness between strains. Fingerprinting techniques (discussed above) can be used for identification, but this strategy is limited by the extensiveness of the particular fingerprinting database. As databases for the different fingerprinting techniques grow, this approach will increase in usefulness. A major advantage of using a fingerprinting approach for typing purposes is its rapidity and, consequently, it is conducive to analyzing a large throughput of unknown isolates. A disadvantage can be the use of fingerprinting techniques with low discriminatory powers, such as ribotyping.

16S rRNA Sequence Analysis. The most accepted means for typing unknown isolates is by sequence analysis of 16S rRNA. This tool for classifying organisms and evaluating their evolutionary relatedness was first developed by Woese and co-workers (reviewed in ref *61*). The available database of rRNA sequences is now

extensive, which allows detailed studies to be made on the phylogenetic position of unknown isolates. This molecular phylogeny approach has revolutionized the field of microbial ecology and has allowed meaningful phylogenetic relationships between microbes in natural ecosystems to be discerned (*62*). Technically, this is very feasible as the PCR can be used to directly amplify the 16S rRNA gene directly from colonies using primers, which are directed at universally conserved regions at both ends of the gene. The entire PCR amplicon, which is ~1.5 kb, can then be directly sequenced and compared to the rRNA database.

This technology has greatly helped our understanding of the phylogenetic relationships between the major genera in the human intestine. The two major genera, *Bacteroides* and *Bifidobacterium*, are very heterogeneous, and the use of 16S rRNA sequence analysis has contributed enormously to their phylogeny (*63*, *64*). Understanding the phylogeny of bifidobacteria is particularly important, as members of this genus are prime candidates for inclusion in probiotic cultures for human consumption. Without comparative studies on the dominant bifidobacteria present in the human intestine, there is limited scientific rationale for selecting specific strains for probiotic purposes.

ITS Sequence Analysis. Within the genus *Bifidobacterium*, the rRNA sequence is highly conserved (*63*) and may not be sensitive enough for the desired level of comparative analysis that is likely needed for selection of worthwhile strains. To complement the rRNA sequence approach, analysis of another molecule, which is not as conserved as 16S RNA but still retains the characteristics of a meaningful phylogenetic marker, is required. Two important criteria for such a molecule are that it is universally present in bacteria and it has high sequence conservation, which illustrates that sequence changes are less influenced by temporary environmental changes. The region between the 16S and 23S rRNA genes, termed the internal transcribed spacer (ITS), has been used for a more detailed analysis of bifidobacteria (*63*). This molecule is universally present in bacteria but exhibits very low sequence conservation (*65*), thus limiting its accuracy as a phylogenetic marker. In addition, the ITS regions within the same bacterial strain can exhibit significant heterogeneity (*66*). However, the molecule is technically very feasible to obtain as PCR can be used to amplify the molecule directly from colonies using primers directed at universally conserved regions within the bordering 16S and 23S rRNA genes. Leblond-Bourget et al. (*63*) evaluated the sequence analysis of this molecule to further characterize bifidobacteria and found that it gave much more sensitivity than the rRNA analysis. However, because of the high heterogeneity of the ITS, this molecule cannot be relied upon for meaningful phylogenetic analysis, particularly during large-scale analysis of intestinal bifidobacteria.

recA Gene Sequence Analysis. Another candidate for sensitive species differentiation within the genus *Bifidobacterium* is a short internal segment of the *recA* gene. It possesses the important criteria of being universally present in bacteria and being highly conserved. The *recA* gene encodes the RecA protein, which plays vital roles in recombination, DNA repair, and SOS response (*67*). Studies have established that meaningful bacterial phylogenetic relationships can be obtained by sequence analysis of the RecA protein (*68*, *69*). These studies highlighted the possibility that a segment of the *recA* gene might be a useful molecule for phylogenetic analysis within a particular genus. This concept was applied to the genus *Bifidobacterium* in a study by Kullen et al. (*70*). The molecule was obtained from both type and intestinal bifidobacteria isolates direct from colony isolates using PCR with primers directed to regions within the *recA* gene, which are universally conserved in bacteria. This approach yielded a fragment of ~300 kb, which could be rapidly sequenced using a single sequence reaction from either end. The phylogenic relationship obtained by sequence analysis of this short segment of the *recA* gene compared favorably with the analysis from the complete rRNA gene. Given the rapidity of obtaining the sequence information of this *recA* molecule, coupled with its theoretical and experimentally substantiated role as a meaningful phylogenetic molecule, it is potentially a very valuable tool for comparative phylogenetic analysis of human intestinal bifidobacteria isolates.

In Situ Tools. The advent of culture-independent molecular techniques for studying microbial communities in situ has revolutionized the field of microbial ecology. Recently, these molecular techniques have begun to be directed to the human intestine and are unveiling many more mysteries of its complex microbial flora. The techniques currently being used include tools for studying the diversity of flora present and tools for analyzing specific bacteria in situ.

Studying the Diversity of Microbes. As many bacteria within natural ecosystems are not detected using standard culturing techniques, an incomplete picture of the true diversity within that ecosystem would result. The ability to be able to directly examine the diversity of a microbial community without culturing can enable a more complete picture of the diversity to be obtained. The tool used for accomplishing this in the human intestine relies on amplifying 16S rRNA directly from fecal samples using PCR. Generally, fecal samples are first enriched for bacterial cells by differential centrifugation and can be used directly in PCR, or total DNA or RNA can first be isolated. As rRNA is often thousands of times more plentiful then rRNA genes, total RNA can be used as a template if a reverse transcriptase treatment is also included. Targeting rRNA, rather than DNA, can be used to preferentially identify bacteria, which are metabolically more dynamic, as faster growing bacteria have greater amounts of rRNA. Once the target has been amplified from fecal material, it consists of a heterogeneous mix of products, within the amplicon. Cloning the amplicon into a standard sequencing vector can result in a bank of individual rRNA gene clones. These clones can then be sequenced and phylogenetically analyzed. Recently this strategy has been applied to the analysis of human intestinal microflora, and indications are that many of the sequences identified were from novel organisms, not previously identified (*71*). This is extremely noteworthy, as it suggests that culture techniques may not be as effective at analyzing the biodiversity of this environment as was generally perceived. Further use of this insightful molecular tool to study the diversity of organisms in the human intestine will have a major impact on the field of probiotics.

The 16S rRNA amplicon from feces can also be used to directly obtain a fingerprint of the microbial diversity within the individual. This can be achieved using

denaturing gradient gel electrophoresis (DGGE), which can separate individual rRNA genes from the universally amplified product. Although all of the individual rRNA species within the amplicon are of the same length, electrophoresis through a linearly increasing gradient of denaturants can separate the products of different sequence (*72*). The principle is based on the melting of rRNA genes at specific denaturing points based on their sequence. Therefore, each individual sequence will begin to melt at a characteristic denaturing point. Urea and formamide are generally used to form the denaturing gradient. However, temperature can also be used, thus creating a temperature gradient gel electrophoresis (TGGE). When an individual rRNA gene begins to melt, its migration slows and it becomes separated from the PCR amplicon. Further migration of the gene through the denaturing gradient, however, could result in the double-stranded DNA becoming denatured into single-stranded DNA products. To prevent this, a GC clamp, consisting of 30−50 "G" and "C" bases attached to the 5′ end of one of the primers used to amplify the rRNA product, can be used. As G/C-rich DNA regions are resilient to melting, this tag can maintain the integrity of the double-stranded rRNA genes. This approach can provide a fingerprint of the complexity of the intestinal flora of an individual. It has been used to monitor overall changes in the diversity of flora in individuals over time (*73*).

There are limitations with these rRNA-based culture-independent techniques, regarding their estimations of biodiversity in natural habitats. One limitation concerns the disparity in the number of rRNA operons in different bacteria. Clearly, an organism with one copy of rRNA genes will be under-represented compared to organisms with eight or more copies. The disparity is magnified if rRNA is used as the template for the PCR. Another limitation concerns the use of universal primers for the amplification of the rRNA product. These primers are not identically homologous to all bacteria and will not amplify all rRNA products with the same efficiency. This can result in a disparity in the biodiversity in favor of those organisms more conducive to PCR with the primers used. To help control for this limitation, different sets of primers targeting different universally conserved regions within the rRNA can be used.

Analyzing Specific Bacteria in Situ. The ability to be able to obtain information on single cells in situ in fecal or intestinal samples is very intriguing. This event is now feasible, primarily due to the development of sensitive fluorescent labels, which enable probes to be visualized by fluorescent microscopy. This technology led to the development of fluorescent in situ hybridization (FISH), which can be used to enumerate bacteria directly in situ in fecal samples by fixing cells on a glass slide (reviewed in ref *74*). Typically, probes based on the 16S rRNA are used, as probes can be designed to be nonselective (for total bacterial counts) or specific for a genus or species. Another advantage of targeting the 16S rRNA is the abundance of target, thus increasing the sensitivity of detection. The use of nonselective FISH has revealed that the total number of bacteria in human fecal samples is ∼10-fold higher than estimated using standard culture techniques (*75*, *76*). However, genus-specific FISH for bifidobacteria has shown this genus to be present at numbers similar to those using culture methods. With the development of better probes, FISH has the potential to uncover the true dominant species in the human intestine (*76*, *77*).

SELECTION OF PROBIOTIC BACTERIA

Although the present knowledge of the diversity of the microbial flora in the human intestine is far from complete, it is widely accepted that members of the genera *Lactobacillus* and *Bifidobacterium* are potential probiotic candidates for the small and large intestines, respectively. However, selection of individual bacterial strains for use as effective probiotics is a complex process, especially as all of the features an isolate should possess for maximum efficacy are not yet known. With a growing knowledge of bacterial interactions in and with the human intestine, this process should become more defined soon. On the basis of the current understanding of these interactions, there are a number of features that are important considerations for strain selection.

Source of Strains. Even though essentially all animals contain an abundance of species/strains of both *Lactobacillus* and *Bifidobacterium*, it is now widely accepted that an effective human probiotic should be of human origin. The underlying reason for this is that human intestines are sufficiently different from those of animals such that the isolates suited to those environments would not necessarily be suited to the human intestine. Currently, an animal isolate of bifidobacteria (*B. animalis*) is widely used commercially in human probiotics, although it is often declared as *B. longum* by the manufacturer (*60*). This strain may not be a very effective probiotic, but it may have some health benefits during its transient passage through the intestine at high numbers. Although not presently researched sufficiently, it is likely there is a large diversity in human intestines depending on age, sex, race, and diet. With a better understanding of these diversities, future probiotic cultures may consist of multiple strains of the same species to account for this diversity and enable beneficial effects in a wider group of people.

Acid/Bile Tolerance. A very practical consideration in the selection of an isolate for use as a probiotic is its ability to survive the transit to its intestinal target via the stomach and duodenum. The high acid conditions of the stomach require that the organism should have a high tolerance to acid. This is frequently measured by evaluating its ability to survive pH 3 or lower for 3 h, an average passage time through the stomach. Alhtough some strains will survive this treatment directly, demonstrating acid resistance, others could tolerate it only if they were first primed at a higher pH. This was demonstrated in my laboratory for some bifidobacteria, lactobacilli, and *Streptococcus thermophilus*, which could not directly tolerate pH 3 (*78*). Exposing organisms to mild stresses can induce tolerances, which can enable the organism to withstand greater stresses. Therefore, potential isolates for probiotic cultures need not necessarily be directly resistant to pH 3, as long as they tolerate it after prior priming at a higher pH. This is easily accomplished when yogurt is used as the delivery vehicle for the probiotic, as the mild acid conditions may be sufficient to effectively prime many isolates.

Similarly, isolates need sufficient tolerance to bile to enable safe passage through the duodenum to their site of action. This is generally measured by simply plating

out isolates on media containing bile salts. This process, however, largely measures direct resistance to bile rather than just tolerance. In vivo, a probiotic culture's stress response will already be strongly induced following passage through the stomach. It is known that exposure to one stress can induce a response that protects cells against multiple stresses (*79*). As the stress response is already induced at that stage, it may be capable of surviving the bile in the duodenum. This is pertinent as many candidate isolates may be overlooked if they do not display direct resistance to bile, when in reality the ability to induce sufficient tolerance is all that is required.

***β*-Galactosidase Activity.** This feature of a probiotic organism is pertinent when the culture is intended to reduce the symptoms of lactose maldigestion. Isolates display large differences in the amounts of this enzyme they produce. It should also be noted that most wild-type isolates have very low levels of this enzyme but have much higher levels following growth in lactose as the sole carbon source. The induced *β*-galactosidase level is therefore the pertinent feature for a probiotic organism intended to aid subjects with lactose digestion. Enzyme activities of potential candidates can also be readily increased up to ~3-fold following a straightforward classical mutagenesis approach (*80*). As this is a food grade, classical approach, it does not cross the realm into the currently used definition for a genetically modified food.

Adherence to Intestinal Cells. To survive and compete successfully in a natural ecosystem, such as the human intestine, which is in constant flux, a bacterium needs to be able to attach to the available attachment sites in the intestine. Without the ability to attach, an organism can only be transient, thus limiting its potential effectiveness. Measuring the adherence ability of isolates, however, is not very easy, given the diversity in human intestines and the diversity of cells within. Currently, adherence is measured primarily using two in vitro cell lines Caco-2 and HT-29. As these essentially represent a single cell from the intestines of two individuals, it is not a thorough test on the true adherence abilities of isolates. Positive attachment to these cell lines, however, can be viewed as a good indicator of their potential to attach. However, it is not scientific to draw negative conclusions about an isolate's adherence potential from a failure to attach to these in vitro cell lines, given the inherent limitations of the assay.

Niche Fitness. This is a measure of the suitability of an organism for survival and competition in an ecosystem. The human intestine is a complex and enclosed ecosystem and is arguably one of the most difficult to study. Consequently, all of the features a probiotic organism should have to compete effectively in this environment are not yet understood. However, from general principles of bacterial competition, some criteria are presently important. Essentially these principles are competition for nutrients and production of antimicrobial compounds. As the carbon sources available in the intestine are quite variable, there is no simple test to screen isolates for effectiveness. However, the area of prebiotics has arisen from a derivation of this concept, that is feeding subjects specific nutrients which can be used only by the target organisms. More specifically, this involves the use of oligosaccharides, such as inulin or fructooligosaccharide (FOS), which are carbohydrates that cannot be absorbed or utilized by the intestinal flora, except for members of *Bifidobacterium*. Members of this genus, which can effectively utilize these carbohydrates, have a competitive advantage over other large intestine flora when other carbon sources are limiting. Screening for bifidobacteria that can efficiently utilize certain prebiotics will therefore enable the probiotic to be ingested with the prebiotic, thus providing a competitive advantage for the organism when both arrive in the nutrient-limiting large intestine. This concept of consuming a probiotic with a prebiotic is referred to as synbiotics (*81*).

The production of antimicrobial compounds gives an organism a competitive advantage if they can inhibit their competitors. Examples of antimicrobial compounds are organic acids, H_2O_2, and bacteriocins. Whereas all of the lactic acid bacteria produce organic acids, only some produce bacteriocins. Therefore, it is considered to be more favorable to select an organism for probiotic purposes if it can produce bacteriocins, which can inhibit deleterious intestinal organisms, such as *Escherichia coli* and clostridia. Numerous bacteriocins of lactic acid bacteria have been described, and they differ in the spectrum of target organisms that they can inhibit (reviewed in ref *82*). Candidate probiotic organisms can be readily screened for the ability to produce broad spectrum bacteriocins.

Another competitive feature for bifidobacteria may be the ability to out-compete other large intestine flora for iron. All known organisms, except certain lactobacilli, require iron for growth (*83*). The extreme insolubility of iron at neutral pH makes it a limiting factor for growth in neutral pH environments (*84*). Because iron plays such a central role in the metabolism of organisms, the expression of many cellular processes has become regulated by iron, making this element a global regulator of gene expression in bacteria (*85*). The primary functions regulated by iron in bacteria are those involved in iron assimilation. Organisms secrete iron-scavenging compounds (siderophores) to enable them to acquire the iron they need for growth (*84*). Dominant colonizers of an environment (especially environments of neutral pH) generally have better iron-scavenging systems and can inhibit the growth of other competing organisms by depriving them of iron (*86*).

Bifidobacteria are superior competitors of their natural environment, the large intestine, which is a neutral pH environment. Do they successfully compete in this environment against other bacteria, such as *E. coli*, by depriving them of iron for growth? Studies on iron uptake by bifidobacteria in vitro suggested they did not secrete iron-binding compounds (*87*). However, these studies were done in batch cultures where the production of organic acids would reduce the pH and make iron more bioavailable. It was, therefore, concluded in my laboratory that the possibility still existed that bifidobacteria produced iron-scavenging proteins. Also, Mevissen-Verhage et al. (*88*) had previously shown that infants fed cow's milk fortified with iron had higher counts of *E. coli* and fewer bifidobacteria in their feces than infants fed unfortified milk. This study would suggest that bifidobacteria use iron scavenging to compete against *E. coli* in the large intestine. To test this hypothesis, we screened recent isolates of bifidobacteria for the ability to produce compounds in a low-iron environment, which could inhibit the growth of other bacteria in an iron reversible fashion. A number

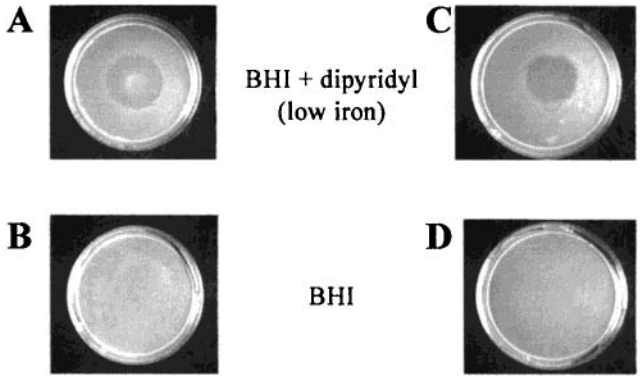

Figure 1. Inhibition of *M. luteus* and *E. coli* by a compound produced by a *B. longum* isolate during prior growth on a filter disk in the center of the plate: (A) inhibition zone of *M. luteus*; (B) no inhibition of *M. luteus*; (C) inhibition zone of *E. coli*; (D) no inhibition of *E. coli* (Ibrahim, Pimental, and O'Sullivan, unpublished data).

of isolates were identified with this characteristic, and the inhibition observed was solely due to iron deprivation (Figure 1). It was also demonstrated that these isolates could out compete *Clostridium perfringens* and *C. difficile* for iron (Ibrahim and O'Sullivan, unpublished results), which are deleterious inhabitants of the large intestine. This iron-scavenging ability is therefore a likely important feature for selection of bifidobacteria isolates for probiotic purposes.

CONCLUSION

Although the probiotic concept is theoretically sound, the present availability of cultures, which can provide probiotic benefits to a human host, is debatable. In recent years the development of molecular tools has resulted in a greater understanding of the intestinal microflora. This is providing a clearer picture of what attributes a successful probiotic organism should have. The culmination of these endeavors will enable the selection of cultures for specific probiotic purposes based on a more complete set of scientific criteria.

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