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Genetic tools for investigating the biology of commensal *lactobacilli*

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1. ABSTRACT

Lactobacilli belong to the genus *Lactobacillus*, the largest genus among the lactic acid bacteria (LAB). They are abundant in plant material and food resources, or they may inhabit niches in or on the bodies of humans and animals, as commensals. Lactobacilli of food origin are commercially important in the production of dairy products, fermented meats, vegetables, and sourdough, and many of their properties have been well studied. Commensal lactobacilli are good candidates for development as probiotics. In recent years, the general biology and host interaction mechanisms of commensal lactobacilli have attracted great interest. Although the metabolic pathways, predicted gene functions, and some phenotypic traits, of commensal lactobacilli can be inferred or deduced to an extent by the growing number of *Lactobacillus* genome sequencing project, various genetic tools are still required to confirm their phenotypic properties and biological traits. The current state of the art with respect to the available complement of genetic tools including genomic resources, and more traditional approaches to investigate the biology of commensal lactobacilli, will now be reviewed.

2. INTRODUCTION

Lactobacilli are Gram-positive, non-spore-forming, acid-tolerant, aerotolerant or anaerobic with low G+C content (1). With a long application history, lactobacilli are important microbes in industry, which contribute to the production of cheese, yogurt, and other fermented products. Apart from being isolated from a broad range of nutrient-rich environments (2-7), lactobacilli are also part of the commensal human microbiota. They are abundant in the vagina (8), and were also found in the oral cavity (9), the small intestine and the large intestine (10). Because of their history of safe use and their natural presence in the human intestinal tract, commensal lactobacilli offer considerable potential as probiotics. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (11). It is known from numerous analyses that probiotics benefit the host through a variety of mechanisms (12-17).

Even more so than other lactic acid bacteria (LAB), *Lactobacillus* species vary greatly in their

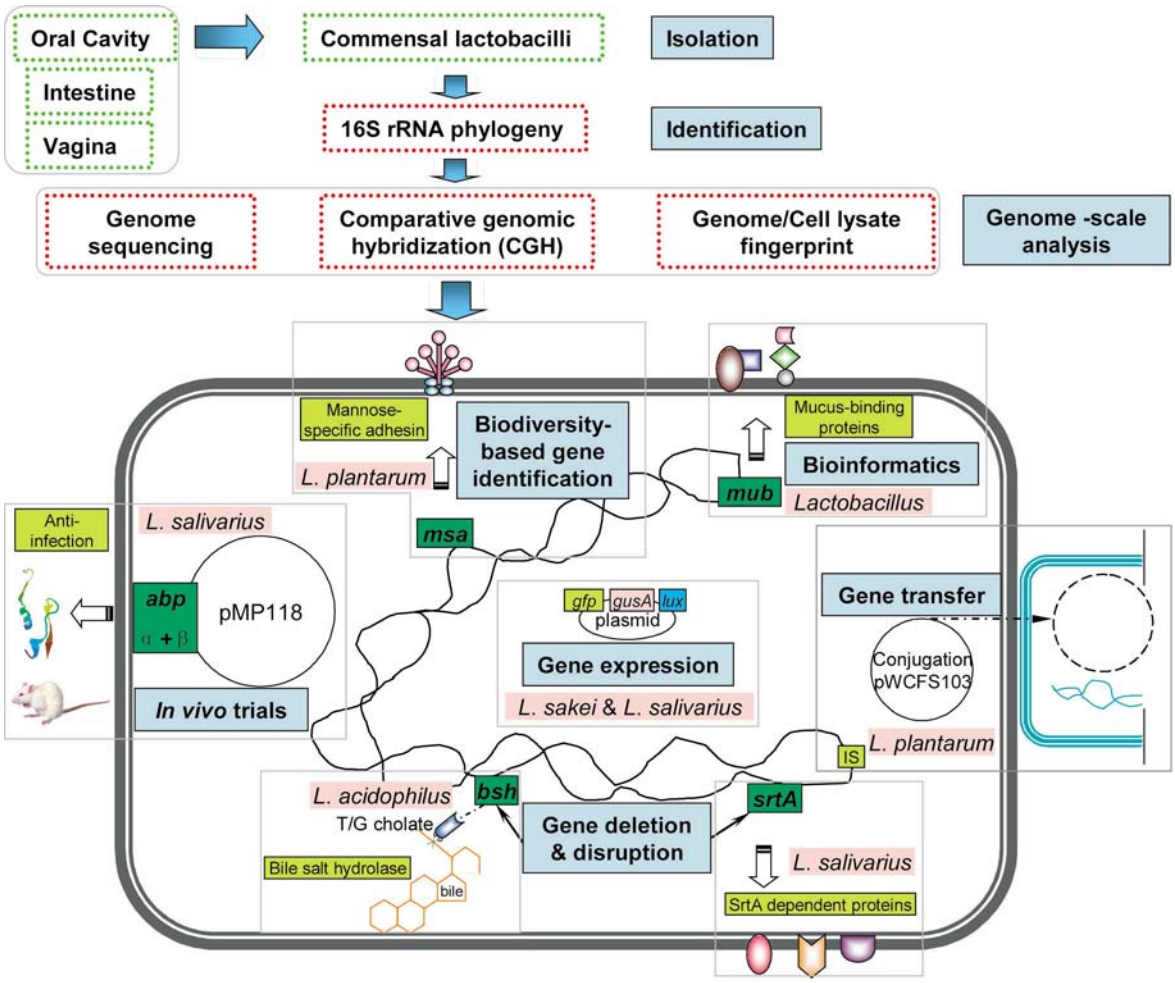

Figure 1. Available genetic tools for functional analysis of candidate probiotic genes in commensal lactobacilli Characterization of mannose-specific proteins in *L. plantarum* WCFS1 by biodiversity screen (21); Bioinformatics analysis of mucus-binding proteins in Lactobacilli (22); Functional analysis of *in vivo L. salivarius* UCC118 anti- *Listeria monocytogenes* activity by gene disruption (23); Functional analysis of sortase and sortase dependent proteins in the interaction with intestinal epithelial cells by gene deletion in *L. salivarius* UCC118 (24); Functional analysis of *bsh* by gene disruption in *L. acidophilus* NCFM (25); Horizontal gene transfer through conjugation in *L. plantarum* WCFS1 (26); Adaptation of endogenous plasmids for construction of gene cloning and expression vectors in *L. salivarius* UCC118 (28).

phenotype, and scientists are faced with an unusually high level of phylogenetic diversity (18). Functional characterization of individual *Lactobacillus* species therefore requires a variety of genetic tools that may need to be adapted or customized for individual species, which usually have distinguishing properties to be investigated. For example, it is established that some lactobacilli benefit the host through modulation of the immune system (19, 20). Colonization (adhesion or persistence) is an expected, but possibly not essential, feature for strains being developed as probiotics. However, strains which are probiotic are expected to have a wider impact on intestinal ecology, which may include production of antimicrobial substances, and usually requires survival in the stressful environment of the GI (gastrointestinal) tract, including resistance to low pH, and bile. The identification of genes or clusters that contribute to these probiotic-related characteristics is an ongoing challenge for scientists. In this context, genetic analysis and manipulation of these bacteria will be of paramount importance to understand their probiotic functionality and for optimizing their performance *in vitro* and *in vivo*. An emerging knowledge of the genomics of *Lactobacillus* species will allow improved understanding of the probiotic characteristics of these strains. However, the development of genetic tools for lactic acid bacteria, especially commensal lactobacilli, has lagged significantly, notwithstanding a number of significant recent studies. Therefore, novel genetic tools are urgently required to understand the biological potential of many *Lactobacillus* species. This review summarizes the current status in this area, and highlights issues of greatest urgency for further advancement. As an accompaniment to the sections which follow, Figure 1 illustrates schematically a selection of genetic tools that have been successfully applied to the study of candidate genes involved in probiotic effects in commensal lactobacilli.

Table 1. Published *Lactobacillus* genomes with relevant properties (January 2008)[1]

LB[2]	Size [3]	pla[4]	IS[5]	Pph[6]	*bsh*[7]	*srt*[8]	R-M[9]	Resource	Reference
La	1.99	0	7	0	2	1	1/II	Human source	29
Lb	2.34	2	1	1	3	1	1/III	Starter culture	30
Lc	2.92	1	4	2	2	3	1/I	Emmental cheese	30
Ld1	1.86	0	5	0	0	1	1/I	Bulgarian yogurt	31
Ld2	1.86	0	2	0	0	1	1/II	French starter	30
Lg	1.89	0	3	1	2	1	1/I	Human GI tract	30
Lh	2.08	0	12	0	0	1	2/I, 1/III	Cheese isolate	32
Lj	1.99	0	3	2	3	1	0	Human isolate	33
Lp	3.31	3	2	2	4	1	1/I	Human saliva	34
Lsk	1.88	0	4	0	1	2	0	French sausage	35
Lsl	1.83	3	10	2	2	1	1/I	Huamn ileum	36
Pp	1.83	0	3	2	1	1	0	Plants	30

[1] Data are derived from published *Lactobacillus* genome information and EGRO™ integrated genomics (Chicargo, USA) [2] *Lactobacillus* strains: La, *L. acidophilus* NCFM; Lb, *L. brevis* ATCC367; Lc, *L. casei* ATCC334; Ld1, *L. delbrueckii* ssp *bulgaricus* ATCC11842; Ld2, *L. delbrueckii* ssp *bulgaricus* ATCCBAA365; Lg, *L. gasseri* ATCC33323; Lh, *L. helveticus* DPC4571; Lj, *L. johnsonii* NCC533; Lp, *L. plantarum* WCFS1; Lsk, *L. sakei* 23K; Lsl, *L. salivarius* UCC118; Pp, *P. pentosaceus* ATCC25745 [3] Genome (chromosome) size (Mb) [4] Number of plasmid in the genome [5] Number of IS family that IS elements from the relevant *Lactobcillus* genome belong to [6] Number of intact prophage in the corresponding *Lactobacillus* genome [7] Number of genes that encoding cholyglycine hydrolase (EC 3.5.1.24) [8] Number of genes that encoding sortase [9] Number/type of intact restiction-modification system in the relevant *Lactobcillus* genome

3. GENOMIC RESOURCES

3.1. Genome databases

Genome sequencing projects provide critical data to underpin investigation of commensal lactobacilli. Twelve *Lactobacillus* genome sequences including five commensal *Lactobacillus* strains have been published by January 2008. The sequenced strains belong to *Lactobacillus acidophilus* (29), *Lactobacillus brevis (*30), *Lactobacillus casei* (30), *Lactobacillus delbrueckii* ssp. *bulgaricus (*31), *Lactobacillus gasseri* (30), *Pediococcus pentosaceus* (30), *Lactobacillus helveticus (*32), *Lactobacillus johnsonii* (33), *Lactobacillus plantarum* (34), *Lactobacillus sakei* (35), and *Lactobacillus salivarius (*36). Their genome properties are described in Table 1. There are another three *Lactobacillus* genome sequencing projects completed but currently unpublished or in press (*Lactobacillus reuteri* F275, *Lactobacillus rhamnosus* ATCC53103 and *Lactobacillus fermentum* IFO3956). More *Lactobacillus* genome sequencing projects are in process according to the genomes online database (GOLD) (37), from the *Lactobacillus* species *amylolyticus*, *antri*, *buchneri*, *coleohominis*, *crispatus*, *iners*, *jensenii*, *reuteri*, *paracasei*, *syntoryeus*, and *ultunensis*. The complete genome resources deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Genome&itool=toolbar) and other databases provide not only complete genome information, but also bioinformatics tools for genome analysis. Websites like KEGG (http://www.genome.jp/kegg/), JGI microbial genomics (http://genome.jgi-psf.org/mic_home.html) offer predicted metabolic pathways or comparative analysis and annotation of publicly available genomes. In addition, the JCVICMR on-line resource from the J. Craig Venter Institute (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi) provides genome tools including genome summary information, graphical displays, genome properties, and sophisticated genome analysis tools (gene position search, protein motif search, restriction digest, pseudo 2D gel, codon usage, putative operon predictions, primer search etc.) as well as comparative tools. The available genome databases will provide information underpinning the understanding of the biology and function of sequenced *Lactobacillus* species as well as those of strains from the same species.

3.2. Microarray-based analysis

Sequenced and annotated *Lactobacillus* genomes provide comprehensive information about predicted gene functions, *in silico*-constructed bacterial metabolic pathways, and especially potential host interaction factors and gene functions in candidate probiotic strains. However, these are all, by definition, merely *predicted* functions, unless and until they are confirmed experimentally. On the basis of sequenced genomes, microarray-based assay is an efficient methodology to measure gene transcription, and it can also be used in comparative genomic hybridization, single nucleotide polymorphism studies, tiling array analyses, and promoter sequence/function studies (38). Among those genome sequences available for commensal lactobacilli, microarray techniques were mainly employed to investigate stress response genes involved in gut persistence such as bile responsive genes (39, 40), acid resistance genes (41) and gut persistence associated genes (42). Targeted analyses of specific operons like those for bile-resistance (43), carbohydrates utilization (44, 45), and also systems involved in regulation of adherence (46) can also be accomplished by microarrays. There are other applications that microarray technology can address. It can track changes in the expression of genes along the digestive tract (47), and can be used to detect gene expression responsive to the intestinal tract (48-50), and to analyze integration and distribution of *Lactobacillus* prophages (51). Microarrays are also being developed which support thousands of 16S ribosomal RNA (rRNA) gene probes that are specific for intestinal bacteria, for community analysis, as described elsewhere in this volume.

3.3. *In silico* modeling of metabolic pathways

Genome sequencing projects can dramatically accelerate the understanding of biological capabilities of lactobacilli. Exemplifying this, genome-scale metabolic

networks can be reconstructed based on the functional annotation of predicted genes, genome and metabolic pathway databases, and targeted experimental verification. For commensal lactobacilli, there are few such networks constructed, with the prominent example of LacplantCyc (52) for the genome sequence of *L. plantarum* WCFS1 potentially serving as a model. The metabolic network links functional genes with metabolic reactions, which can be used to investigate cellular properties of organisms and help scientists to understand the diversity of their capabilities, roles and interactions. Recently, an AUTOGRAPH-method (Automatic Transfer by Orthology of Gene Reaction Associations for Pathway Heuristics) (53) has been developed to accelerate the process of metabolic reconstruction. However, the quality of the metabolic network derived from it depends on the availability and quality of manually curated metabolic networks and the orthology detection. Given the critical role of metabolism of lactic acid bacteria in carbohydrate and protein catabolism, the extension of *in silico* metabolic mapping to other species is highly desirable, reinforced by experimental validation where appropriate. Considering an individual *Lactobacillus* strain as a cell factory (54), metabolic models will help to build cellular signaling and regulatory networks.

3.4. Codon usage and operon structure

Access to complete genome sequences of commensal lactobacilli provides empowering information for rational design of cloning experiments, for example to facilitate heterologous gene expression. Thus, there is an accompanying requirement to characterize properties such as codon usage, for trouble-shooting and optimizing protein production. Bioinformatic tools like Artemis (Sanger, Cambridge, UK) and the JCVICMR on-line genome tools can derive codon usage patterns for a bacterium whose genome sequence is available. However, despite the high bias of the codon usage in lactobacilli (55), there are surprisingly no meta-analyses of this topic in the post-genomic era.

Given the high coding density typical of prokaryotes, and utilization of single promoters for co-transcription of complex operons, many biologically interesting genes in commensal lactobacilli are likely subject to co-regulation and transcriptional dependence on contiguous genes. The burgeoning availability of genome sequences, improved tools for motif detection such as deployment of Hidden Markov models (HMM) (56), and the increasing power of comparative genomics (57) will collectively ensure that powerful tools are available to identify genes, operons, promoters and regulatory sequences.

4. *LACTOBACILLUS* IDENTIFICATION TOOLS

Identification of lactobacillus species has been historically contentious, and some high-profile strains have been re-classified. Meaningful biological investigations of commensal lactobacilli benefit from robust identification and phylogenetic positioning of strains that are often isolated from very complex environments. The classification of lactobacilli is complex and controversial: Identification of commensal lactobacilli is difficult as they belong to a large extremely diverse genus *Lactobacillus* which contains 147 recognized species to date (58). Genetic investigations and tools therefore provide important adjuncts to taxonomy tools based on physiological, biochemical and molecular profiles available for differentiation of *Lactobacillus* as described in Table 2. The classic approach such as employment of API 50 CH strips (bioMérieux, Inc., Marcy l'Etoile, France) (59) based on carbohydrates fermentation profiles is common and efficient for the identification of lactobacilli. However, due to the diversity of *Lactobacillus* and lack of database profiles for species including *L. gasseri*, *L. jensenii*, *L. iners* and *Lactobacillus vaginalis*, identification of commensal *Lactobacillus* species by API 50 CH can lead to misidentification or uninterpretable results (60). Complicating identification are persistent errors in classification; for example, it is not robust to divide some species into subspecies (61) either by molecular techniques, or by reference to carbohydrate utilization profiles in the API 50 CH database. Therefore, differentiation of *Lactobacillus* species according to physiological and biochemical criteria alone can be unreliable.

Molecular approaches are more reliable, and have been used to distinguish lactobacilli at the species or strain level. Most of these molecular approaches are based on analyzing differences in PCR amplicons derived from fragments or regions including 16S ribosomal RNA (rRNA) gene (62), 16S-23S rRNA intergenic spacer region (63, 64), *groEL* (*hsp*60 PCR-RFLP) (65) or random DNA (randomly amplified polymorphic DNA, RAPD) (66). These strain identification tools are efficient and can distinguish lactobacilli at species level. For differentiating genotypically close *Lactobacillus* species or distinguishing at strain level, it is necessary to employ identification tools that couple PCR with restriction or electrophoresis analysis, such as amplified ribosomal DNA restriction analysis (ARDRA) (67, 68), PCR-coupled temperature or denaturing gradient gel electrophoresis (PCR-TGGE and PCR-DGGE (69), respectively), transferred-DNA (tDNA) intergenic spacer PCR (tDNA-PCR) (70) and multiplex RAPD-PCR (71). The choice of application of these approaches mainly depends on the requirement for differentiation. Methods based on whole genome polymorphisms such as renaturing SDS-PAGE (72), pulsed-field gel electrophoresis (PFGE) (73), amplified fragment length polymorphism (AFLP) (74) and temporal temperature gradient gel electrophoresis (TTGE) (75) are also useful to determine the relatedness of *Lactobacillus* isolates.

Of great interest, some methods such as typing IS (insertion sequence) elements have been used to group lactobacilli of the same species into clusters which correlated with the isolation source (76). Other identification tools based on microarrays proved useful for the characterization of lactobacilli. For example, genome-probing microarrays (GPM) (77) detected a linear relationship between the hybridization signal intensity and target genome quantity. This method showed high

Table 2. Approaches for the identification of *lactobacilli*

Approach	Principle	Discrimination level and application	Reference
API 50 CH strips	Identify species based on their carbohydrates fermentation profiles	Species level	59
16S rRNA[1] sequence	Differentiate species based on their 16S phylogeny	Species level	62
16S-23S rRNA ISRs[2]	Multiplex PCR assay based on strain 16S-23S rRNA intergenic spacer region and its 23S rRNA gene flanking region	Can identify human stool samples and distinguish lactobacilli at species level	63, 64
tDNA-PCR[3]	Identify species on their unique tDNA fingerprint patterns	Species level	70
hsp60 PCR-RFLP[4]	Differentiate strains from their *groEL* PCR-restriction fragment length polymorphism patterns	Can differentiate 110 Lactobacillus isolated from cheese and dry-fermented sausages	65
RAPD[5]	Differentiate strains from the random amplified polymorphic DNA pattern	Used for typing *L. plantarum* strains	66
Multiplex RAPD-PCR	Based on PCR generated unique DNA profiles	Clustered lactobacilli gastrointestinal isolates. Can characterize and infer the relatedness of *Lactobacillus* isolates	71
RAPD-PCR and AFLP[6]	Differentiate strains on the RAPD-PCR and AFLP generated genomic fingerprint patterns	Can differentiate phenotypically highly similar species (eg., *L. gasseri* and *L. johnsonni*, *L. amylovorus* and *L. gallinarum*)	74
TTGE[7]	Differentiate variation in 16S rRNA sequence of lactobacilli by electrophoresis	Can distinguish closely-related target species and reveal sequence heterogeneities in the 16S rRNA genes	75
ARDRA[8]	Differentiate strains from their amplified ribosomal DNA restriction patterns	Differentiated *Lactobacillus* from gastrointestinal, food and birds isolates at species level	67, 68
Renaturing SDS-PAGE[9]	Identify *Lactobacillus* from their electrophoretic pattern of peptidoglycan hydrolases	Distinguished phylogenetically close species from food isolates	72
DGGE[10] and specific primers	Identify strains by the combination of gel electrophoresis of DNA PCR fragments and 16S-23S rRNA intergenic spacer region	Can identify *Lactobacillus* from human and procine GI isolates at species level	69
FAFLP[11]	PCR based whole genome DNA fingerprint with selective amplification of restriction fragments	Can delineate *L. rhamnosus* strains from food and human isolates at intraspecific level	73
PFGE[12]	Differentiate by electrophoresis patterns of restricted whole genome	Can determine *L. rhamnosus* genotypic relatedness at strain level when combine with FAFLP	73
GPM[13]	Based on whole genome sequence	Species level; can quantitatively identify LAB[14] community from food	77
Genome microarray	Based on whole genome sequence	Can distinguish to strain level and reveal the diversity of a species	78

Abbreviations: [1] ribosomal RNA, [2] intergenic spacer regions, [3] tDNA intergenic spacer polymerase chain reaction, [4] PCR-restriction fragment length polymorphism, [5] randomly amplified polymorphic DNA, [6] amplified fragment length polymorphism, [7] temporal temperature gradient gel electrophoresis, [8] amplified ribosomal DNA restriction analysis, [9] sodium dodecyl sulfate polyacrylamide gel electrophoresis, [10] denaturing gradient gel electrophoresis, [11] fluorescent amplified fragment length polymorphism, [12] pulsed-field gel electrophoresis of macrorestriction fragments, [13] genome-probing microarray, [14] lactic acid bacteria

sensitivity for identifying *Lactobacillus* strains in a community background. (0.25% prevalence in the total community, testing 2.5 total ng DNA, was still detectable). Another genome-typing approach identified regions of variance among *L. plantarum* strains (78). This method was able to distinguish *L. plantarum* into two groups. Robust phylogenetic positioning of lactobacilli, complemented by phylogenomics, will be useful for ecological studies of environments such as the gut that contain many members of this genus.

5. GENE TRANSFER

Although genetic manipulation of strains for usage in food preparation is prohibited in many jurisdictions, there is still a great deal to be learned by studying *Lactobacillus* gene function in a heterologous background. Thus it is essential to develop methods for transferring genes between strains and model hosts.

5.1. Transformation

Replicons derived from different plasmids that have been demonstrated to replicate in lactobacilli are listed in Table 3. Electroporation has been widely used for plasmid transfer and cloning in many lactobacilli. For most species, transformation occurs at reasonable frequencies, namely 10^4 transformants per μg of DNA or greater, though this transformation efficiency is lower than that possible with lactoccoci. However, the transformation efficiencies for some *Lactobacillus* species like *L. crispatus*, *L. delbrueckii*, and *L. helveticus* are quite low, at approximately 30-50 transformants per μg of DNA, and many strains of those species are non-transformable (79). The low efficiency of transformation could be due to the presence of incompatible plasmids (80, 81) in the recipient. Therefore, either improvement of cell competence preparation protocols, or development of new plasmid replicons, is required for gene transfer via electroporation in these species. An additional barrier to transformation is the presence of diverse restriction/modification systems, which are evident from genome sequences of lactobacilli such as *L. acidophilus*, *L. brevis*, *L. casei*, *L. delbrueckii* ssp *bulgaricus*, *L. gasseri*, *L. helveticus*, *L. plantarum* and *L. salivarius* (Table 1).

5.2. Conjugation

Although electroporation is widely used for gene transfer and cloning in lactobacilli, some species of this genus are poorly transformable or non-transformable. Conjugation is an alternative approach for the introduction

Table 3. Replicons that may be transformed into *Lactobacillus*

Replicon	Type	Ts[1]/μg DNA	Host	Reference
pSH71	RC[2]	10^5 10^4 10^5 $>10^2$	*L. salivarius* UCC118 *L. acidophilus* *L. helveticus* KU107 *L. delbrueckii* subsp. *bulgaricus* VI104	82-84
pWV01	RC	10^4 10^7 $>10^2$	*L. salivarius* UCC118 *L. salivarius* Sn1 *L. delbrueckii* subsp. *bulgaricus* VI104	82, 84, 85
pAMβ1	Theta	10^4	*L. salivarius* Sn1	85
pLC494	theta	10^5 10^3	*L. casei* L-49-4 *L. acidophilus* NIAIL-54	86
pRV500	theta	10^1-10^6	*L. sakei*	87
pLEM3	RC	10^3-10^4	*L. delbrueckii* subsp. *bulgaricus* VI104	84
pBUL1	ND[3]	$>10^3$	*L. delbrueckii* subsp. *bulgaricus* VI104	84
pWS58	ND	$>10^3$	*L. delbrueckii* subsp. *bulgaricus* VI104	84
pLTK2	RC	10^4	*L. plantarum* NCL21	88
pFX1	RC	10^4	*L. salivarius* Sn1	85
pLP825	ND	10^6-10^7	*L. casei* ATCC 393 *L. plantarum* NCDO 1193	81
pLP825	ND	10^2-10^3	*L. acidophilus* NCK 89 *L. brevis* VK3 *L. fermentum* NCK127 *L. pentosus* MD353 *L. plantarum* ATCC 14917	81

Abbreviations: [1] transformants, [2]rolling cycle, [3] not determined

of novel DNA into poorly-transformable lactobacilli (79). Conjugal gene transfer can be achieved by conjugative plasmids (26), or comobilization of a nonconjugative plasmid with a conjugative plasmid (89). For instance, conjugal gene transfer was used to introduce a heterologous gene encoding beta-glucanase into a non-transformable strain of the species *L. helveticus (*90). Conjugation can also be used to transfer genes between different species. Therefore, development of a recombinant plasmid transfer system based on conjugation may be a useful and potentially broad-range tool for gene transfer in lactobacilli. Normally, the source vector for conjugal gene transfer comes from existing conjugative plasmids. Construction of a conjugal plasmid can alternatively be accomplished by cloning the mobilization region into a *Lactobacillus* cloning vector. Some *Lactobacillus* species harbour transmissible or conjugative plasmids. For example, the sequenced *L. plantarum* strain WCFS1 harbours a conjugative plasmid pWCFS103 (26). The megaplasmid of *L. salivarius* UCC118 contains a tract of genes related to conjugation (36), though they appear to be genetically corrupt (28). Genes encoding TraA related to transmission were also found in a small plasmid present in the same strain (28). This plasmid formed the basis for development of a vector that could be mobilized into *L. salivarius* and various other species. This emphasizes the potential of adapting endogenous plasmids for developing new genetic tools for lactobacilli.

5.3. Transduction

Another gene transfer method is transduction, bacteriophage-mediated chromosomal or plasmid DNA transfer. Transduction has been infrequently reported for gene transfer in lactobacilli compared with transformation, electroporation and conjugation. To our knowledge, transduction was only employed in four *Lactobacillus* strains, *L. delbrueckii* (91), *L. gasseri* (92) *L. acidophilus* (93) and *L. salivarius* (94) with bacteriophage LL-H/S, Φadh and PLS-1, respectively. The role of transduction in gene mapping and linkage studies is redundant in the post-genomic era, but transduction remains a useful alternative method, albeit one requiring time investment for isolating appropriate phage.

6. GENE EXPRESSION AND CONTROL

6.1. Available plasmids replicons and construction of gene cloning vectors

Cloning vectors for lactobacilli can be divided into three classes (95): Promiscuous plasmids based on rolling cycle replication (RCR) replicons such as pWV01 (96) derivatives; plasmids with replication origins for both *E. coli* and gram-positive bacteria such as pAMβ1 (97) or pSH71 (98) based replicons; and native *Lactobacillus* vectors with selectable markers and alternative replication origins for gram-negative bacteria. Presence of plasmids is a common feature in *Lactobacillus* species (80). This characteristic of *Lactobacillus* supplies a large reservoir of plasmids which can be exploited for the construction of the third class of *Lactobacillus* vector, and for diverse recombinant applications. Since *repA*-based plasmid replicons replicate via the theta mode of replication, which confers stability, derivatives of this replicons type have been widely used (86-88, 99-101) to construct vectors for *L. casei*, *L. plantarum*, *L. delbrueckii*, *L. fermentum*, and *L. sakei* . Some of the constructed vectors have a broad host range. For example, the shuttle vector pLP825 could be introduced by electroporation into *L. casei*, *L. pentosus*, *L. plantarum*, *L. acidophilus*, *L. fermentum*, and *L. brevis* strains with similar efficiencies (81). Derivatives of pLP1, a 2.1 kb plasmid extracted from *L. plantarum* CCM 1904 (ATCC 8014) can replicate in *L. curvatus*, *L. sakei*, *Carnobacterium*, and *Leuconostoc mesenteroides* (102). *L. salivarius* UCC118 harbours 3 plasmids (36), a megaplasmid pMP118 and two endogenous plasmids pSF118-44 and pSF118-20 which putatively replicate via a theta replication mechanism. The *repA* of pSF118-20 has high sequence similarity with *repA* of plasmids from different *Lactobacillus* species. This suggests it has the potential to be used to develop vectors with broader host range among the lactobacilli (28).

Table 4. Bacteriocin-controlled gene expression accomplished in lactobacilli

Strain	Gene/Protein	Inducer	Reference
L. brevis	chimeric S-layer	nisin	113
L. casei ATCC393	virus VP60	nisin	114
L. gasseri	*pepI*	nisin	115
L. helveticus CNRZ32	*gusA*	nisin	104
L. paracasei NFBC 33*8*	*groEL*	nisin	116
L. plantarum	*gusA, alr,* TTFC[1]	nisin	103, 117
L. rahmnosus GG	GFP[2]	nisin	118
L. reuteri	*amyL*	nisin	105
L. sakei/plantarum	*gusA, pepN*	nisin, sakacin	109, 119
L. salivarius UCC118	*betL*	nisin	111

Abbreviations: [1] the C subunit of the tetanus toxin, [2] green fluorescent protein

6.2. Gene expression and control of expression

Among the available gene expression systems for application in *Lactobacillus*, the *Lactococcus* nisin-controlled gene expression system may be the most widely used. As listed in Table 4, the nisin regulated gene expression system has been used for gene expression in *L. plantarum* (103), *L. helveticus* (104) and *L. reuteri (*105). Gene expression under constitutive promoters e.g. *pldh (*106, 107) or through integration upstream of a native promoter (108) has also been employed in lactobacilli. There are other systems such as sakacin (109) or lactose inducible (110) gene expression systems available for conditional gene expression in lactobacilli. The nisin controlled gene expression system has been employed in *L. salivarius* UCC118 for expressing the osmoprotective glycine-betaine transporter *betL (*111) by adaptation of the pNZ8048 and pNZ9530 (112) system. However, this system requires addition of multiple antibiotics, which could either induce stressful effects on the host or prevent gene expression in strains that already harbour an antibiotic resistance marker. In addition, low level expression of *betL* without induction was detected when using the pNZ8048 and pNZ9530 nisin regulated gene expression system. Therefore, optimization of inducible expression and perhaps integration of the *nisRK* gene into the chromosome of *L. salivarius* UCC118 (and other species) is desirable. Moreover, constitutive gene expression systems would be useful for many species, as a complement to inducible expression systems.

6.3. Genetic markers, promoters

Commensal lactobacilli, by virtue of their source niches, are suitable candidates to be explored and developed as probiotic agents. The probiotic characteristics and safety aspects of such strains must be ratified before their employment as food supplements or functional adjuncts. In this context, the development of genetic makers that can be used as tags to investigate the biology of commensal lactobacilli, and the mechanism of their interaction with the host *in vivo,* is desirable. Various reporter systems have been used to identify regulatory sequences and to monitor gene expression for commensal lactobacilli in the past 10 years. Those based on the emission of light may be a particularly suitable monitoring system for commensal lactobacilli as light emission can be detected in real time, and its monitoring is less laborious compared with other reporter systems.

Genes encoding enzymes that produce light include *lux* (which encodes bacterial luciferase) from *Photorhabus luminescens, Vibrio harveyi* or *Vibrio fisheri*, and *gfp* (that encodes Green Fluorescent Protein) from *Aequorea victoria*. The bioluminescence reporter system was widely used in *L. casei* (120, 121), *L. jensenii* (122), *L. plantarum* and *L. gasseri* (123) for various utilities. However, due to the requirement for $FMNH_2$ (reduced riboflavin mononucleotide), which is involved in the emission of luminescence by bacteria harbouring the *lux* genes, the NAD (P)H:FMN oxidoreductase (EC 1.6.8.1) is required to generate a constant level of $FMNH_2$ for continuous emission of light (124). Therefore, use of bioluminecence as a genetic marker may not be possible in *Lactobacillus* strains or species that lack NAD (P)H:FMN oxidoreductase.

Expression of *gfp* was employed in *L. acidophilus (*83), *L. reuteri* (105), *L. casei (*110), *L. hevelticus (*79), *L. sakei* (125) and *L. plantarum* (103) by either integration into the chromosome or gene fusion in an expression vector. GFP tagging was also used to track *L. dekbrueckii* and *L. fructosus* for localization on the GI epithelium (107) and to monitor the behavior of *L. rhamnosus* GG *in vivo* (118).These and other markers will soon be deployed to monitor commensal bacteria *in vivo*, which is highly desirable. For example, *L. salivarius* has demonstrated probiotic properties in human trials and animal models (126-129). However, very little is known about the fate of live *L. salivarius* cells when administered *in vivo*, or about the interaction of these microorganisms with the gastrointestinal ecosystem. For future applications, it would be extremely useful to have reporter systems (*gfp* or *lux* fusions) to monitor bacterial cell distribution and gene expression by *L. salivarius* and other commensal lactobacilli in animal models. In order to investigate the biology of commensal lactobacilli, availability of defined promoters would be highly advantageous for multiple applications. For a specific microorganism, most promoters required could be isolated and identified from a genome library. Promoters regulated by e.g. bacteriocin induction peptides could be used as inducible promoters. Microarray based transcriptional analysis is an efficient method to characterize strong promoters, and promoters which function at different bacterial growth phases. Among commensal lactobacilli, a synthetic promoter library was constructed for *L. plantarum* WCFS1 and was then evaluated by expression of GusA and PepN (130). Further studies on *Lactobacillus* promoter sequences, in a broader range of species, are clearly warranted.

7. MUTAGENESIS SYSTEMS

Generally, there are two methods for generating genetic mutants in lactobacilli for targeted and random mutagenesis. The first is integration, which is rec-dependent recombination of cloned DNA with a homologous locus. The second is rec-independent, which involves transposons or insertion sequences.

7.1. Integration and insertion systems

Integration of genes or vectors into the chromosome of bacteria is a critical genetic tool. Integration may be required in manipulation of gene stabilization, fusion, amplification, deletion, insertional mutagenesis, and creation of physical/genetic maps. A number of techniques for integration in *Lactobacillus* have been developed to date. Homologous recombination in lactobacilli can be achieved by one plasmid or two plasmid systems. One plasmid systems have been widely used in LAB. They are based on either temperature-sensitive integrated vectors such as pG+host (pWV01 derivative (131)), pIP501 (pSA3 derivative (132)), pTN1 (115) and an unstable replicon pGID (pE194 derivative) (133) or non-replicative plasmids such as pUC18/19 (134) and pBluescript SK^- (108, 135). The two plasmid systems include the pORI28 system (136, 137) and its derivative systems pORI19-pVE6007 (138) and pORI28-pTRK669 (139). pORI28-pTRK669 system is an expansion of pORI28 for use in themophilic lactobacilli, by site specific DNA replacement (140, 141). These systems are all based on the conditional replication of the lactococcal pWV01-derived vector pORI. Combining the system with a temperature-sensitive pWV01 derivative increased the frequency of Campbell-type recombinants. The pORI19-pVE6007 gene integration system has been successfully adapted to delete a gene encoding sortase (24), multiple genes for surface proteins (24), and a gene which encodes a bacteriocin transporter on the megaplasmid pMP118 in *L. salivarius* UCC118 (23). However, for deletion or disruption of genes on a genome-wide scale, pVE6007 and pORI is not a efficient integration system. Powerful and universal gene deletion or disruption tools need to be developed for such a specific requirement. A novel method for the directed genetic manipulation of the *Bacillus subtilis* chromosome free of any selection markers has been developed recently (142). This method employed the *Escherichia coli* toxin gene *mazF* as a counter-selectable marker, which makes it possible to repeatedly and successfully inactivate a specific gene, to introduce a gene of interest, and to accomplish the in-frame deletion of a target gene in the same strain. In addition, there are other new mutagenesis systems, such as that using extracellular endoglucanase A (*celA*) of *Clostridium thermocellum* as a screening marker (143) and the Cre-*lox*-based system (144) which has been developed and employed in *L. plantarum*.

In addition to homologous recombination strategies via suicide or temperature-sensitive replicons, prophages constitute a substantial proportion of the mobile DNA of their bacterial hosts. Two temperate phages from *L. delbrueckii* (145, 146) and *L. gasseri* (147) were used to construct site-specific integration vectors. A DNA sequence was stably introduced into the chromosome of *L. casei* by application of prophage integration and it could be subsequently excised (148). Sequence analysis revealed that *L. plantarum* (34), *L. gasseri* (149), *L. casei (*149), *L. johnsonii* (51), and *L. salivarius* (149) contain 2 (Lp1and Lp2a), 1 (Lgal), 1 (Lca1), 2 (Lj928 and Lj965), and 2 (Sal1 and Sal2) intact prophage sequences, respectively. Thus, genomes of sequenced *Lactobacillus* species provide the potential for establishing efficient and site-specific integration systems for commensal lactobacilli.

7.2. Random mutagenesis systems

Random mutagenesis is a valuable genetic tool to study genes and their regulators. However, it requires generation of a genuinely random i.e. non-biased mutant library. *In vivo* expression technology (IVET) (150) can be used to detect bacterial genes that are specifically induced in their host. IVET and R-IVET ((resolvase-based) *in vivo* expression technology) were used to detect *L. reuteri* 100-23 (151) and *L. plantarum* WCFS1 (152) genes specifically induced in the murine gut. Both transposon mutagenesis and insertion sequences (IS) are common approaches for generating a mutant library. However, there are only a few transposon-based mutagenesis systems available for lactobacilli. The conjugative transposon Tn916 family and transposon Tn917 from *Enterococcus faecalis* have been used for random insertional mutagenesis of LAB (153-156), with the latter example being in *L. plantarum*. However, both systems appear to have limitations in their utility for generating mutant libraries for LAB, due to low transfer and integration frequencies and limited transposon carriers. Therefore, the most frequently used mutagenesis approach for lactobacilli is IS-based mutagenesis. IS-elements can be used for construction of suicide insertion vectors. 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. The insertion of IS*1223* into pSA3 (157), and IS*1223* or IS*1201* into pIP501 (158) exemplify the successful usage of IS elements for random mutagenesis in lactobacilli. The addition of the lactococcal IS*S1* to the thermosensitive replicon pG^+host allowed construction of a food grade random mutagenesis system which has been employed for generating stable mutants in *L. plantarum* (159) and other gram-positive bacteria (160). Patnaik (161) accomplished genome shuffling by recursive pool-wise protoplast fusion and successfully identified shuffled strains that produced more lactic acid that the wild type. It will be interesting to see if this approach can be used to improve host association, or intestinal persistence, in commensal lactobacilli.

8. SUMMARY AND PESPECTIVES

It is now five years since the first genome of a *Lactobacillus* was definitively elucidated by sequencing. Primary molecular genetic tools like gene cloning, expression and disruption have been constructed for investigating traits including probiotic characteristics. Though most of these are derivatives of lactococcal genetic tools, some customized systems for use in lactobacilli have been developed recently. Following genetic

characterization, genetically modified organisms (GMOs) will not ultimately be used for human consumption in most jurisdictions; thus there is a parallel requirement for development of *in vivo* assays and appropriate animal models, to demonstrate proof of concept or functionality in genes that have been modified or altered. However, considering the heterogeneity of this genus, a novel greatly-expanded repertoire of genetic tools is required. Approaches are also desirable for DNA manipulation in *Lactobacillus* strains with atypical genome architectures. For example, megaplasmids are widely present in *L. salivarius* strains and a few other species (162). Thus, efficient gene disruption systems need to be developed to inactivate genes of interest which are located on megaplasmids. Plasmid Toxin-antitoxin (TA) addiction systems (163) that contribute to plasmid maintenance or stability in the relevant host were detected in *L. plantarum* (164) and *L. salivarius (*28). However, plasmid TA systems are not well understood in *Lactobacillus* e.g. the mode of toxin action. Given the fact that curing a resident plasmid has been accomplished by production of the relevant antitoxin *in trans (*28), adaptation of plasmid TA systems for plasmid curing or for recombinant plasmid retention will become feasible and desirable, as more manipulations are required in natural isolates with complex plasmid profiles. Following the leading example of *L. plantarum* WCFS1 (165), analytical tools like proteomics should be employed for detecting stress response, looking for biomarkers, and for detecting secreted proteins of commensal lactobacilli. With the discovery of presumptive contingency metabolic genes in some genomes, lactobacillus metabolomic studies coupled with *in silico* modelling will be another fruitful approach for studying the adaptation of strains to environmental conditions. For providing extended safety validation of probiotics for human consumption, systems approaches that are underpinned by genetic tools will provide additional supporting data on individual gene function, and overall strain phenotypes. Following patterns in the development of genetic systems for microbial pathogens, and well-characterized lactic acid bacteria such as *Lactococcus*, we anticipate that the next decade will witness establishment of a more comprehensive set of genetic tools for the lactobacilli, which will be broadly welcomed.

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Abbreviations:ATCC: American type culture collection; AUTOGRAPH: automatic transfer by orthology of gene reaction associations for pathway heuristics; DPC: moorepark culture collection, dairy products research centre, Ireland; $FMNH_2$: reduced riboflavin mononucleotide; GFP: green fluorescent protein; GI:

gastrointestinal; GMOs: genetically modified organisms; GOLD: genomes online database; GPM: genome-probing microarrays; HMM: Hidden Markov models; IS: insertion sequence; IVET*: in vivo* expression technology; JCVICMR: J. Craig Venter Institute comprehensive microbial resource; JGI: Doe Joint Genome Institute; KEGG: kyoto encyclopedia of genes and genomes; LAB: lactic acid bacteria (LAB); NAD (P)H: reduced nicotinamide adenine dinucleotide (phosphate); NCBI: national centre for biotechnology information; NCC: nestlé culture collection, Switzerland; NCIMB: national collections of industrial food and marine bacteria, UK; R-IVET: resolvase-based *in vivo* expression technology; rRNA; TA: toxin-antitoxin; UCC: University College Cork, Ireland; WCFS: Wageningen centre for food sciences, The Netherlands

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