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Selection and design of probiotics

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Abstract

Over the past 5 years the probiotic field has exploded with a number of new cultures, each purported to elicit a variety of benefits. Lists of functional characteristics and benefits, in vivo, are now commonplace to any presentation on probiotics. Scientifically established health claims remain among the highest priorities to companies who seek to establish solid health benefits that will promote their particular probiotic. The scientific community faces a greater challenge and must objectively seek cause and effect relationships for many potential and currently investigated probiotic species and strain combinations. Rational selection and design of probiotics remains an important challenge and will require a platform of basic information about the physiology and genetics of candidate strains relevant to their intestinal roles, functional activities, and interactions with other resident microflora. In this context, genetic characterization of probiotic cultures is essential to unequivocally define their contributions to the intestinal microbiota and ultimately identify the genotypes that control any unique and beneficial properties. Strain selection and differentiation, based on the genetic complement and programming of a candidate probiotic, then becomes feasible. Looking ahead, it will be vital to the development of this exploding field to correlate important characteristics in probiotics with known genotypes and regulatory controls that are likely to affect functionality and beneficial outcomes, in vivo.

Keywords: Probiotics; *Lactobacillus*; *Bifidobacterium*; Lactic acid bacteria

1. Introduction

The food industry in the United States is the country's largest manufacturing industry with revenues exceeding 500 billion dollars per year. The key research priorities of this industry that are targeted for the next decade include:

1. increase processing efficiencies with a reduction of environmental impact;
2. expand development of value-added technologies;
3. understand and utilize component interactions in formulated food systems;
4. develop and promote strategies to control food borne illnesses;
5. increase development of foods that promote health and well being.

Issues and topics of food microbiology are paramount throughout this group of emerging research priorities. Among these, the exploding area of

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functional foods and probiotics shows considerable promise to expand the industry into new arenas, through developments likely to arise in at least four of these priorities. The economic impact of the projected US functional foods market is significant, recently estimated at 134 billion (Sanders, 1998) and spans foods including natural functional foods (cranberry juice, green tea), FOSHU foods/ingredients (*Foods and ingredients for specified health use*), formulas (infant and elderly), medical foods, nutraceuticals, and drug foods. Within this continuum between food and drug, there are seemingly unlimited niches for the development of food systems that promote optimal nutriture, health, and general well being. In the face of these exploding developments, the challenges for tomorrow's food microbiologist will be more exciting and span issues on food safety, preservation, bioprocessing, and most certainly, probiotics.

2. Probiotics — the quest

A probiotic is a 'live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). This definition was broadened by Havenaar and Huis in't Veld (1992) to a 'mono- or mixed-culture of live microorganisms which benefits man or animals by improving the properties of the indigenous microflora'. The gastrointestinal tract of vertebrate animals is the most densely colonized region of the human body (Tannock, 1995a,b). There are $\sim 10^{12}$ bacteria per gram of contents in the large intestine, which is estimated to contain several hundred bacterial species (Savage, 1977). It is widely accepted that this collection of microbes has a powerful influence on the host in which it resides. It is implicit in the definition of probiotics that consumption of probiotic cultures positively affect the composition of this microflora and extends a range of host benefits (Sanders, 1998; Tannock, 1999a), including:

1. pathogen interference, exclusion, and antagonism;
2. immunostimulation and immunomodulation;
3. anticarcinogenic and antimutagenic activities;
4. alleviation of symptoms of lactose intolerance;
5. reduction in serum cholesterol;
6. reduction in blood pressure;
7. decreased incidence and duration of diarrhoea (antibiotic associated diarrhoea, *Clostridium difficile*, travelers, and rotaviral);
8. prevention of vaginitis;
9. maintenance of mucosal integrity.

Many of the specific effects attributed to the ingestion of probiotics, however, remain convoluted and scientifically unsubstantiated (O'Sullivan et al., 1992), and it is rare that specific health claims can be made (Sanders, 1993). Over decades of work, the science supporting the probiotic concept remains remarkably weak (Sanders, 1993; Tannock, 1999a), owing mostly to three main issues. First is our lack of understanding about the complexity of the gastrointestinal environment and failure to recognize the thousands of potential species that impact the microecology of the GI tract. Measurement of the impact of probiotics, focused currently on lactobacilli and bifidobacteria, must now be extended to the larger microbial communities that are affected and, moreover, are also likely to effect overall probiotic functionality. Second, confusion over the identity, viability, and activity of probiotic strains has been a historical problem which has contributed to the misidentification of cultures under laboratory and clinical investigations. Third, single probiotic strains are often proposed to contribute a multitude of benefits across many individuals in a test population. This situation is aggravated by the high costs of clinical trials that force consideration of 'one strain versus one placebo' experiments in attempts to prove efficacy of the simplest probiotic concepts in limited subject populations.

Nevertheless, industrial interest in developing probiotics and probiotic-functional foods is thriving, driven largely by the market potential for foods that target general health and well being (Sanders, 1998). In this regard, funds to establish clinical efficacy have increased dramatically in the past few years, fueled by the promise of concept substantiation and label claims.

3. Probiotic science: developments

In recent years, advances in microbiology and molecular biology advances have stabilized a number of fronts that have been historically problematic for

probiotics. First, the availability of molecular tools to properly identify probiotic species and individual strains has virtually eliminated confusion over strain identity and ancestry. While classical microbiological approaches remain very important for culture propagation, selection, enumeration, and phenotypic characterization (e.g. fermentation profiles; temperature/salt/pH tolerances), these properties do not unequivocally classify a culture taxonomically. Singularly, phenotype characterization is problematic for identification of closely-related species, like those in the *Lactobacillus acidophilus* complex, that occupy similar ecological niches and are likely to play similar functional roles (Fig. 1). Phylogenetic analysis is the most powerful tool for taxonomic classification of bacterial cultures (Woese, 1987). The accumulating information on ribosomal RNA sequences provides a growing resource for comparative identification of probiotic cultures, both established candidates and potentially new candidates. Lactobacilli and bifidobacteria constitute the two most important probiotic groups under consideration, owing to their recognition as members of the indigenous microflora of humans, their history of safe use and the general body of evidence that supports their positive roles. At this juncture, phylogenetics has recognized 54 species of lactobacilli, 18 of which are considered to be of some interest in probiotics; and 31 species of *Bifidobacterium*, 11 of which have been detected in human feces (summarized from Tannock, 1999b, Table 1).

Phylogenetic analysis can be conducted in varying degrees and combined with other characteristics (phenotypes) as needed to make definitive taxonomic classifications. A number of sequence-based typing systems have been used to analyze conserved regions of the ribosomal RNA operon or other conserved genes in probiotic cultures (O'Sullivan, 1999):

1. PCR amplification and sequencing of ~1500 bp of the 16S rRNA gene;
2. PCR amplification and sequencing of ~450 bp of the internal transcribed spacer region;
3. PCR amplification and sequencing of alternative genes that are universally present and highly conserved; e.g. *recA* gene of bifidobacteria (Kullen et al., 1997b);
4. PCR amplification and sequencing of ~50 bp variable region of 16S rRNA to identify members of the *L. acidophilus* complex (Fig. 2, Kullen, Sanozky-Dawes, Crowell and Klaenhammer, unpublished).

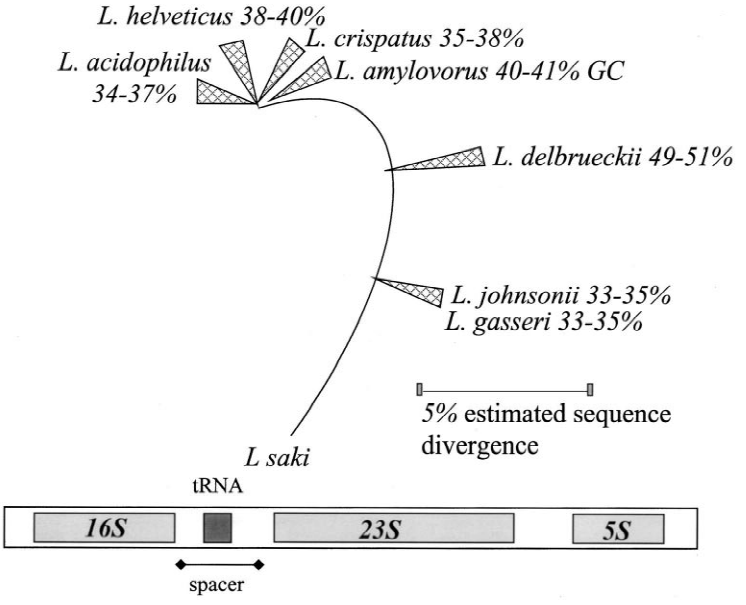

Fig. 1. Phylogenetic relationships among members of the *Lactobacillus acidophilus* complex. Adapted from: Schleifer et al., 1995. Phylogenetics for the genus lactobacillus and related genera. Syst. Appl. Microbiol. 18, 461–467.

Table 1
Primary species of lactic acid bacteria used as human probiotics[a]

Lactobacillus species	*acidophilus*	*amylovorus*	*casei*	*crispatus*
	gallinarum	*gasseri*	*johnsonii*	*plantarum*
	reuteri	*rhamnosus*	*salivarius*	
Bifidobacterium	*animalis*	*bifidum*	*breve*	*infantis*
	longum	*lactis (animalis)*		
Streptococcus	*thermophilus*			
Enterococcus	*faecium*			

[a] Collins et al., 1998; Tannock, 1999b.

There are a number of alternative taxonomic classification methods available, most notably including hybridization with species-specific probes (Pot et al., 1993) and generation of profile PCR amplicons by species-specific primers (Tilsala-Timisjarvi and Alatossava, 1997).

While these methods have been used successfully to identify species, the outcomes can be highly variable and the results less definitive than if the ribosomal RNA genes were sequenced directly. In many laboratories, direct sequencing of selected rRNA regions is now the preferred method for species identification of new probiotic cultures. The availability of efficient and cost-effective commercial sequencing facilities has now made bacterial species identification by phylogenetic analysis commonplace. The ability to properly identify probiotic species provides the first giant step toward eliminating any confusion over strain identity and ancestry. Furthermore, the practice is uncovering many potentially new probiotic species that have been hidden below the surface of traditional taxonomic descriptions.

The second key development step in probiotic science has been the availability of molecular methods for identification and tracking of individual strains. Historically, methods used for detection of probiotics in the human gastrointestinal tract include identification by colony morphology (Robins-Browne and Levine, 1981; Goldin et al., 1992; Nielsen et al., 1994), fermentation patterns (Goldin et al., 1992; Johansson et al., 1993), serotyping (Stotzer et al., 1996), or some combination of these methods (Lidbeck et al., 1987; Alander et al., 1997). Despite their limitations, traditional methods continue to be used (Ahrne et al., 1998). Advances in molecular typing techniques now provide reliable methods to identify and differentiate bacterial strains based on the composition of biological molecules such as fatty acids, proteins, enzymes and nucleic acids. The most powerful of these are genetic based molecular techniques used to fingerprint specific

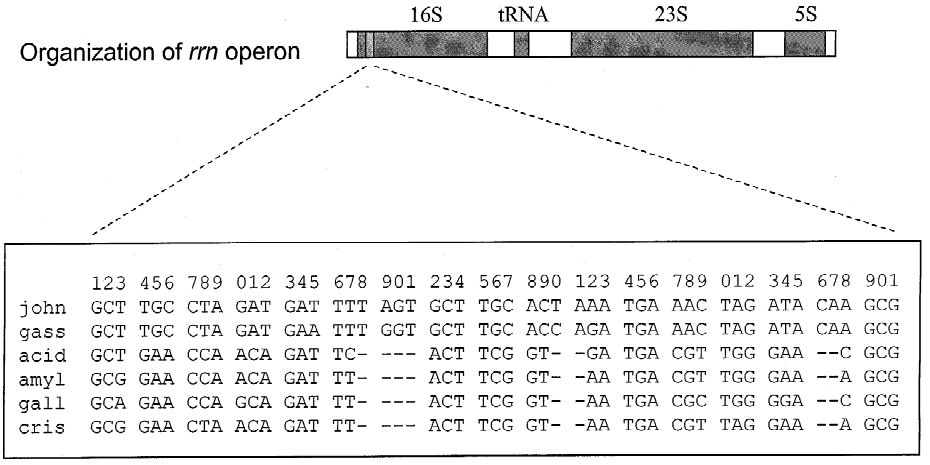

Fig. 2. Variable region (V1) of the 16S rRNA of the six species in the *L. acidophilus* complex.

DNA patterns that are characteristic for a single strain. Methods sporadically applied to probiotic cultures over the past 5 years include randomly amplified polymorphic DNA (RAPD) (Ahrne et al., 1995; Tilsala-Timisjarvi and Alatossava, 1998), plasmid profiling (Johansson et al., 1993), ribotyping (Rodtong and Tannock, 1993; Zhong et al., 1998), and analysis of restriction fragment length polymorphisms of genomic DNA (Johansson et al., 1993; Ahrne et al., 1995; Sanders et al., 1996) and 16S rDNA (Kullen et al., 1997a).

An excellent technical overview of the major fingerprinting methods and their applications in probiotics has been summarized recently by O'Sullivan (1999) and include:

1. ribotyping;
2. restriction fragment length polymorphisms and pulsed-field gel electrophoresis;
3. random amplified polymorphic DNA: including multiplex-PCR, arbitrary primed PCR and triplet arbitrary primed PCR.

All three of these approaches have been used successfully to identify the gastrointestinal survival and passage of a fed probiotic strain in humans (Kimura et al., 1997; Crowell, 1998; Alander et al., 1999). In a recent study, Alander et al. (1999) used a combination of methods to identify the probable presence of *Lactobacillus rhamnosus* GG in the feces and colons of human volunteers. Identification of GG was based on its colony morphology and PCR amplification of an *L. rhamnosus* species-specific amplicon, using primers designed against the 16S rRNA gene. Two particularly interesting results were reported. First, *L. rhamnosus* GG-like colonies could be detected in colon biopsies and feces 14 days after feeding was halted, indicating retention in the colon. Second, whereas GG-like colonies were cleared from the feces by 28 days after feeding, two of seven biopsies were still positive suggesting that the culture persisted at low, but detectable levels in the colonic microflora. This is an interesting report that provides strong evidence for detection of 'GG-like' colony morphologies and amplicons that are specific for *L. rhamnosus* species. However, the methods used fail to unequivocally establish the presence of GG. The molecular tools that are available in genomic fingerprinting are excellent methodologies that can be used to unequivocally link the fed probiotic culture with the strain recovered.

In a study conducted in collaboration with Dunn and Simenhoff of Thomas Jefferson University and our group at NCSU, *L. acidophilus* NCFM was fed to five healthy humans for 3 months (Crowell, 1998). Fecal samples before, during, and following the cessation of capsule feeding were analyzed for total lactobacilli. The presence of NCFM was evaluated by genomic fingerprinting of the predominant colony types in pulsed-field electrophoresis gels, using *Sma*I profiles. The study showed that *Lactobacillus* counts on LBS agar (containing tomato juice) increased upon feeding. At the end of 3 months of feeding, NCFM was confirmed as the predominate *Lactobacillus* genotype enumerated in all five volunteers. After the cessation of feeding, *Lactobacillus* counts dropped to pre-feeding levels. *L. acidophilus* NCFM was recovered from two of five patients after 1 week, and was not recovered after 2 weeks post feeding. In one of the human volunteers the predominant *Lactobacillus* genotype found prior to feeding was again the dominant type recovered after NCFM feeding was halted. Use of genomic fingerprints unequivocally established the presence of NCFM in the fecal samples of the human volunteers fed the probiotic and, further, identified and differentiated other predominant lactobacilli that increased or decreased over the study. Similar to the study of Alander et al. (1999), clearing of the probiotic occurred after cessation of feeding. Restriction enzyme digestion of 16S rRNA genes was used to monitor the fate of an ingested bifidobacteria through the human GIT (Kullen et al., 1997a). In this study, a *Hae*III-generated RFLP of a PCR-amplified portion of the 16S rRNA gene allowed for the differentiation of an experimental, ingested bifidobacteria from endogenous bifidobacteria. Using this means of discrimination, it was determined that during the period of bididobacteria ingestion, the experimental organism became the predominant bifidobacteria in the feces of subjects. However, after feeding stopped, the presence of the organism diminished rapidly and became undetectable after 8 days. Taken together, these studies support assertions that probiotics are not likely to colonize the GI tract permanently (Tannock, 1999a) and require continuous delivery to maintain their presence. A requirement for long term and continuous administration is

certainly a desirable criteria for culture manufacturers, and functional food industries developing vehicles to deliver probiotics.

4. Complex interactions

At this juncture, tracking probiotic strains through food, GI tract or body cavity can be accomplished definitively using traditional methods for selection and enumeration, combined with molecular technologies that identify an individual strain's genetic fingerprint by a variety of approaches. Research on the presence, survival, and retention of probiotics in various scenarios should flourish in the next few years and provide a platform of quality information on strain relationships, ancestries, and potential for survival and competition in the varying environments of food carriers and the GI tract. Forthcoming will be applications for non-culture techniques that will allow in situ detection of a specific probiotic culture within mixed microbial populations (O'Sullivan, 1999). Both prokaryotic in situ PCR and FISH (fluorescent in situ hybridization) rely on primers that will amplify specific sequences in the target organism or group within a milieu of microorganisms. Application of these techniques will ultimately depend upon the availability of unique sequences that are either identified in the genome, or introduced by genetic techniques within the probiotic species. The knowledge and availability of unique sequences for in situ analysis will be an important consideration in the selection and design of probiotic cultures destined for clinical analysis and investigation in complex environments.

The probiotic concept predisposes that the culture(s) delivered will impact the local microflora, which itself is highly complex and variable. Assessing the effect of probiotic cultures on the native microflora by standard microbiological techniques presents many obstacles and has by all scientific accounts, failed to consider the silent majority of non-culturable organisms that are known to dominate in complex microbial communities. Research addressing the impact of probiotics on the native microflora has recently entered a new and exciting dimension, following the development of molecular techniques that can amplify, separate, and then identify major rRNA species present in mixed populations (Felske et al., 1998; Muyzer et al., 1993). The technique employs highly conserved primers for the 16S rRNA gene and uses PCR to amply this region from the collection of bacteria present in a sample (food, environmental, gastrointestinal). The amplicons of different organisms vary in their internal 16S rRNA nucleotide sequence and base composition and, thus, can be separated by thermal or denaturing gel electrophoresis (TGGE or DGGE, respectively). The patterns generated can detect predominant species increasing or decreasing in a mixed microbial population by observing the appearance or disappearance of specific amplicons in the denaturing gel. Major amplicons can be cloned and sequenced in an attempt to identify, by phylogenetic analysis, the responsible microorganisms. This is a major development that will promote characterization of the microbes within any complex ecosystem. Of special interest to probiotic science is the ability to better analyze the microbial world of the gastrointestinal tract and potentially identify new candidates that can exert positive functional roles. Moreover, these rRNA-based technologies allow a quantitative assessment that (i) follows the introduction of a probiotic culture into the GI tract communities, (ii) determines its relative level within the population, and (iii) identifies its potential to alter the presence and/or level of residing microbes.

Given the developments in molecular techniques over the past decade, many of the key issues which have hindered scientific progress in probiotics are now removed. Exacting methods for the identification, tracking, and analysis of probiotic cultures within complex microbial ecosystems are now available and promise to revolutionize our understanding of their functional roles, and in vivo effects.

5. Selection of functional probiotics

The perceived desirable traits for selection of functional probiotics are many. Table 2 presents a list of selection criteria that have been collated from published reports and recommendations that span over 20 years. All the detailed criteria fall into four basic categories — Appropriateness, Technological suitability, Competitiveness, Performance and functionality.

Table 2
Selection criteria for probiotic strains[a]

Appropriateness	
i.	Accurate taxonomic identification
ii.	Normal inhabitant of the species targeted: human origin for human probiotics
iii.	Nontoxic, nonpathogenic, GRAS status
Technological suitability	
iv.	Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution
v.	Viability at high populations (preferred at 10^6–10^8)
vi.	Stability of desired characteristics during culture preparation, storage, and delivery
vii.	Provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes
viii.	Genetically stable
ix.	Genetically amenable
Competitiveness	
x.	Capable of survival, proliferation, and metabolic activity at the target site in vivo
xi.	Resistant to bile
xii.	Resistant to acid
xiii.	Able to compete with the normal microflora, including the same or closely related species; potentially resistant to bacteriocins, acid, and other antimicrobials produced by residing microflora
xiv.	Adherence and colonization potential preferred
Performance and functionality	
xv.	Able to exert one or more clinically documented health benefits (e.g. lactose tolerance)
xvi.	Antagonistic toward pathogenic/cariogenic bacteria
xvii.	Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids, or other inhibitory compounds)
xviii.	Immunostimulatory
xix.	Antimutagenic
xx.	Anticarcinogenic
xxi.	Production of bioactive compounds (enzymes, vaccines, peptides)

[a] Compiled from Crowell (1998), Collins et al. (1998), Conway (1989), Fuller (1989), Gilliland (1990), Havenaar and Huis in't Veld (1992), Johnson et al. (1987), Klaenhammer (1982, 1995), Kullen and Klaenhammer (1999a), Salminen et al. (1996), Sanders (1993) and Tannock (1997).

Many of these criteria are sensibly based on extensive experience with microbial selection, propagation (viability, technological suitability), and safe use of lactic acid bacteria in foods (non pathogenic; non-toxic, genetically stable, normal inhabitant of target species, viability). However, those selection criteria that address competitiveness and performance issues remain controversial because the underlying mechanisms by which probiotics exert functional roles in vivo are not generally understood. Three excellent examples of this are as follows.

Bile tolerance and bile salt hydrolase (BSH) activity. Probiotic bacteria vary considerably in their levels of bile tolerance. The mechanism of tolerance is not understood and the minimum acceptable level of bile tolerance for a candidate probiotic remains unknown. The lactobacilli are also the largest contributors of BSH to the GI tract, but any presumed positive effects (cholesterol metabolism, small bowel colonization) or negative effects (toxicity of deconjugated bile acids in the small bowel) are not understood (Tannock, 1998).

Adjuvant activity and immunostimulation. Substantial documentation has now been presented that lactobacilli can act as immunoadjuvants and immunostimulants, leading to increased levels of IgA and IgM antibodies (Tannock, 1998; Mercenier, 1999). These properties are highly variable among strains and it remains to be determined what features of the cell or cell surface are responsible for exerting these effects. It is also pertinent to investigate how probiotic cultures affect and respond to the intestinal mucosa of the host.

Antimicrobials. On every list of probiotic criteria is a requirement for antimicrobial activity that targets enteric undesirables and pathogens. Not unexpected are reports of suspect antimicrobials among probiotics that are promoted to have inhibitory effects in vivo (Bernet-Camard et al., 1997; Hudault et al., 1997; Cassas et al., 1999). Bacteriocins lead the criteria list as candidates for in vivo activity (Klaenhammer, 1998; Ouwehand, 1999), by inhibiting closely related bacteria competing for similar intestinal niches, or antagonizing a wider range of intestinal undesirables (e.g. *Clostridium*, *Enterococcus*, *Listeria*). Whether or not these antimicrobials or bacteriocins are produced in the gut, or have a functional impact remains to be investigated.

Understanding the mechanisms of how these criteria impact in vivo functionality will present one of the major scientific challenges for probiotics in the coming decade. Genetic content and controls underlie each mechanism and dictate the performance of probiotic cultures across their expected roles. Therefore, this field is perfectly poised to exploit the recent progress in sequencing capacity and functional genomics toward the investigation of these bacteria and their probiotic capabilities (Klaenhammer, 1998). There is now a myriad of possible probiotic strains, coupled with a diverse set of phenotypes, which are being increasingly linked to a variety of benefits. It has become clear that defining and screening important genetic traits, which confer functional probiotic activities, offers considerable promise to attack the insurmountable task of selecting superior strains and building combinations that can elicit unique or multiple effects.

6. Functional genomics: structural and responsive gene systems

Genetic work on probiotic cultures is in its infancy but promises to be a rapidly moving field that will reap rich benefits as knowledge accumulates and new discoveries support practical applications. At this juncture, the most critical research involves the correlation of genotypes to phenotypes that are believed to impact probiotic functionality. The availability of the genome sequences of model probiotic species will greatly accelerate the task of correlating genotypes with the capabilities and behavior of probiotic strains. Genome projects have been initiated on *Lactobacillus acidophilus* and *Lactobacillus johnsonii* (Klaenhammer, 1998). Additional candidates for genome projects are now being considered as a result of the powerful automated sequencers that have now become available and affordable. A genome project on *Bifidobacterium* should be initiated with a model human species, such as the *B. longum*/*B. infantis* group. The first genome projects on probiotic lactobacilli should be completed by the end of 2000. The outcomes are expected to establish a mechanistic understanding of the capabilities of lactobacilli in the gastrointestinal tract, promote new applications for probiotics, lead to improved functionality for existing probiotics, and enhance our ability to select functional probiotic strains and strain combinations using genetic screening.

Genome sequencing and analysis of probiotic cultures promises to identify two major categories of gene systems. First are those that will be *required* for survival and activity in grossly contrasting and changing environmental scenarios (food versus the GI tract). Second are *responsive* gene systems that react to the varied stimuli encountered within the food-carrier or GI tract, (Fig. 3). Induced expression of critical gene systems (e.g. adherence, bacteriocin production, acid tolerance) is likely to be vital among many key functions that determine the survival, activity, and colonization potential of probiotic cultures. The expected roles of probiotics in these highly contrasting environmental scenarios are distinct. Initially, through the food-carrier, it is survival of high probiotic populations with minimal injury. Eventually, in the GI tract, it is passage and survival through a gauntlet of varying stresses (acid, bile) and

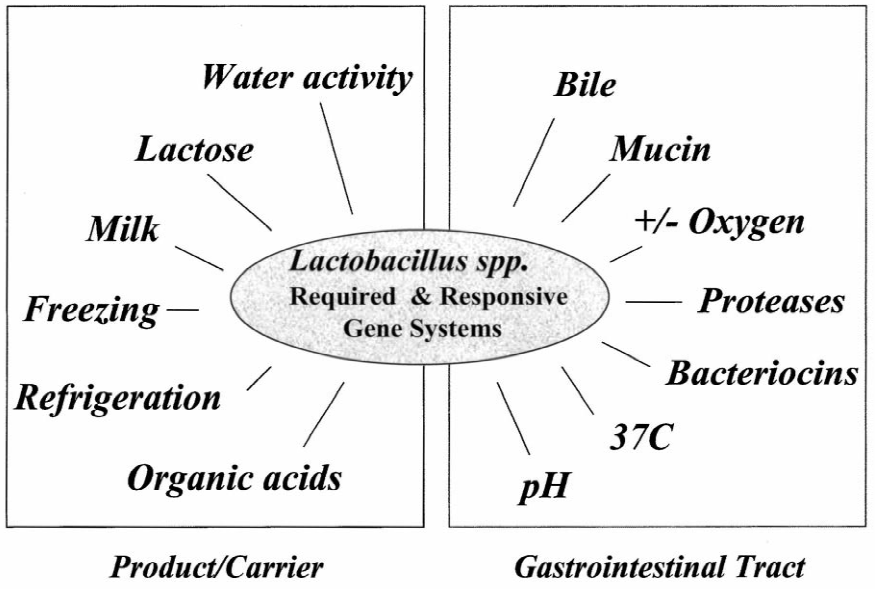

Fig. 3. Stimuli encountered by probiotics in two key environments.

functional probiotic activity (competitiveness and performance) at the targeted in vivo locations. Systematic investigation of behavior under such adverse conditions is an impossible task to manage empirically.

Approaches in functional genomics have now appeared that can identify the required gene systems in probiotic cultures and elucidate inducible gene expression systems and traits that are likely to direct essential probiotic activities and functions, across these contrasting environments. In one example, *L. acidophilus* is being investigated for gene systems that respond to stimuli encountered in either the GI tract or in dairy products used to deliver probiotic cultures (Kullen and Klaenhammer, 1998). Conditionally expressed genes were identified by differen-

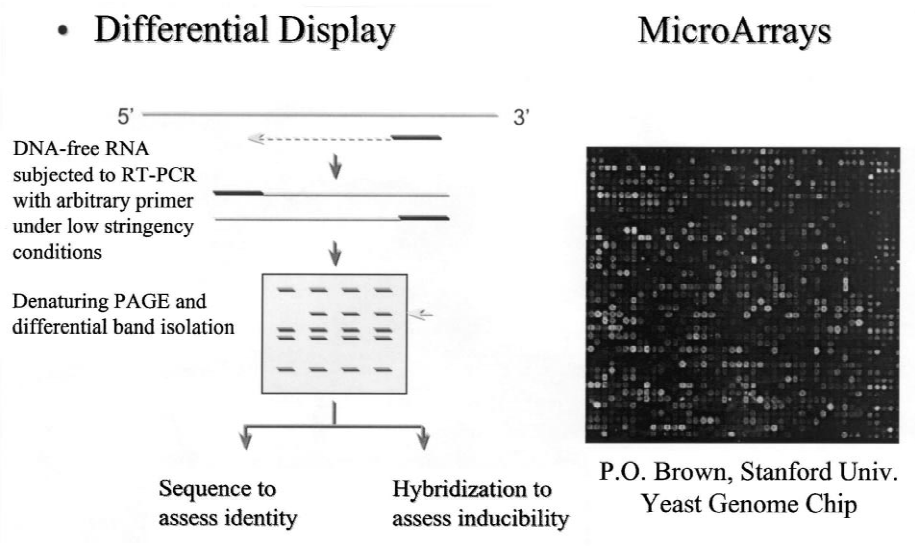

Fig. 4. Identification of conditionally expressed genes.

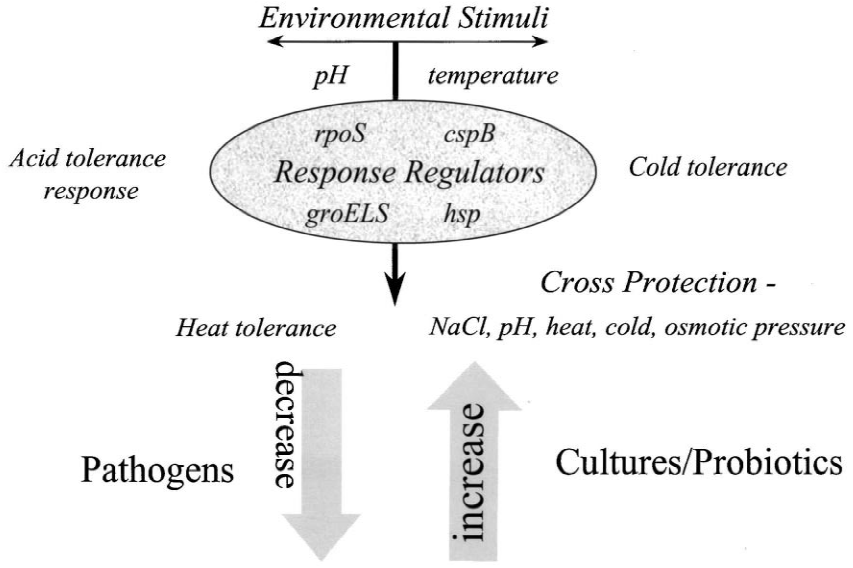

Fig. 5. Modulating stress responses: pathogens versus probiotics.

tial display analysis (Fislage, 1998). RNAs expressed over a time course, following exposure to the stimulus, were subjected to randomly primed RT-PCR amplification and cDNAs separated on denaturing PAGE gels, (Fig. 4). Unique bands appearing after the exposure can be extracted, cloned and sequenced to identify conditionally expressed genes. While false-positives are routinely found for abundantly expressed RNAs, the technique has been successfully used to identify several responsive gene systems in *L. acidophilus* (Kullen and Klaenhammer, 1999b; 1999c).

Over the last few years, genomic science has emerged with a powerful set of tools for comparing genomes and examining differentially expressed genes. Microarray analysis offers the potential to examine differential gene expression across the entire genome (Chetverin and Kramer, 1994; Ramsay, 1998; Lipshutz et al., 1999). As genome sequences for probiotic cultures become available, microarray analysis will be a valuable tool to assess coordinated gene expression by the bacteria across the varying conditions to which they are exposed.

Varying stresses and stimuli are known to induce coordinated gene expression in microorganisms that lead to tolerance states. Sublethal exposure to acid, heat, cold, or salt, as well as entrance into stationary phase of growth, are all known to induce genetic responses in bacteria that can lead to elevated tolerance and cross protection against other stresses, (Fig. 5). Excellent examples have already been illustrated in lactic acid bacteria (Hartke et al., 1994; Kets et al., 1996; Wouters et al., 1998). Stress responsive gene systems are being identified in probiotic cultures and can be exploited to gauge preconditioning treatments that can improve their survival in foods, during storage, and upon delivery to the stomach and GI tract (Walker and Klaenhammer, 1996).

7. Future developments

Moving the probiotic field into the functional genomics area will yield a mechanistic view of the potential for probiotic cultures to be used in both traditional and novel roles. In this regard, molecular tools and gene transfer systems to support self cloning will be needed to advance genetic analysis and modification of probiotic cultures. Efforts should be intensified to construct cloning, expression, and integration vectors that have general utility in probiotic species. The efficiency of these systems is still far below that needed to carry out genetic studies with most probiotic cultures (Kullen and Klaenhammer, 1999a).

There will be many opportunities to enhance existing traits and develop novel properties by genetic modification of probiotic cultures. Targets for genetic modification and improvement include: immunostimulation and oral vaccine development; antimicrobials and bacteriocins; vitamin synthesis and production; adhesions and colonization determinants; production and delivery of digestive enzymes; and metabolic engineering to alter products (e.g. polysaccharides; organic acids) or link cultures with specialty prebiotics designed to enhance the performance of a probiotic in vivo (Kullen and Klaenhammer, 1999a).

Most importantly, probiotic science will benefit from molecular tools that can be used to investigate the complex microbial interactions that occur between the delivered cultures and the residing microflora. It is an arena where fundamental genetic knowledge of the players will guide future efforts to unravel the many roles and activities of probiotic cultures. Tannock (1999a) pointed out that mechanisms of drug action are actively sought out and the knowledge gained is used to guide applications and future developments across the pharmaceutical field. Validity of probiotic science will be derived by the same process where mechanisms can be linked directly to efficacy and applications.

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