

# Genetic Modification of Intestinal Lactobacilli and Bifidobacteria

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## Abstract

**Lactobacilli and bifidobacteria are important members of the gastrointestinal microflora of man and animals. There is a substantial and growing body of evidence that these microbes provide benefits to the host in which they reside. Understanding the roles of these two groups of bacteria in the intestine continues to be a significant challenge. To this end, genetic characterisation and manipulation of intestinal lactobacilli and bifidobacteria is essential to define their contributions to the intestinal microflora, and to potentially exploit any beneficial or unique properties. This review will describe the tools and strategies currently available for the genetic manipulation of lactobacilli and bifidobacteria. Additionally, the ramifications and opportunities that may arise as a result of the genetic manipulation of probiotic lactobacilli and bifidobacteria will be addressed.**

## Introduction

The gastrointestinal tract of vertebrate animals is the most densely colonized region of the human body (112, 127). There are approximately  $10^{12}$  bacteria per gram of contents in the large intestine, which is estimated to contain several hundred bacterial species (99). The accumulated evidence indicates that this collection of microbes has a powerful influence on the host in which it resides. Comparisons between germfree and conventional animals have shown that many biochemical, physiological and immunological functions are influenced by the presence of the diverse and metabolically active bacterial community residing in the gastrointestinal tract (80, 111, 112).

The use of live microbes as dietary adjuncts or probiotics has received considerable commercial and scientific attention (127). Fuller (34) defined a probiotic as a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." This definition was broadened by Havenaar and Huis in't Veld (41) to a "mono- or mixed-culture of live microorganisms" which benefits man or animals by improving the properties of the indigenous microflora. A number of excellent overviews are available on probiotics (8, 27, 36, 37, 60, 81, 94, 95). The potential health benefits attributed to probiotics include the following:

- maintenance of the normal microflora
- pathogen interference, exclusion, and antagonism
- immunostimulation and immunomodulation
- anticarcinogenic and antimutagenic activities
- alleviation of symptoms of lactose intolerance
- reduction in serum cholesterol

Many of the effects attributed to the ingestion of probiotics, however, remain convoluted and scientifically unsubstantiated (81), and it is rare that specific health claims can be made (96). This situation could be resolved by providing exact descriptions of the probiotic microorganisms involved and developing an understanding of the control mechanisms for those functional properties that are vital to the survival and activity of these organisms in the human gastrointestinal tract. However, the perceived desirable qualities of probiotics are many (Table 1), and it is highly unlikely that any one strain will harbor all the qualities or provide the multitude of proposed benefits. There is a myriad of possible probiotic strains, coupled with a highly diverse set of phenotypes and potential benefits. Therefore, screening genetic traits offers considerable promise in attacking the almost insurmountable task of surveying for functional probiotic properties, or building combinations of probiotic strains that can elicit multiple effects. Moreover, genetic modification of probiotic bacteria offers the added developmental potential to annex new beneficial activities (e.g. vaccine presentation) or improve the effectiveness of existing properties (e.g. bacteriocin levels).

Lactobacilli and bifidobacteria constitute two extremely important groups of probiotic bacteria. As members of the normal microflora of the gastrointestinal tract of humans, they offer considerable potential as probiotics because of their history of safe use and the general body of evidence that supports their positive roles. Genetic analysis and manipulation of these bacteria will be paramount to understanding their probiotic roles and maximising their performance *in vitro* and *in vivo*. The field is now poised to exploit genetic approaches in the investigation of these bacteria and their probiotic capabilities. However, recent surges in the development of genetic tools for lactic acid bacteria have lagged significantly for bifidobacteria and intestinal lactobacilli. Genetic work on bifidobacteria is in its infancy. For the lactobacilli, there have been a number of significant developments (reviewed in 53, 86). However, these efforts have been dispersed over a collection of newly recognized, and collectively significant probiotic lactobacilli (e.g. *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*) resulting in only incremental steps in our understanding of the genetic programming and potential of the *Lactobacillus* species, overall. The following is intended to overview the tools available for genetic manipulation of lactobacilli and bifidobacteria and discuss opportunities for genetic analysis and modification of these two key groups of probiotic bacteria.

## Lactobacilli

Members of the genus *Lactobacillus* constitute an extremely diverse and important group of organisms, of

Table 1. Characteristics Expected of Potential Probiotic Strains

1	accurate taxonomic identification
2	normal inhabitant of the species targeted: human origin for human probiotics
3	nontoxic and nonpathogenic
4	genetically stable
5	capable of survival, proliferation, and metabolic activity at the target site
6	adherence and colonization potential preferred
7	stability of desired characteristics during culture preparation, storage, and delivery
8	viability at high populations preferred at $10^6$ - $10^8$
9	production of antimicrobial substances, including bacteriocins, hydrogen peroxide, and organic acids
10	antagonistic toward pathogenic/cariogenic bacteria
11	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
12	resistant to bile
13	resistant to acid
14	immunostimulatory
15	able to exert one or more clinically documented health benefits
16	amenable to production processing: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution
17	provision of desirable organoleptic qualities (or no undesirable qualities) when included in fermentation processes

Compiled by Crowell, 1998 (23) from: Conway, 1989 (21), Fuller, 1989 (34), Gilliland, 1990 (39), Havenaar and Huis in't Veld, 1992 (42), Havenaar *et al.*, 1992 (41), Johnson *et al.*, 1987 (48), Klaenhammer, 1982 (51), Salminen *et al.*, 1996 (95), Sanders, 1993 (96), and Tannock, 1997 (113).

which some are members of the colonic microflora. *L. acidophilus* has been considered the most important of the gastrointestinal lactobacilli, but in 1980 (58), the group of organisms previously known as "*L. acidophilus*" was shown to be highly heterogeneous. The species was, subsequently, separated into the DNA homology groups, A and B, which now form six separate species: *L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *L. gasseri* and *L. johnsonii* (33, 47, 58). While the name of *acidophilus* was retained by the neotype strain, ATCC 4356, this species is not the dominant lactobacillus found in the intestine of man and other vertebrate animals (75). In addition to the species comprising the *L. acidophilus* group, *Lactobacillus salivarius*, *L. casei*, *L. plantarum*, *L. reuteri* and *Lactobacillus brevis* are found in the gastrointestinal tract of humans and other animals, including avians.

### Lactobacillus Plasmids and Vectors

The genetic analysis and modification of lactobacilli was ushered in by the discovery of broad host-range plasmids and the development of electroporation procedures for DNA transformation. Current cloning vectors for lactobacilli fall into three classes: promiscuous plasmids based on RCR (rolling circle replication) replicons, plasmids with two replication origins for *Escherichia coli* and gram-positive bacteria, and native *Lactobacillus* vectors with selectable markers and alternative replication origins for gram-negative bacteria. The prototype vector of the RCR replicon class is pGK12, which is based upon pWVO1 and contains erythromycin (Em<sup>r</sup>) and chloramphenicol (Cm<sup>r</sup>) resistance markers which were selectable in lactococci, *E. coli*, *Bacillus subtilis* (55), and most *Lactobacillus* species including *L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L. plantarum*, *L. casei*, *Lactobacillus fermentum*, and *L. reuteri* (65). The second category of vectors contains two origins, one functional in *E. coli* and the second in gram-positive bacteria. The most widely used vector of this class is pSA3 (24), which has proven to be particularly useful both as cloning and integration vector in *L. johnsonii*, *L. gasseri*, and *Lactobacillus helveticus* (11, 87, 121). A pair of high- and low-copy number vectors (pTRKL/H), functional in lactobacilli, were constructed using the broad gram-positive host range pAMβ1-based replicons pIL252 (low copy) and

pIL253 (high copy) (109) and an *E. coli* P15A origin (82). pTRKL/H series vectors replicate via a theta mechanism which is broadly functional across lactobacilli and provides more structural stability to recombinant plasmids since a ssDNA intermediate is not involved. These vectors work well in *L. johnsonii* and *L. gasseri*, but not in *L. acidophilus*. The third vector design incorporates selectable markers and alternative replication origins for gram-negative bacteria to small cryptic *Lactobacillus* plasmids, a number of which have been completely sequenced (13, 15, 65, 73, 86, 98). Plasmid vectors based on RCR *Lactobacillus* replicons marked with Em<sup>r</sup> and/or Cm<sup>r</sup>, *lacZ* or *xyI* (xylose catabolism) have been compiled by Pouwels and Leer (86) and continue to appear (50, 54, 120). In some cases, the host range of *Lactobacillus*-based vectors includes lactobacilli and other gram-positive bacteria, but not *E. coli* (85, 86). However, several replicons of plasmids isolated from *Lactobacillus* have now been found that have a broader host range, allowing replication in *E. coli*, *Bacillus*, and various lactic acid-producing bacteria (LAB) and include: pPSC20/pPSC22 (20), pLC2 (54), pGT633 (115), pA1 (120) and pLA106 (98). Vectors of this type may be useful for genetic modification and analysis of intestinal lactobacilli, in which other RCR or theta replicons are unstable. In contrast, plasmids with limited host range replicons have been identified in *L. reuteri* (1) and *L. crispatus* (86). As noted by Pouwels and Leer (86), these vectors may be particularly useful in the development of food/vaccine-grade vectors as their small host range makes them much less likely to promote horizontal gene transfer to other bacterial species.

### Electrotransformation

Electroporation has been used widely for gene transfer and cloning in many lactobacilli (6, 10, 17, 65, 85). Transformation of intestinal lactobacilli at reasonable frequencies has been reported widely for *L. gasseri*, *L. johnsonii*, and *L. reuteri* (1, 31, 50, 64, 65, 66, 77, 115), and these species appear highly amenable for DNA manipulation and gene transfer. However, electroporation protocols that are effective for many lactobacilli have not yielded transformants of type A1, *L. acidophilus* strains. Early reports of electroporation of "*L. acidophilus*" from our laboratory (64, 65) actually involved strains of *L. gasseri* and *L. johnsonii* strains which were reclassified on the basis

of the new taxonomy for the "*L. acidophilus*" group (33, 57). Kanatani *et al.* (50) constructed an Em<sup>r</sup>-vector using a small plasmid from *L. acidophilus* TK8912 and noted transformation frequencies near 10<sup>7</sup>/μg DNA. The taxonomy of this strain has not been reported with reference to the new classifications. Recently, we have successfully transformed two type-A1 *L. acidophilus* strains with minor modifications to the method developed by Bhowmick and Steele (10) for *L. helveticus* (122). *L. acidophilus* ATCC 4356 (A1-neotype) and ATCC 700396 (NCFM/N2) were transformed with pGK12 and pGhost (67) at frequencies ranging from 10 - 10<sup>2</sup> transformants/μg DNA. While these frequencies are low when compared to other species transformable at 10<sup>5</sup>-10<sup>7</sup>/μg, they provide opportunities for genetic modification and experimentation with these two important *L. acidophilus* strains, one the neotype and the second, a commercial strain distributed widely in acidophilus milk and a variety of dietary-health products.

### Conjugation

While broad host range vectors like pAMβ1 and pVA797 are transmissible from heterologous donors (*Lactococcus* and *Enterococcus*) to lactobacilli, reports of native conjugation systems in lactobacilli are few. *L. gasseri* transconjugants did not act as donors for second round transfers (64) whereas *L. reuteri* transconjugants could transfer pAMβ1 to *L. reuteri* and *E. faecalis* (114). Conjugal transfer of plasmid-associated lactose fermenting ability in *L. casei* was reported by Chassy and Rokaw (19). Since this initial report, however, the only evidence which has appeared for a native conjugal system in *Lactobacillus* species is in *L. johnsonii* (76). Bacteriocin (lactacin F) production and immunity in *L. johnsonii* VPI11088 (NCK88) is chromosomally encoded on a conjugative episomal element (30, 77). This conjugation system was employed to mobilize pSA3 in conjugation experiments from *L. johnsonii* (pSA3) donors leading to the recovery of a pSA3 resolution product carrying IS1223 (121). This IS-element is functional in *L. gasseri*, *L. johnsonii*, and *L. acidophilus*. While it is clear that conjugation is a mechanism for gene transfer in lactobacilli, the extent and usefulness of conjugative systems have not been examined to a significant degree. Because many of the type A1 strains of the *L. acidophilus* group are recalcitrant to electroporation, conjugation may be a more useful and potentially broader range tool for gene transfer in lactobacilli.

### Integration and Insertion Systems

Integration of genes/vectors into the bacterial chromosome is a critical genetic tool for insertional mutagenesis, creation of physical/genetic maps, and directed manipulations such as gene stabilization, fusion, amplification, deletion, and replacement. In the lactobacilli, a number of new technologies are emerging that utilize IS-elements, *attP*/integrase systems, or homologous recombination strategies via suicide or temperature-sensitive replicons.

Several different IS elements have been found in lactobacilli (108, 110, 121, 126). One of these IS elements, IS1223, has been used for construction of suicide integration vectors using pSA3 (121) and pGhost replicons (Aoyama, Walker and Klaenhammer, unpublished). Integration experiments have demonstrated that IS1223 directs random insertions in *L. acidophilus* and *L. gasseri* chromosomes where there is no detectable homology for

the IS element. In contrast, insertions in *L. johnsonii*, where resident copies of IS1223 are found, show a site preference. While their use as stable integration vectors would be limited due to their propensity for additional transposition events, the discovery of functional IS elements in lactobacilli should aid in the development of functional mutagenesis and insertional vectors for a variety of intestinal lactobacilli.

Establishment of the prophage state by temperate bacteriophages is a highly efficient and site-specific integration system. Insertion at a specific chromosomal location (*attB*) is mediated by a small region of homology on the phage (*attP*) and a phage-encoded integrase (*int*). The *attP* and *intG* of the *L. gasseri* temperate phage φadh have been cloned, sequenced, and used for construction of site-specific suicide integration vectors (31, 87). While the vectors are functional across strains of *L. gasseri*, the *attB* sequence is not conserved in other intestinal lactobacilli (DeAntoni, Fremaux, Raya, and Klaenhammer, unpublished). The *attP* and *intG* of the *L. delbrueckii* subsp. *bulgaricus* temperate phage mv4 have also been used in the construction of a site-specific integration vector (7). A particularly attractive aspect of this system is that the integration site for the vector is at the 3' end of a tRNA<sup>Ser</sup> gene and occurs without inactivation of this gene in lactobacilli. There are several advantages that phage-based integration systems offer for the genetic manipulation of lactobacilli for food or medical applications: insertions are stable and at single copy; large fragments encoding complex operons could be integrated; and, insertions occur at a specific, non-essential site that should not disrupt culture viability or activity.

Homologous recombination has been used extensively in the construction of integration vectors for accomplishing gene disruption, amplification, replacement, and insertions in a variety of bacteria. An important element for integration experiments is a reasonable frequency of electroporation since generation of integrants relies on both successful transformation and recombination events. As noted earlier, transformation via electroporation can be applied throughout the lactobacilli, but many species are transformable at frequencies that are only marginal for many integration experiments. To circumvent problems with low efficiencies, autoreplicating or temperature-sensitive plasmids, that replicate conditionally or are unstable in the absence of selection, should be employed. While the pGhost series of vectors contain temperature sensitive origins (67), these plasmids are problematic in lactobacilli because outgrowth of transformants at the permissive temperature (28°C) is very inefficient. Both pSA3 (3, 10, 88, Walker and Klaenhammer, unpublished) and pGK12-type plasmids (28, 44) exhibit features of conditional replication in lactobacilli and these plasmids have been exploited for various integration experiments via homologous recombination. In one report of homologous recombination in an intestinal *Lactobacillus* strain, disruption of the *lafI* gene occurred via a gene disruption cassette on pSA3, which was introduced into *L. johnsonii* (3). In this instance, conditional replication of pSA3 was directed by manipulation of the temperature and antibiotic concentration. Disruption of the *cbh* gene, which codes for conjugate bile salt hydrolase, was accomplished by delivering a chloramphenicol resistance gene-containing, disruption cassette on a ColE1 replicon in *L. plantarum*

(61). Similarly, Fitzsimons *et al.*, (28) integrated an active fragment of the  $\alpha$ -amylase gene into the chromosome of *L. plantarum* at the *cbh* locus by utilizing an autoreplicative plasmid. Aside from these reports, the exploitation of homologous recombination for genetic manipulation for the intestinal lactobacilli has not occurred.

### Gene Expression

Cloning and characterisation of *Lactobacillus* genes has been accompanied by physical and phenotypic analysis of their expression signals. Many *Lactobacillus* genes are expressed in *E. coli* indicating that their expression signals are similar enough to be recognized in other bacteria (86). Moreover, phenotypic selection or complementation in *E. coli* has proven to be a successful strategy for cloning a number of genes which have been characterized to date (124, 105). The DNA-dependent RNA polymerase from *L. acidophilus* transcribed some, but not all *E. coli* promoters, *in vitro* (79). *L. acidophilus* promoters were also transcribed, and sequence analysis identified a -10 region similar to the *E. coli* consensus promoter sequence. Surveys of the transcriptional and translational signals of lactobacilli have revealed the following consensus sequence features (18, 86):

Promoters	-35	-10
	TTGACA < 17 bp ave > TATAAT	
	Ribosomal Binding Site	Start Stop
	AGGAGG < 6-10 nt ave > AUG	UAA

It was noted that these should be weighted cautiously due to known exceptions (e.g. rare start codons GUG & UUG),

variation among species in %GC content, and the relatively small number of expression regions that have been sufficiently characterized. There is some limited information about regulated promoters, RNA processing, inducers, repressors, and antiterminators in select systems described in *L. casei* (regulation of *lacEGF* operon transcription via transcription antitermination), *L. actobacillus pentosus* for xylose metabolism (negative control of *xylA/B* operon by a negative repressor, xylose inducer, and catabolite repression), *L. plantarum* (two component regulatory system for plantaricin), *L. amylovorus* (glucose repression of  $\alpha$ -amylase expression/secretion signals), and *L. helveticus* (SOS-like induction of helveticin J) (25, 44, 29; reviewed in 73, 86). Polycistronic operons have been described with contiguous and overlapping genes which can increase the efficiency of translation of downstream genes through translational coupling (reviewed in 86). Control of gene expression will be vital to food and medical applications now envisioned for the lactobacilli. In this regard, accelerated characterisation of genetic operons, expression systems, and their sensing and regulatory machinery is needed, particularly for the intestinal species.

Heterologous genes from a diverse group of microorganisms have been expressed in lactobacilli under the control of their own native heterologous promoter, a *Lactobacillus* promoter, or another promoter (Table 2). The current list includes genes and expression signals from gram-negative and gram-positive bacteria, sporeformers, and fungi. While expression may occur, the level can vary dramatically based on the gene, promoter, and expression host. Heterologous gene expression will allow construction of novel lactobacilli with potentially valuable properties. However, additional efforts are needed to isolate strong, weak, and regulated promoters from intestinal lactobacilli where gene expression can be controlled under the conditions in the gastrointestinal tract.

Table 2. Expression of Heterologous Genes Among Gastrointestinal Lactobacilli

Protein	Expression Host	Origin	Pomoter	Reference
Acidocin B	<i>L. plantarum</i>	<i>L. acidophilus</i>	native	62
Alcohol dehydrogenase	<i>L. casei</i>	<i>Zymomonas mobilis</i>	P32 - <i>Lc. lactis</i>	40
$\alpha$ -Amylase	<i>L. plantarum</i>	<i>L. amylovorus</i>	amyA	28
	<i>L. plantarum</i>	<i>B. leicheniformis</i>	amyL & <i>L. plantarum</i>	44
	<i>L. plantarum</i>	<i>B. stearothermophilus</i>	native	103
$\beta$ -Galactosidase	<i>L. plantarum</i>	<i>B. amylooliquefaciens</i>	native	49
	<i>L. casei</i>	<i>E. coli</i>	cbh	86
$\beta$ -Lactamase	<i>L. casei</i>	<i>E. coli</i>	slpA - <i>L. brevis</i>	100
	<i>L. gasseri</i>			
	<i>L. plantarum</i>			
Cellulase	<i>L. plantarum</i>	<i>Cl. thermocellum</i>	native	104
Chitinase	<i>L. plantarum</i>	<i>Serratia marcescens</i>	P32 - <i>Lc. lactis</i>	16
Chloramphenicol acetyltransferase	<i>L. casei</i>	pC194	amyA, L-ldh	86
Cholesterol oxidase	<i>L. casei</i>	<i>Streptomyces spp.</i>	native	116
Endoglucanase	<i>L. plantarum</i>	<i>Cl. thermocellum</i>	native	104
	<i>L. reuteri</i>	<i>Bacillus macerans</i>	native	43
EZZ-VD4	<i>L. plantarum</i>	<i>Staph. aureus</i>	spa - <i>S. aureus</i>	93
Lafi immunity protein	<i>L. acidophilus</i>	<i>L. johnsonii</i>	P6 - <i>L. acidophilus</i>	3
	<i>L. gasseri</i>			
Levanase	<i>L. casei</i>	<i>Bacillus subtilis</i>	native	123
	<i>L. plantarum</i>			
Lysostaphin	<i>L. casei</i>	<i>Staph. simulans</i>	native	38
M6-gp41E	<i>L. plantarum</i>	<i>Strep. pyogenes</i>	P25 - <i>S. thermophilus</i>	45
Pyruvate decarboxylase	<i>L. casei</i>	<i>Zymomonas mobilis</i>	pMGE36e - <i>Lc. lactis</i>	40
Restriction complex Llal	<i>L. acidophilus</i>	<i>Lc. lactis</i>	P6 - <i>L. acidophilus</i>	26
	<i>L. gasseri</i>			
	<i>L. johnsonii</i>			
	<i>L. plantarum</i>			
Superoxide dismutase	<i>L. gasseri</i>	<i>E. coli</i>	P32- <i>Lc. lactis</i>	92
Xylose isomerase	<i>L. casei</i>	<i>L. pentosus</i>	xylA	84
Xylanase	<i>L. plantarum</i>	<i>Cl. thermocellum</i>	native	104

Heterologous gene expression in lactobacilli has developed a critical need to investigate and control excretion and secretion processes in order to export proteins, enzymes, and potentially antigenic epitopes. The S-layer genes of *L. brevis* (119) and *L. acidophilus* (12) have been cloned and sequenced. The regulatory and secretion signals of the *L. brevis* S-layer gene has been used in the design of highly efficient synthesis and export system for heterologous proteins and epitopes. Gene fusions to TEM- $\beta$ -lactamase showed that the S-layer gene promoters and secretion signals functioned well in *Lactococcus (Lc.) lactis*, *L. plantarum*, and *L. brevis*, while in *L. casei* and *L. gasseri*, the recognition of these signals was less efficient (100). Using the *Bacillus licheniformis*  $\alpha$ -amylase gene as a reporter gene, Hols *et al.*, (44) successfully cloned a number of *L. plantarum* expression and secretion signals capable of directing extracellular  $\alpha$ -amylase. The regulatory and secretion signals for  $\alpha$ -amylase from *L. amylovorus* are also functional in *L. plantarum* (28). An expression system in *L. plantarum* and *L. fermentum*, based upon the regulatory signals of protein A of *Staphylococcus aureus* was used to direct the synthesis and export of a gene fusion product, containing a region of a chlamydial major outer-membrane protein gene (93). Similarly, Hols *et al.*, (45) designed an expression system containing the P25 promoter of *S. thermophilus*, *IdhD* RBS from *L. pentosus*, and the secretion signal of M6 protein of *Streptococcus pyogenes*. This system was capable of the expression and secretion of a large quantity (approx. 10 mg/L culture medium) of the model antigen, M6-gp41E. Lastly, small N-terminal leader sequences with a Gly-Gly processing motif are believed to direct the excretion and activation of class II-bacteriocins (52, 117). LAB can use bacteriocin ABC transporters to export heterologous peptide bacteriocins, using either homologous or heterologous N-terminal extensions (2, 4, 117). Whether or not gene fusions with these leader sequences would direct the export of heterologous, non-bacteriocin peptides or proteins has yet to be established. It is apparent, however, that signal peptide recognition and processing systems are present in lactobacilli which recognize, cleave, and secrete heterologous prepeptides.

### Bifidobacteria

Bacteria in the genus *Bifidobacterium* were initially described by Tissier around the turn of the century. Since this time, the classification of this group has been a point of some contention and confusion. The initial classification of bifidobacteria as *Bacillus* plus the phenotypic and morphological attributes that they share with many lactobacilli led to the popular belief that they belonged to the genus *Lactobacillus* (9). Classification by molecular methodologies have provided evidence that this is indeed a distinct genus and is more closely related to the *Actinomycetaceae* family than to *Lactobacillaceae* (59, 102). As a result of their time spent in a poorly defined taxonomic position, little is known about the genetics of bifidobacteria and application of recombinant DNA technology to these organisms has been slow to occur. At present, there are 26 species of the genus *Bifidobacterium* (22, 101), but those commonly observed in the gastrointestinal tract are: *B. adolescentis*, *B. animalis*, *B.*

*angulatum*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* (32, 56, 71, 101). Sanders *et al.*, (97) and Yaeshima *et al.*, (125) have demonstrated the widespread presence of *B. animalis* in a variety of commercial dairy products distributed world-wide. Meile *et al.*, (72) recently made a description and proposal of a new species, *B. lactis*, which was isolated from a commercial yogurt. Given this strain's immediate habitat (fermented milk), very close phylogenetic proximity to *B. animalis* and the high degree of identity between the 16S rRNA sequences (98.6% over more than 1.4 kb) of this strain with *B. animalis*, it is likely that *B. lactis* is represented in the *B. animalis* isolates analyzed by Sanders *et al.*, (97) and Yaeshima *et al.*, (125). Because of its widespread occurrence in fermented dairy foods, *B. animalis*, under any name, is certainly of significance to probiotics research from an industrial perspective.

### Bifidobacterial Plasmids and Vectors

Plasmids in members of the genus *Bifidobacterium* were initially reported by Sgorbati *et al.*, (106). Similar to the lactobacilli, plasmids are detected only in a few species. From bifidobacteria isolated from the human gastrointestinal tract, only *B. longum* (83, 106, 107, 70) and *B. breve* (14, 46) have been shown to harbor plasmids. These plasmids are all cryptic and most have not been characterised beyond restriction mapping. One cryptic plasmid, pMB1, has been sequenced and appears to encode essential replication proteins (91). The two putative replication proteins, whose genes have been designated *orf1* and *orf2*, encoded on pMB1 show similarity to proteins encoded by plasmids pXZ10142 of *Corynebacterium glutamicum* and pAL500 of *Mycobacterium fortuitum*. It is noteworthy that, like *Bifidobacterium*, the genera *Corynebacterium* and *Mycobacterium* are members of the family *Actinomycetaceae*.

Since these first observations of plasmids, several innovations in the plasmid biology of bifidobacteria have occurred. Vectors, capable of transforming a variety of bifidobacteria, are of the general utility type with distinct replication origins and markers selectable in bifidobacteria and *Escherichia coli*. *E. coli*-*Bifidobacterium* shuttle vectors derived from the small cryptic plasmid described above, pMB1 (90, 91, 74), and from other less well defined plasmids (69) have now been constructed. The first of these, pRM2 (74), contains spectinomycin ( $Sp^r$ ) and ampicillin ( $Ap^r$ ) resistance determinants for selection in *B. longum* and *E. coli*, respectively. pMR2 also harbors a useful multiple cloning site, the putative origin of pMB1 and a ColE1 replication origin for *B. longum* and *E. coli*, respectively. Recently, new varieties of pMB1-based vectors have been constructed with  $Sp^r$ ,  $Em^r$  and  $Cm^r$  determinants for selection in bifidobacteria and the  $Ap^r$  determinant for *E. coli* (90). A small (2.8 kb) pMB1-based vector, pTRE3, which contains no cryptic DNA, a MCS and  $Cm^r$  was also introduced with this series of shuttle vectors. While pTRE3 does not replicate in *E. coli*, it demonstrates excellent segregational stability (>95% cells harbor plasmids after 100 generations without selection) in *B. animalis* (90). In addition to the pMB1-based vectors, *E. coli*-*Corynebacterium* shuttle vectors have been shown to replicate in *B. animalis* (5), which is in agreement with phylogenetic positioning of these two genera. It has also been demonstrated (5) that plasmid pLP825 (85), which harbors a replicon from *L. plantarum*, and the broad host

range *Lactococcus* plasmid, pGK12 (55), are incapable of transforming *B. animalis*, suggesting that the replication functions of these AT-rich plasmids are poorly recognized by bifidobacteria.

Initially, transformation efficiencies of a variety of species of bifidobacteria with these plasmids were poor and irreproducible (74), owing primarily to the fact that the procedures were designed for other bacteria. However, recent developments in transformation protocols by modification of growth medium and electric field strength (89), modification of the electroporation buffer, and preincubation of cells at 4°C prior to addition of DNA and electroporation (5) have dramatically increased transformation efficiency for all strains tested thus far, including those significant to probiotic research.

### Genetically Modified Microbes

While there is considerable potential for genetic modification and improvement of probiotic bacteria, there are formidable barriers that will limit commercial use, public acceptance and environmental release of genetically modified organisms (GMOs), particularly those capable of colonising body cavities and mucosal surfaces. Foremost among these barriers will be public acceptance of probiotic GMOs. This is an important issue that will be faced in every instance when a probiotic GMO is considered for release. Drawing comparisons to the success of the plant biotechnology industry in the U.S.A, it could be expected that public acceptance of probiotic GMOs will improve concurrently with incremental exposure to microbial products of biotechnology that are advantageous and provide tangible benefits. Various studies have already shown that consumers in developed countries will accept products manufactured or containing GMOs, if they offer benefits to health and product quality that the consumer can clearly recognize (118). In this regard, because probiotics by definition, offer health benefits, consumers may willingly accept and use genetically modified probiotics if there are substantial and tangible benefits provided over their traditional counterparts.

Industries that manufacture lactic cultures and probiotics may also view GMOs favourably if self-imposed guidelines that adhere strictly to regulatory positions are evoked. The industry should be expected to favourably consider manufacturing GMOs if they offer a clear competitive advantage over their traditional counterparts. These advantages may reflect processing and economic gains, but the stakes can become significantly higher if health-based benefits can be associated with the probiotic GMO, or the product used to deliver it. Again, any linkage to health and well being has the potential to favour industrial positions on probiotic GMOs if clear and tangible benefits can be realized. At this point in time, any recombinant DNA technology used to create probiotic GMOs would require strict adherence to the concepts of self cloning (using DNA originating only from the same strain/species; sequence analysis of any modifications - e.g. deletions, additions - to ensure that new reading frames are not generated and that antibiotic resistance markers are not present in GMO). Ultimately, any GMO would require regulatory review and safety approval. In the U.S.A., regulatory scrutiny is focused on the safety of the GMO, not the process by which it was

constructed. Indeed, biotechnology occurs via precise genetic modifications where the specific genetic changes are defined and the safety of the GMO can be quickly and rationally assessed. This approach is generally applauded by scientists over "black box" mutation and selection strategies, where the genetic changes are often not known or defined. In fact, such "fry and try" strategies may be viewed in considerably less favour if in the course of genome sequencing projects, any remnants of undesirable genes (toxins, virulence factors) are uncovered in generally regarded as safe (GRAS) lactic acid bacteria. Nevertheless, probiotics are well positioned in the "natural" and "functional" food categories. Any industries aligned with manufacturing of natural and organic products would be expected to adamantly exclude GMOs and favour traditional probiotics as a more natural approach.

### Possible Developments

Genetic work on probiotic lactobacilli and bifidobacteria 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, some of the more critical research areas are as follows:

- Phenotype/genotype correlation of microbial characteristics that impact on probiotic functionality
- Genome sequencing of lactobacilli and bifidobacteria
- Molecular tools and gene transfer systems to support genetic modification and self cloning
- Genetic modification to enhance existing characteristics and develop novel properties
- Molecular signatures and tags
- Investigate gene transfer and dissemination

### Phenotype/Genotype Correlation of Microbial Characteristics that Impact Probiotic Functionality

Evidence supporting the *in vivo* roles of probiotics is needed to support the development of this industry. As such, demonstration of critical properties, genetic controls, and how they impact on *in vivo* functionality will be important. In this regard, the availability of the genome sequences of model probiotic species will facilitate correlation of genotypes with the capabilities and behaviour of probiotic strains. The genome project on *Lactobacillus acidophilus* will be completed by 2000 and is expected to fuel important efforts to understand the capabilities of lactobacilli in the gastrointestinal tract. Similarly, a genome project on *Bifidobacterium* should be initiated, with a model human species being a member of the *B. longum/B. infantis* group.

### Molecular Tools and Gene Transfer Systems to Support Self Cloning

These techniques will be needed to support genetic analysis and modification of probiotic cultures. Efforts should be intensified to construct cloning, expression, and integration vectors that are of general utility in probiotic species. In conjunction, the availability of plasmid replicons, genetic markers, promoters, and terminators that are "self", originating from the targeted probiotic species, need to be developed concurrently. It is emphasized that efficient gene delivery systems (transformation, conjugation, transduction) are vital in the conduct of any genetic analysis

or modification of probiotic strains. The general utility and efficiency of these systems is still far below that needed to carry out genetic studies with many probiotic cultures.

### Opportunities to Enhance Existing Characteristics and Develop Novel Properties by Genetic Modification of Probiotic Cultures

Potential targets for genetic modification and improvement include: immunostimulation and oral vaccine development; antimicrobials and bacteriocins; vitamin synthesis and production; adhesins and colonisation 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*. In this regard, one attractive genetic target is to create molecular signatures or tags on the genomes of probiotic cultures. Modifications in the DNA sequence of an individual probiotic strain can be designed to allow rapid detection by specialised probes, PCR primers, or fingerprinting patterns. Molecular tags will not only allow definitive identification of novel probiotic strains, but further allow tracking their survival and dissemination through the environment or host organism. In this regard, use of new molecular methods to assess changes in the residing microbial communities (63, 78), upon delivery of probiotics, will be another important research area.

Lastly, it will be vitally important to begin work on gene transfer and dissemination, *in vivo*. Probiotic cultures are often delivered in high daily doses into the oral and intestinal cavities. Little information is currently available on gene transfer from, or to, probiotic cultures, *in vivo*. Investigation of the genetic routes and transfer mechanisms available for probiotics in the gastrointestinal tract, as well as the capacity for probiotics to disseminate genes, will be an important area for future research. These studies will be a vital component in the portfolio of work needed to assess the safety of genetically modified probiotics in the host and the environment.

### Conclusions

Genetic manipulation of intestinal lactobacilli and bifidobacteria presents the opportunity to investigate, determine, add and improve the traits considered important for their functional roles as probiotics. The potential to present bacteria, proteins, enzymes, and antigenic epitopes in selected intestinal locations via a bacterial delivery and expression system is quickly becoming a reality. Equally important may be genetic modifications that improve their *in vivo* effects or allow molecular tracking of fed probiotic strains in the human gastrointestinal tract. While substantial progress has been made in this exciting field over the past decade, there remain many barriers to be overcome in defining gene transfer, expression, and control, in lactobacilli and bifidobacteria of gastrointestinal origin.

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### Further Reading

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