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Discovering lactic acid bacteria by genomics

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Abstract

This review summarizes a collection of lactic acid bacteria that are now undergoing genomic sequencing and analysis. Summaries are presented on twenty different species, with each overview discussing the organisms fundamental and practical significance, environmental habitat, and its role in fermentation, bioprocessing, or probiotics. For those projects where genome sequence data were available by March 2002, summaries include

a listing of key statistics and interesting genomic features. These efforts will revolutionize our molecular view of Gram-positive bacteria, as up to 15 genomes from the low GC content lactic acid bacteria are expected to be available in the public domain by the end of 2003. Our collective view of the lactic acid bacteria will be fundamentally changed as we rediscover the relationships and capabilities of these organisms through genomics.

Introduction (contributed by Todd Klaenhammer)

The lactic acid bacteria (LAB) represent a group of bacteria that are functionally related by their ability to produce lactic acid during homo- or heterofermentative metabolism. The acidification and enzymatic processes accompanying the growth of LAB impart the key flavor, texture, and preservative qualities to a variety of fermented foods. Industrial applications of the LAB rely on six key beneficial and nonpathogenic species; *Lactococcus* (milk), *Lactobacillus* (milk, meat, vegetables, cereal), *Leuconostoc* (vegetables, milk), *Pediococcus* (vegetables, meat), *Oenococcus oeni* (wine) and *Streptococcus thermophilus* (milk). Other members of the LAB, notably lactobacilli, occupy important niches in the gastrointestinal tracts of humans and animals and are considered to offer a number of probiotic benefits to general health and well being. These benefits include a positive influence on the normal microflora, competitive exclusion of pathogens, and stimulation/modulation of mucosal immunity. More recently, LAB are being used in the production of industrial chemical and biological products including biopolymers (*Leuconostoc* spp.), bulk enzymes (*Lactobacillus brevis*), ethanol, and lactic acid (*Lactobacillus casei, lactis, delbrueckii, brevis*) (Gold et al. 1992; Hofvendahl & Hahn-Hagerdal 2000). The LAB are also strong candidates for development as oral delivery vehicles for digestive enzymes and vaccine antigens (Wells et al. 1996; Pouwels et al. 1998; Steidler et al. 2000). Their innate acid tolerance, ability to survive gastric passage, and safety record during human consumption, are key features that can be exploited to effectively deliver biologics to targeted locations and tissues.

Realizing their practical significance in fermentation, bioprocessing, agriculture, food, and more recently, medicine, the LAB have been the subject of considerable research and commercial development over the past decade. Contributing to this explosion have been the recent efforts to determine the genome sequences of a representative collection of LAB species and strains. The first complete genome of the LAB group was published on *Lactococcus lactis* subsp. *lactis* IL1403 by Bolotin et al. (2001). The genome was 2.4 Mb in size and revealed a number of unexpected findings: biosynthetic pathways for all 20 amino acids, albeit not all are functional, a complete set of late competence genes, five complete prophages, and partial components for aerobic metabolism. Noting that some of these systems are not functional or complete, the genomic analysis of *Lactococcus* suggests an evolutionary trend toward minimization of the chromosome and elimination of unnecessary systems during adaptation to nutritionally complex environments, such as milk. At this writing, four other LAB genomes have been completed, (*L. plantarum, L. johnsonii, L. acidophilus, S. thermophilus*), and >20 more are in progress (Table 1) with expected completions for sequencing by the end of 2002. Additional genome sequencing is underway for microbes that are not considered as members of the LAB, but contribute important LAB-like properties either as probiotics (*Bifidobacterium longum, B. breve, and Brevibacterium linens*) or flavor adjuncts (*B. linens, Propionibacterium freudenreichii*). Among the total genome projects ongoing, there are several cases where genome sequences will become available for multiple strains of the same species, notably *L. lactis* (three strains), *L. casei* (two strains), *L. delbrueckii* (three strains), *S. thermophilus* (three strains), *Oenococcus oeni* (two strains) and *B. longum* (two strains).

Of the 29 genomes listed in Table 1 on LAB-type microbes, 11 genomes are being sequenced by the Department of Energy–Joint Genome Institute (JGI) in collaboration with the Lactic Acid Bacteria Genome Consortium (LABGC), composed of 10 US scientists representing seven universities in the U.S. As part of their microbial genomes program (see http://www.jgi.doe.gov/JGI_microbial/html/index.html) JGI will carry out genome sequencing of LAB species representing considerable diversity in ecological habitat (milk, meat, plants, GI tract) and roles (probiotic versus fermentation). All of the microbial genomes will undergo the same processing; first to generate a 10× coverage of each genome with a random shotgun small-insert library (3 kb), and to supplement this with ~5× coverage of a large insert (40 kb) cosmid library. These data will be incorporated into an assembly and the assembled scaffolds

Table 1. Genome sequencing projects of lactic acid bacteria

Species	Strain	genome size (Mb)	Contact person	Institution
Sequencing completed				
Lactococcus lactis	IL1403	2.3	A. Sorokin	INRA and Genoscope, FR
Lactobacillus plantarum	WCFS1	3.3	M. Kleerebezem	WCFS, NL
Lactobacillus johnsonii	NCC533	2.0	D.Pridmore	Nestlé, CH
Lactobacillus acidophilus	ATCC700396 NCFM	2.0	T. Klaenhammer, R. Cano	North Carolina State University and Cal-Poly Technical University, USA
Bifidobacterium longum	NCC2705	2.3	F. Arigoni	Nestlé, CH
Sequencing ongoing				
Lactococcus lactis ssp. *cremoris*	MG1363	2.6	O.Kuipers, M.Gasson, D. van Sinderen	University of Groningen, NL; IFR, UK; UCC, IRL
Lactococcus lactis ssp. *cremoris*	MG1363	2.4	A. Sorokin, A. Bolotin	INRA, FR
Lactobacillus delbrueckii ssp. *bulgaricus*	ATCC11842	2.3	E. Maguin, M.vd Guchte	Genoscope and INRA, FR.
Lactobacillus delbrueckii ssp. *bulgaricus*	DN-100107	2.1	T. Smokvina	Danone Vitapole, FR
Lactobacillus sakei	23K	1.9	M. Zagorec, S. Chaillou	INRA, FR.
Lactobacillus casei	BL23	2.6	J. Deutscher, A. Hartke	INRA/CNRS, Caen University, FR
Lactobacillus helveticus	CNRZ32	2.4	J. Steele	University of Wisconsin, USA
Lactobacillus rhamnosus	HN001	2.4	M. Lubbers	Fonterra Research Center (formerly NZDRI) and ViaLactia BioSciences, NZ
Streptococcus thermophilus.	LMG18311	1.9	P. Hols	UCL, Belgium
Streptococcus thermophilus	CNRZ1066	1.8	A.Bolotin	INRA, FR, Integrated Genomics Inc, USA
Oenococcus oeni	IOEB84.13	1.8	J. Guzzo	Universities Dijon and Bordeaux-INRA-GENOME Express, FR
Bifidobacterium breve.	NCIMB8807	2.4	D. van Sinderen	University College Cork (UCC), IRL
Propionibacterium freudenreichii	ATCC6207	2.6	H. Pel, J. Sikkema	DSM Food Specialties, Friesland Coberco Dairy Foods, NL
Joint Genome Institute/Lactic Acid Bacteria Genome Consortium (JGI/LABGC)				
Lactobacillus gasseri	ATCC33323	1.8	T. Klaenhammer	North Carolina State University, USA
Lactobacillus casei	ATCC334	>2.2	J. Broadbent	Utah State University, USA
Lactobacillus delbrueckii ssp. *bulgaricus*	ATCCBAA-365	2.3	J. Steele	University of Wisconsin, Madison, USA
Lactobacillus brevis	ATCC367	2.0	M. Saier	University of California, San Diego, USA
Lactococcus lactis ssp. *cremoris*	SK11	2.3	B. Weimer, L. McKay	Utah State University and University of Minnesota, USA
Leuconostoc mesenteroides	ATCC 8293		F. Breidt, H.P. Fleming	USDA, North Carolina State University, USA
Oenococcus oeni	PSU1 ATCC BAA-331	1.8	D. Mills	University of California, Davis, USA
Pediococcus pentosaceus	ATCC25745	2.0	J. Steele, J. Broadbent	University of Wisconsin, Madison, Utah State University, USA
Streptococcus thermophilus	ATCC BAA-491	1.8	R. Hutkins	University of Nebraska, USA
Brevibacterium linens	BL2/ ATCC 9174	3.0	B. Weimer	Utah State University, USA
Bifidobacterium longum	DJ010A	2.1	D. O'Sullivan	University of Minnesota, USA

ordered by PCR primer walking to fill gaps. The scaffolds will undergo an automated annotation which will be performed by Oak Ridge National Laboratories.

It is noteworthy that as these sequences are generated, they will be placed in the public domain on the JGI website for common use. The timely and public availability of genome information for various LAB species will catapult our collective efforts to carry on with comparative and functional genomic analyses of the LAB group.

The explosion of available genome sequences for LAB will accelerate their exploitation in both traditional and non-traditional arenas. While phylogenetically closely related by their small genomes (~2–4 Mb), the LAB occupy a diverse set of ecological niches suggesting that considerable genetic adaptation has occurred during their evolution. Comparison of the genome sequences of multiple LAB species and strains is expected to provide a critical view of microbial adaptation and genetic events leading to their adaptation to specialized environments. Comparative genomics among the microbes sequenced thus far has already illustrated that essential housekeeping gene functions are widely conserved among microbes and horizontal gene transfer commonly occurs. An expected outcome of comparative genomics of LAB will be the definition of conserved and unique genetic functions in LAB that enable core functions, e.g., production of lactic acid, proteolytic and peptidase activities, survival at low pH , stress tolerance, production of antimicrobials, transport systems, cell signaling, and attachment/retention in dynamically mobile environments.

It is anticipated that IS-elements, bacteriophages, and mobile genetic elements provide the major routes through which horizontal gene transfer occurs, and the roadmap to the most interesting and practically significant genetic regions that underscore the unique and beneficial properties of the LAB. It is well documented that the LAB undergo conjugation, exist in phage-contaminated environments where gene transfer may occur by transduction, and harbor sets or remnants of competence genes for transformation (Bolotin et al. 2001). Understanding gene transfer, particularly in environments where LAB coexist, or compete, will provide one important view of their evolution, adaptation, and potential for unique applications. Conjugation has played a key role in the evolution and adaptation of *L. lactis* to a milk environment noting that many attributes for growth in milk, including lactose and casein utilization and bacteriophage resistance, are encoded by conjugative plasmid DNA in this species (Broadbent 2001).

With the availability of genomic maps of more than 25 LAB, comparative genomic analysis will identify critical similarities and differences and is expected to identify 'islands of adaptability', defined as key genetic regions that may be instrumental in the evolution of the various species to their specialized habitats, and their functions within those environments. Comparisons of food-grade LAB with other related Gram-positive pathogens (*Enterococcus faecalis*, *Streptococcus agalactiae*, *Streptococcus equi*, *Streptococcus pneumoniae*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Listeria monocytogenes*) has already revealed many common features and will most certainly define the essential genetic differences between pathogens, non-pathogens, and commensals. Over the next year, our scientific community will be fundamentally empowered by the availability of numerous related, yet distinct genomes, to make these comparative analyses and define the similarities and differences that characterize the genomes of LAB.

This chapter presents a collection of summaries on the LAB organisms that are now in genomic sequencing or analysis. Each summary describes the organism and its roles in the environment or bioprocessing, the status of the sequencing effort as of March 2002 and, for some, selected interesting features that have been uncovered (Tables 2 and 3). Our view of the LAB will be fundamentally changed as we discover the relationships and capabilities of these organisms through genomics.

SUMMARIES OF SEQUENCING PROJECTS

Lactococcus lactis subsp. *cremoris* SK11 (contributed by Larry L. McKay and Bart Weimer)

Lactococci are mesophilic LAB that were first isolated from green plants. However, today they are used extensively in food fermentations, which represent about 20% of the total economic value of fermented foods produced throughout the world. This group of bacteria, previously designated the lactic streptococci (*Streptococcus lactis* subsp. *lactis* or *S. lactis* subsp. *cremoris*) was placed in this new taxon in 1987 by Schleifer. Lactococci gained notable interest because many of their functions important for successful fermentations are linked to plasmid DNA

Table 2. Genome statistics (March 2002)

Species	Strain	Genome			ORFs	Prophages			IS elements	RNA			Plasmids
		Size (Mb)	Contigs	%GC		Intact	Remnant	Total intact	Different types	rRNA operons	tRNA loci	tRNA number	Size kb (%GC)
L. lactis	IL1403	2.366	1	35.4	2310	5	1	43	6 (IS981, IS982, IS983, IS904, IS905, IS1077)	6		62	None
L. lactis	MG1363	~2.5	458 (>1 kb)	37.1	~2500	0	6	62	8 (IS981, IS982, IS905, IS904, IS946, IS1077G,ISS1, IS1675)	6	?	?	None
L. plantarum	WCFS1	3.308	1	44.5	~3050	2	2	12	2 (IS1165, IS1182)	5	5	62	1.9 (39.5%) 2.4 (34.3%) 36.1 (40.8%)
L.johnsonii	NCC533	2.022	1	34.6	1857	3	0	15	6 (ISL3, IS30)	6	4	87 ?	None
L. acidophilus	ATCC700396	1.99	1	34.7	1979	0	3	6	1 (IS3)	6			None
L. gasseri	ATCC33323	1.84	67	35.1	1803	1	1	3	3(ISL3, IS1397, IS1167)	>2		20	None
L. helveticus	CNRZ32	~2.4	~1000	37.1	> 2,300	?	≥ 1	?	> 5 (IS1201, ISL3 ISLh1, IS3, IS30)	≥3	?	?	None
L. delbrueckii ssp. *bulgaricus*	ATCC11842	1.8	3	50	?	?	?	Several		?	6 or 7	?	None
L. sakei	23K	1.772 (96%)	<150	41.4	>1792	?	?	12	3 (IS1520-types, new IS30-like, new IS150-like)	7	6	>58	None
L. casei	BL23	~ 2.6	563	46	?	?	Several	18	5 (IS1, IS3, IS30)	≥5	?	?	None
O. oeni	IOEB84.13	1.754	63	37.9	1784	4		3		2	2	43	3.9
P.freudenreichii	ATCC6207	2.64	12	67.4	2552	?		Many	?	2 (?)	2	44	None
B. breve	NCIMB8807 ATCC9174	~ 2.43	140	~ 53	?	0		~ 35	(IS2606,IS1383, IS1088,IS10,IS21)	~4	?	?	None
B. longum	NCC2705	2.256	1	60.1	~1730		Some?	16	5(IS3,IS21,IS30, IS256,IS200/IS605)	4	3	57	3.6 (64.8%)

Table 3. Encoded features

		L.lactis	*L.lactis*	*L. plantarum*	*L. rhamnosus*	*L. johnsonii*	*L. acidophilus*	*L. gasseri*	*L. helveticus*	*L. casei*	*B. longum*
strain		IL1403	MG1363	WCFS1	HN001	NCC533	ATCC700396	ATCC33323	CNRZ32	BL23	NCC2705
Biosynthesis pathways (de novo)											
Amino acids		all	all except Gln/Glu, Leu, Val, Ile, Met, His	all except Ile, Leu, Val	all except Ala, Leu, Lle, Val, Phe, Tyr	None	?	?	?	all except Ile, Leu, Val	all except Met and maybe Trp
Vitamins, cofactors		folate, menaquinone, riboflavin, thioredoxin	?	folate, molybdenum cofactor	folate, thiamin, heme		?	?	?	?	folate, nicotinate, thiamin
Nucleotides	Purines	present	present	present	present	absent	?	?	?	present	present
	Pyrimidines	present	present	present	present	present				present	present
Polysaccharides	gene clusters	1 ?	1 (?)	4	?	1	?	>2	1	?	1?
Bacteriocins	type	none	none	plantaricin	none ?	lactacin F	helveticin J	helveticin J, lactacin B	helveticin J-like	?	none
Bile salt hydrolases	number	2 ?	1 ?	4	none	3	3	2	1	1	1
Proteolytic system											
Cell-envelope proteinase		0	0	0	1	1	1	0	> 2	?	0
Peptidases		13	~14	19	29	25	>20	19	> 18	21	>20
Sugar transport											
PTS complete (EIIA+B+C)		6	?	25	?	?	?	9	> 6	31	1
Secreted proteins											
Lipoproteins		39	~30	~50	?	~40	?	?	?	~45	59
LP×TG-anchor proteins		6	~4	~25	~8	~15	?	?	?	?	8

(McKay 1985). Plasmids are commonly exchanged between strains via conjugation (McKay 1985; Dunny & McKay 1999) and with the chromosome by IS elements (Hughes 2000). Presumably, these exchanges and rearrangements mediate rapid strain adaptation and evolution but also add to the instability of important metabolic functions.

These bacteria are selected for use in fermentations based on their metabolic stability, their resistance to bacteriophage, and their ability to produce unique compounds – often from amino acid catabolism. The study of their physiology in adverse conditions such as low pH and high NaCl indicates that they adapt to these environments quickly and change their metabolism as a result of carbohydrate starvation (Stuart & Weimer 1998). Recent genome studies and physical maps indicate that bacterial genomes are very dynamic (Hughes 2000). These rearrangements are mediated by IS elements and result in gene duplication, translocation, inversion, deletion and horizontal transfer events. For example, an inversion encompassing approximately one-half of the chromosome of *L. lactis* ML3 occurred by homologous recombination between two copies of IS*905* (Daveran-Mingot et al. 1998). The response to these stresses, particularly to exposure to bacteriophage (Forde & Fitzgerald 1999), highlights the plasticity of the genome (Le Bourgeois et al. 1995; Delorme et al. 1994; Davidson et al. 1996). Establishing the links between environmental conditions, genome organization, and cellular physiology in lactococci will provide new and exciting information about the molecular mechanisms of these important bacteria. Advances that define the fundamental knowledge of the genetics, molecular biology, physiology, and biochemistry of lactococci will provide new insights and applications for these bacteria.

The importance of lactococci, specifically *L. lactis* subsp. *cremoris,* is demonstrated by its continual use in food fermentations (Garvie et al. 1981; Beimfohr et al. 1997). *L. lactis* subsp. *cremoris* strains are preferred over *L. lactis* subsp. *lactis* strains because of their superior contribution to product flavor via unique metabolic mechanisms (Sandine, 1988; Salama et al. 1991). The DNA sequence divergence between the subspecies is estimated to be between 20 and 30% (Godon et al. 1992). Of the many lactococcal strains used, *L. lactis* subsp. *cremoris* SK11 is recognized for the beneficial flavor compounds it produces (Lawrence et al. 1976). Although some progress in unlocking this strain's genetic secrets has been made, much more can be accomplished by using a genomics/proteomics approach. With this genome sequence, it will be possible to confirm the metabolic and evolutionary differences between subspecies of lactococci in order to identify the important characteristics that define this genus.

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Lactococcus lactis ssp. *cremoris* MG1363 (contributed by Douwe van Sinderen, Mike Gasson, Jan Kok and Oscar Kuipers)

Lactococcus lactis ssp. *cremoris* MG1363 is a cured isolate of strain NCDO 712 (Gasson, 1983), and is the most widely used model *Lactococcus* strain in genetic, physiological and applied research all over the world. *L. lactis* is a prominent bacterium in cheese production and can also be used for various other applications such as a vehicle for oral vaccines or delivery of health-promoting factors via the GI tract. Even before the genome sequencing projects started already over 10% of its gene content had been identified and characterized by conventional methods. Recently the complete genome sequence of *L. lactis* ssp. *lactis* IL1403 was published (Bolotin et al. 2001), providing a great opportunity to compare the two genomes, with respect to their similarities and differences. Many important features, such as pathways leading to the formation of flavour compounds, carbon and nitrogen metabolic routes, gene regulation mechanisms, prophage or sex-factor occurrence, and stress responses can be compared when the genome sequence of MG1363 is also known. Functional studies using transcriptome and proteome analyses can also be applied in order to speed up characterisation of important novel targets.

Genome sequencing status and results

To obtain the complete nucleotide sequence of the genome of *Lactococcus lactis* subsp. *cremoris* MG1363 a shotgun sequencing approach was used. The sequence of 15 000 ends of inserts of pUC clones, ranging in size from 1 to 3 kb, have been determined, yielding 9 million bp of sequence. The obtained

sequences were aligned with known sequences of MG1363 present in the NCBI nucleotide database (± 270 kb) and sequences present within the consortium (± 150 kb) using the Staden and Sequencer software packages. As the construction of a random library with inserts larger than 20 kb failed, the genome sequence of *L. lactis* subsp. *lactis* IL1403 was used as a template to start completing the genome sequence. After positioning of all the contigs on IL1403, forward and reverse primers were designed using an in-house developed software tool, in the ends of the contigs. The nucleotide sequences of the PCR fragments are currently being used to close the gaps. Annotation of the nucleotide sequence is being done with Glimmer, Blast, PFAM, Blocks.

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Lactococcus lactis ssp. *cremoris* MG1363 (contributed by Alexei Sorokin and Alexander Bolotin)

Two strains, IL1403 and MG1363, isolated from industrial sources and cured from plasmids, are commonly used to study genetics, physiology and molecular biology of *Lactococcus lactis*. These strains belong to two *L. lactis* strain clusters, called *lactis* and *cremoris*, distinct by the spectra of dairy fermentation products. We sequenced the entire genome of the strain IL1403 (Bolotin et al. 1999, 2001). To compare genomes of the two strains in the simplest way we decided to perform a co-linear scaffolding of the chromosome of strain MG1363. Co-linear scaffolding consists in random sequencing of a limited number of clones and Long Range PCR mapping of the co-linear regions of two genomes, one of which was completely sequenced earlier. In the co-linear regions, established by PCR, gene content and order should be approximately the same. The established co-linear scaffold of the MG1363 genome, which shares 85% identity with that of IL1403, allows easy access to 50% of the genes from this strain.

Genome sequencing status and results

Total DNA of *L. lactis* MG1363 was randomized by *Alu*I in conditions of non-complete digestion or by sonication and extracted after separation in an 0.8% agarose gel to give an average fragment size of 1.5 kb. It was then cloned into vector pSGMU2 cut by SmaI and the inserts from approx. 1500 random clones were sequenced by forward and reverse primers. Sequences of 513 of these clones, having a total non-redundant length of 0.3 Mb, were used to construct a co-linear scaffold of the strain MG1363 over IL1403. The total size of the co-linear genome parts was estimated to be around 1100 kb, that is approximately 45% of the entire genome (Bolotin et al. 2002). Oligonucleotide-directed sequencing over gaps amplified by LR PCR fragments of MG1363 and the entire sequence of a few regions of interest resulted in the accumulation of 1038 kb non-redundant sequence distributed in 733 contigs. The contigs were compared for homology, using BLASTx, with the complete protein set of *L. lactis* IL1403. Sequences of 523 contigs encode proteins that are more than 80% identical to proteins from IL1403. The sequences of the 733 contigs are available from GenBank as dbGSS (acc. Nos. BH770319-BH771051).

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Lactobacillus plantarum WCFS1 (contributed by Michiel Kleerebezem and Roland Siezen)

The genus *Lactobacillus* encompasses a large number of different species that display a relatively large degree of diversity. Among these, *Lactobacillus plantarum* is a flexible and versatile species that is encountered in a large variety of environmental niches, including some dairy, and many vegetable or plant fermentations. Some strains of *Lactobacillus* are found as natural commensals of the gastrointestinal tract (GI tract), the oral cavity and the female uro-genital tract

of animals and humans. The ability to adhere to specific regions of the GI tract is a property which has stimulated research aimed at the use of lactobacilli as delivery-vehicles for therapeutic compounds such as immunomodulators, antibodies, enzymes and vaccines (Marteau & Rambaud 1993 Hols et al. 1997; Slos et al. 1998). *Lactobacillus plantarum* arrives in the small intestine in an active state (Vesa et al. 2000) and is frequently encountered as a natural inhabitant of the human gastro-intestinal tract (Ahrne et al. 1998). Some strains are being marketed as probiotic. Previous studies using gel electrophoresis have indicated that *L. plantarum* has one of the largest genomes known among lactic acid bacteria (Chevalier et al. 1994; Daniel 1995). The Wageningen Centre for Food Sciences (WCFS) in collaboration with Greenomics in Wageningen, has determined the complete genome sequence of *L. plantarum* WCFS1, a single colony isolate of strain NCIMB8826, a strain of human origin.

Genome sequencing status and results

Sequencing was accomplished using a shotgun approach, while λ-clones and multiplex PCR were used to fill the gaps. The circular chromosome of *L. plantarum* WCFS1 consists of 3 308 274 bp with an average G+C content of 44.5%, and is among the largest of lactic acid bacteria. In addition, the strain harbours three plasmids of 36 069 bp (G+C content: 40.8%), 2365 bp (G+C content: 34.3%), and 1917 bp (G+C content: 39.5%), respectively. Automated prediction software (Glimmer, Genemark) was used to obtain a primary prediction of protein encoding genes, which was manually improved to result in a current list containing about 3050 genes.

The orientation of the majority of the genes is highly organized from the origin to the terminus of replication for both halves of the chromosome. Five rRNA-encoding operons, with very few sequence polymorphisms, could be identified scattered around the chromosome. Automated ORF analysis and functional annotation was performed using BioScout (LION) and Pedant-Pro (BioMax) software packages, followed by extensive manual curation. Functions were predicted for over 65% of the gene products. Major categories are proteins involved in energy metabolism (8%), cell envelope (8%), transport (13%) and regulation (9%). Over 2300 encoded proteins have expect scores better than 1e-10 to database entries, and nearly 90% of these are most similar to proteins of other Gram-positive bacteria, predominantly those encoded in the genomes of *Listeria, Lactococcus, Bacillus* and *Streptococcus*.

L. plantarum is a versatile and flexible organism and is able to grow on a wide variety of sugar sources. This phenotypic trait is reflected by the high number of predicted PEP-dependent sugar phosphotransferase systems (PTS; 25 complete) and other sugar transporters encoded by WCFS1. Strikingly, over 60% of these sugar transporters are located within 250 kb in both directions from the origin of replication.

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Lactobacillus rhamnosus HN001 (contributed by James Dekker and Mark Lubbers)

Lactobacillus rhamnosus is one of the few species of *Lactobacillus* that have been used as probiotic organisms in functional foods. In addition, this species is one of the two most common non-starter lactic acid bacteria found in New Zealand cheddar cheese; the other is *L. paracasei*. We have identified a strain of *L. rhamnosus*, designated HN001, that has both flavour-enhancing and probiotic attributes. It can be used as an adjunct during cheese manufacture to reduce adventitious microflora, accelerate cheese ripening, and improve cheese flavour. We have also demonstrated that HN001 has the 'prerequisite' properties of a probiotic, including confirmation of taxonomic classification, acid and bile resistance, adherance to intestinal cells, transient colonisation, and lack of any toxicity or detrimental effects (Prasad et al. 1998; Zhou et al. 2000a,b, 2001; Gopal et al. 2001; Sheih et al. 2001). A number of *in vitro* and *in vivo* tests, including and human clinical trials, have demonstrated the ability of HN001 to influence specific cytokines, NK cell activity, T cell stimulatory capacity and phagocytic ability, and influence the balance of the intestinal microflora in humans (Gill et al. 2000, 2001a; Tannock et al. 2000). HN001 also has antimicrobial activity against important gut pathogens (Gill et al. 2001b). *L. rhamnosus* HN001 is genetically accessible and we have developed tools and techniques for efficient gene disruption and overexpression. Therefore, HN001 is an ideal candidate for

an in-depth study of factors that contribute to probiotic and flavour-enhancing attributes of lactobacilli.

Genome sequencing status and results

A draft genome sequence of HN001 was obtained by shotgun sequencing. Analysis of the data indicates a genome size of about 2.4 Mb, and an average G+C content of approximately 46.4%, which is within the range reported for other *Lb. rhamnosus* species. In addition, HN001 harbours two plasmids of 8754 bp (G+C content: 41.9%) and approximately 40 000 bp (G+C content: 43.7%). Automated ORF prediction and annotation were not used. Rather, the whole genome sequence was compared against publicly available databases and the output manually examined for genes of specific interest. In addition, metabolic pathways of particular interest were also identified. To date, over 900 candidate genes have been examined and almost 300 genes with potential involvement in flavour, survival and probiotic activity (e.g. gut adhesion, immune and anti-microbial activity) have been identified. Of these, 39 HN001 genes have been functionally characterised using a number of approaches, including gene knockout by disruption, overexpression, gene complementation and biochemical assay of purified recombinant protein.

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Lactobacillus johnsonii La1 (NCC2761) (contributed by David Pridmore)

Lactobacillus johnsonii strains have been mainly isolated from the feces of humans and animals (Johnson et al. 1980; Fujisawa et al. 1992), suggesting that these bacteria constitute part of the natural intestinal flora. *L. johnsonii* La1 (formerly *Lactobacillus acidophilus* La1) is a Nestlé proprietary strain that has been extensively studied for its probiotic properties and is commercialised in the LC[1] fermented milk products. La1 shows immunomodulatory properties (Link-Amster et al. 1994; Schiffrin et al. 1995; Haller et al. 2000a,b), antipathogenic properties (Bernet et al. 1994; Bernet-Camard et al. 1997; Felley et al. 2001; Pérez et al. 2001) and the ability to interact with the host (Granato et al. 1999; Neeser et al. 2000). The determination and analysis of the complete genome sequence of *L. johnsonii* La1 has provided valuable targets for the investigation of the above interactions. *L. johnsonii* La1 is amenable to genetic manipulation, an important consideration in view of the wealth of information revealed in the genome sequence and the interest to investigate the probiotic features of this bacterium at the genetic level. Efficient transformation by plasmids, the ability to use pG+host9 and its temperature sensitive replication to produce genetic disruption of selected genes and the application of pG+host9:IS*S1* (Maguin et al. 1996) to produce random mutations are some of the tools available, making *L. johnsonii* La1 an important host for advanced genetic analysis.

Genome sequencing status and results

Random shotgun cloning and sequencing was supplemented with sequencing of BAC clones and long-range PCR products to finally produce a circular chromosome of approximately 2.022 Mbp. The computer-predicted *Sma*I digest closely matches the physical digest pattern. Bioinformatic analysis has identified the *L. johnsonii* La1 counterparts of the best-studied genes and pathways. The initial analysis of these results has not revealed any major surprises, except for the complete lack of amino acid biosynthetic pathways which appears to be compensated for by an increased number of amino acid transporters. Given the limited literature describing the natural habitat of this bacterium, a more profound analysis of the genetic potential of *L. johnsonii* La1 could provide important clues as to the nutritional composition of its environment. The structure of the *L. johnsonii* La1 chromosome is unusual in that both the plot of GC skew and the direction of the transcription of the majority of genes do not place the proposed terminus opposite the origin (approximately 1000 kb), but close to 1300 kb. This chromosome structure may be explained by the structure, orientation and position of two genes encoding large cell-wall anchored proteins in *L. johnsonii* La1. Both genes predict proteins with more than 100 copies of a conserved 10 amino acid repeat. This repeat is reflected in the gene sequences and may be the target for recombination. Inversion of the approximately 600 kb of sequence between the repeat would position

the terminus opposite the origin. A second example of *L. johnsonii* La1 genome rearrangement has been observed by defining the direct repeats produced by IS element transposition. One pair of elements showed a perfect exchange of the direct repeat sequences. Confirmed by PCR, this event has produced an inversion of approximately 800 kb of the *L. johnsonii* La1 genome. Extension of this analysis to other *L. johnsonii* isolates has allowed the identification of some of the intermediates in the events leading to the *L. johnsonii* La1 genome configuration.

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Lactobacillus acidophilus ATCC700396 / NCFM (contributed by Todd R. Klaenhammer and Raul Cano)

In 1900, Moro first isolated *Lactobacillus acidophilus* from infant feces. Meaning 'acid loving', *acidophilus* can be found in the intestinal tract of humans and animals, as well as infants consuming high milk, lactose, or dextran diets. Metchnikoff's 1906 work on *The prolongation of life: optimistic studies*, implicated a lactic acid bacillus, found in Bulgarian yoghurts, as the agent responsible for deterring intestinal putrefaction and aging. Later, *Lactobacillus acidophilus* was considered to be the most likely species to fulfill the base criteria expected of a probiotic cultures; survival through the GI tract, bile tolerance, acid tolerance, and antimicrobial production. Over the last century a considerable amount of research has been carried out on *L. acidophilus*, and a group of closely-related species clustered in a group known as the 'acidophilus complex' (Klaenhammer & Russell, 1999). Of the six species in the group, *L. acidophilus* continues to be the bacterium most often implicated in providing probiotic benefits and remains to be the species most commonly found in foods or supplements that contain probiotic cultures. In a recent survey, Clark et al. (2001) found that when *L. acidophilus* was recovered from probiotics samples, the majority of confirmed strains showed a genetic fingerprint resembling that of NCFM. The *L. acidophilus* NCFM culture has been used commercially since 1972 in a variety of products and probiotic dairy foods, like Sweet Acidophilus^{TM} milk. Its long history of safe use in commercial products for and human consumption, and its close genetic relationship to the neotype strain, ATCC4356 (Walker et al. 1996), were two major reasons that the *L. acidophilus* NCFM strain (deposited as ATCC700396) was selected for genome sequencing.

L. acidophilus is an obligate homofermenter and metabolizes hexoses primarily to lactic acid. Both the D- and L-isomers of lactate are produced. *L. acidophilus* NCFM metabolizes fructo-oligosaccharides (Kaplin & Hutkins 2000), which are prebiotic compounds that support the growth of beneficial gut bacterial. Some of the functional properties of this organism have been reviewed (Sanders & Klaenhammer 2001) and include: survival through the GI tract (Conway et al. 1982), reduction of fecal mutagenic enzymes (Goldin & Gorbach 1980; Goldin et al. 1980), adherence to intestinal tissues (Conway et al. 1987; Greene & Klaenhammer 1994), stimulation of IgA in mice by a culture cocktail that includes *L. acidophilus* NCFM (Tejada-Simon & Pestka 1999), and production of the bacteriocin, lactacin B (Barefoot & Klaenhammer 1983). In contrast to the other members of the acidophilus complex, there is considerable information on *L. acidophilus* NCFM related to its transformability by electroporation (Luchansky et al. 1988; Walker et al. 1996) and the development of genetic tools, that will be used for the functional genomic analysis of this species (Kullen & Klaenhammer 1999; Russell & Klaenhammer 2001a,b).

Genome sequencing status and results

Genome sequencing was accomplished using a shotgun random library approach and pUC18. PCR and direct genome sequencing (Fidelity Systems, Inc.) were used to fill gaps and polish selected regions. The genome has been assembled into a single contig and consists of 1.99 Mbp with an average GC content of 34.6%. The genome was automatically annotated by the prediction, database, and annotation software GAMOLA (Global annotation of multiplexed on-site blasted DNA sequences) to identify 1979 ORFs in a coding density of 89.9%. Predicted ORFs were manually verified against the on-site Blast and Pfam databases. Some features encoded in the NCFM genome are six copies of an IS3-type element, two bacteriocins, three distinct regions encoding competence genes, two *ldh*

genes, two bile salt hydrolase genes, two operons for fructo-oligosaccharide (FOS) utilization, and no intact prophages. There is also a collection of genome sequence information available in public databases for various strains of the *L. acidophilus* species, including heat shock operons *groELS* and *dnaK*, a sigma factor *ropD*, bacteriocins acidocins 8912 and B, bile salt hydrolase, and β- and phospho-β-galactosidases. Comparison of the NCFM genome sequence with these available database sequences has revealed a number of interesting similarities and marked differences.

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Lactobacillus gasseri ATCC33323, neotype (contributed by Todd R. Klaenhammer, Eric Altermann and Rodolphe Barrangou)

Recent developments in molecular taxonomy have revealed six different *Lactobacillus* species that comprise the 'acidophilus' group, which are widely considered as model probiotic organisms (Klaenhammer & Russell 2000). Among these, *Lactobacillus gasseri* appears to represent the major homofermentative *Lactobacillus* species that occupies the human GI tract (Mitsuoka, 1992; Kullen et al. 2000; Heilig et al. 2002). *L. gasseri* demonstrates good survival in the GI tract (Conway et al. 1982; Pedrosa et al. 1995) and has been associated with a variety of probiotic activities and roles including reduction of fecal mutagenic enzymes (Pedrosa et al. 1995), adherence to intestinal tissues (Conway et al. 1987; Greene & Klaenhammer 1994), stimulation of macrophages (Kitazawa et al. 1994; Kirjavainen et al. 1999; Kitazawa et al. 1994), and production of bacteriocins (Itoh et al. 1995).

Data are available on the transformability by electroporation (Russell & Klaenhammer 2001a,b), and expression of heterologous genes in *L. gasseri* (Cho et al. 2000), as well as on the genome sequence of one of its temperate bacteriophage (Altermann et al. 1999). *L. gasseri* is more amenable to DNA introduction and manipulation than other members of the acidophilus complex, leading to the development of genetic tools that will be essential to functional genomic analysis of this species (Kullen & Klaenhammer 1999; Russell & Klaenhammer 2001a,b).

Genome sequencing status and results

Genome sequencing of the *L. gasseri* neotype strain, ATCC33323, was carried out by the Department of Energy–Joint Genome Institute in collaboration with the Lactic Acid Bacterial Genomics Consortium. The strategy for genome sequencing was based on a shotgun random sequencing of a small genomic DNA insert (average 2.5 kb) library cloned into pUC18 at approximately 8-fold coverage, complimented by sequencing a large insert (35–45 kb) Fosmid library at a lower coverage. Gap closing is ongoing using a multiplex PCR approach. The genome assembly of *L. gasseri* currently consists of 67 contigs, representing a total of 1.84 Mbp, with an average G+C content of 35.1%. The sequence information is posted on the JGI Microbial Genomes web site at: (http://www.jgi.doe.gov/JGI_microbial/html/ lactobacillus_gas/lactob_gas_homepage.html) GAMOLA (*G*lobal *A*nnotation of *O*n-site *B*asted DN*A*-sequences) software was developed by E. Altermann to automatically predict coding open reading frames (ORFs) and annotate the draft sequence (http://www.cals.ncsu.edu/food_science/trk/main.html). GAMOLA relies on the available software Glimmer2 (Delcher et al. 1999), NCBI toolkit (National Center for Biotechnology Information Bldg 38A, NIH 8600 Rockville Pike Bethesda, MD 20894), Primer3 (Rozen & Skaletsky, 1997) and HMMER2.2g (Eddy 1998) and combines the results into functionally annotated DNA sequences in Genbank format.

For the *L. gasseri* sequence, the automated annotation predicted 1803 ORFs larger than 100 bp. The average gene length is 930 bp with a coding percentage of 89.1%. After the initial automated annotation, the genome was predicted to encode: 31 ABC transporters, 14 ion-ATPase transporters and two proton-antiporters; 21 PTS-systems (some partial), 16 stress responsive genes (sigma factor, chaperones, heat shock operons and chaperones), one intact prophage, 14 mobile elements (transposases, integrases and IS-elements), 32 genes involved in cell-cycle or cell-shape, eight genes implied in aggregation/adhesion, seven genes related to late competence, 40 peptidases (proteolytic system plus general peptidases), 20 tRNA genes, and at least two rRNA operons. Manual curation of the automated annotation will be carried out

upon completion of genome sequencing and closure of the major gaps.

This project has revealed a number of gene systems that are likely to be important in the gastrointestinal survival and activity of this human probiotic species. The public availability of the *L. gasseri* genome will promote comparative genomic analysis among LAB.

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Lactobacillus helveticus CNRZ32 (contributed by: Jim Steele and Jeff Broadbent)

Within the genus *Lactobacillus, L. helveticus* is part of the obligately homofermentative ('Group I') cluster, which can produce D- and L-lactic acid from hexose sugars via the Embden-Meyerhof pathway and is incapable of fermenting pentoses (Axelsson 1998). *L. helveticus* grows on a relatively restricted number of carbohydrates that includes lactose and galactose and typically requires riboflavin, pantothenic acid and pyridoxal for growth (Hammes & Vogel 1995).

Phylogenetically, *L. helveticus* is quite closely related (<10% sequence divergence) to *L. amylovorus*, *L. acidophilus*, *L. delbrueckii*, *L. acetotolerans*, *L. gasseri*, and *L. amylophilus*. Among these species, *L. helveticus* is most closely related (<2% sequence divergence) to *L. amylovorus* and *L. acidophilus* (Schleifer & Ludwig 1995). The genome size of *L. helveticus* has been determined to be 2.4 Mb by pulse-field gel electrophoresis (unpublished data). Approximately 40 chromosomal genes and four plasmids have been sequenced from *L. helveticus*. Methods for electroporation and gene replacement have been developed for *L. helveticus* CNRZ32 (Bhowmik & Steele 1993; Bhowmik et al. 1993); however, attempts to utilize these methods with other strains of *L. helveticus* have been unsuccessful.

L. helveticus is a component of 'thermophilic' starter cultures used in the manufacture of a number of fermented dairy products (Hassan & Frank, 2001). These cultures have an optimum growth temperature of approximately 42 °C and contain *Streptococcus thermophilus* with *L. delbrueckii* subsp. *bulgaricus* and/or *L. helveticus*. These cultures are utilized in the manufacture of Swiss-type and Italian-type cheese varieties. There has been a 325% increase in the past 20 years in the production of Mozzarella cheese in the United States to more than 2244 million pounds, with an economic value of nearly $1 billion (National Cheese Institute 1998). Additionally, strains of *L. helveticus* are commonly used as flavor adjunct cultures, where they are added to bacterial ripened cheese varieties to reduce bitterness and accelerate the development of beneficial flavors.

To have utility in the manufacture of fermented dairy products strains must be resistant to bacteriophage, have stable fermentation properties, and consistently produce products with acceptable flavor and texture attributes. A significant body of research is available concerning the proteolytic system of these organisms, with at least 15 components of the proteolytic system characterized at the nucleotide level and numerous single and double mutants lacking specific components studied in detail (Christensen et al. 1999). Limited information is available concerning a number of other industrially relevant metabolic pathways (i.e., carbohydrate fermentation) and enzymes (i.e., esterases). Genomic sequence analysis of *L. helveticus* will allow researchers to fill the significant gaps present in our understanding of the physiology of this organism by providing a comprehensive view of the enzymes and metabolic pathways potentially involved in industrially relevant phenotypes. This knowledge will allow researchers to develop more effective strategies to enhance the utility of these organisms in the manufacture Swiss-type and Italian-type cheese varieties as well as their utility as flavor adjunct cultures.

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Lactobacillus delbrueckii subsp. *bulgaricus* ATCC BAA-365 (contributed by James Steele)

Within the genus *Lactobacillus, L. delbrueckii* is part of the obligately homofermentative ('Group I') cluster, which can produce D-lactic acid from hexose sugars via the Embden–Meyerhof pathway and is incapable of fermenting pentoses (Axelsson 1998). The *L. delbrueckii* species contains three subspecies, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis*, and *L. delbrueckii* subsp. *bulgaricus*. *L. delbrueckii* subsp. *bulgaricus* grows on a relatively restricted number of carbohydrates and typically requires pantothenic acid and niacin (Hammes & Vogel 1995).

Phylogenetically, *L. delbrueckii* subsp. *bulgaricus* is closely related (<10% sequence divergence) to *L. amylovorus*, *L. acidophilus*, *L. helveticus*, *L. acetotolerans*, *L. gasseri*, and *L. amylophilus* (Schleifer & Ludwig 1995). The GC ratio of *L. delbrueckii* subsp. b*ulgaricus* (49–51%) is somewhat higher than that found among other species (34–46%) within this phylogenetic tree (Hammes & Vogel 1995). The genome size of *L. delbrueckii* subsp. *bulgaricus* has been determined to be 2.3 Mb by pulse-field gel electrophoresis (Leong-Morgenthaler 1990). Very few chromosomal genes (<15) have been sequenced from *L. delbrueckii* subsp. *bulgaricus*, however the complete sequence of a small cryptic plasmid and the partial sequence of a bacteriophage are known. Gene transfer systems for *L. delbrueckii* subsp. *bulgaricus* include two conjugation-based gene transfer systems (Rantsiou et al. 1999; Thompson et al. 1999) and electroporation (Serror et al. 2002).

L. delbrueckii subsp. *bulgaricus* is a component of 'thermophillic' starter cultures used in the manufacture of a number of fermented dairy products (Hassan & Frank 2001). These cultures have an optimum growth temperature of approximately 42 °C and contain *Streptococcus thermophilus* with *L. delbrueckii* subsp. *bulgaricus* and/or *L. helveticus*. These cultures are utilized in the yogurt, Swiss-type and Italian-type cheese varieties. There has been a 240% increase in the past 20 years in the production of yogurt in the United States to 1371 million pounds in 1997, with a wholesale value of over $1.1 billion (Milk Industry Foundation 1998).

Acknowledgments

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Lactobacillus delbrueckii ssp. *bulgaricus* ATCC11842 (contributed by Maarten van de Guchte and Emanuelle Maguin)

Lactobacillus delbrueckii ssp. *bulgaricus* is a thermophilic lactic acid bacterium that is principally known for its use in yogurt production, where it assures milk fermentation in conjunction with *Streptococcus thermophilus*. As such, *L. bulgaricus* is one of the economically most important LAB.

For the type strain ATCC11842 a reproducible transformation procedure is available (Serror et al. 2002) as well as gene inactivation systems (unpublished) that will allow the exploitation of genome data. A Proteome reference map has been established (unpublished) which will facilitate and benefit from a genome analysis. In this context, the determination of the complete genome sequence of this strain was undertaken in a joint effort of the Centre National de Séquençage (CNS, Genoscope, Evry Fr) and the Institut National de la Recherche Agronomique (INRA).

Genome sequencing

Although the genome size of *L. bulgaricus* was previously estimated to be 2.3 Mbp, recent pulse field electrophoresis analysis revealed that the genome size of the type strain ATCC11842 was about 1.8 Mbp. The genome sequence has been determined using a shotgun sequencing approach. Contigs were assembled after sequencing of two plasmid libraries with differently sized inserts. Sequences from a mini-BAC library were subsequently used for scaffolding. The scaffold structure was validated by long range PCR, and links between the initial scaffolds were established by multiplex long accurate PCR. The average GC content of the *L. bulgaricus* genome is 50%, while regions that presumably result from horizontal transfer can be detected with GC contents as low as 31%.

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Lactobacillus sakei 23K (contributed by Stéphane Chaillou and Monique Zagorec)

Lactobacillus sakei is the predominant LAB found on fresh meat and is widely used as starter in fermented meat products in Western Europe. It is also occasionally found on silage, sourdough and smoked fish. The physiology of *L. sakei* is still poorly understood when compared to dairy LAB. Recent studies seem to indicate that *L. sakei* has developed original metabolic and physiological traits to adapt to a meat environment (for a review see Champomier-Verges et al. 2002). To better understand the ability and fitness of *L. sakei* to grow on meat we have chosen the genomic approach. The *L. sakei* genome sequencing project was launched in year 2000, using strain 23K, isolated from sausage, as a model.

Genome sequencing status and results

A physical and genetic mapping of the chromosome was first established by pulse-field gel electrophoresis after digestion with rare-cutting enzymes (Dudez et al. 2002). This preliminary map allowed to estimate the chromosome size to be about 1.85 Mb. Seven *rrn* operons including one doublet were identified and their flanking regions characterised. The sequencing of 90% of the genome was accomplished by a classical shotgun strategy, where 8000 clones were first sequenced on their forward side, followed by reads on the reverse side for 2000 clones flanking contigs end, and 500 internal reads. The gap closure phase was carried out using ligation-mediated PCR and several customised BLAST strategies against related genomes such as *Bacillus subtilis*, *Bacillus halodurans* and *Listeria monocytogenes*. Remaining gaps are currently analysed with a set of other strategies such as multiplex PCR, sequencing on chromosomal DNA, and contigs ordering based on the physical map. Currently, 96% of the genome has been sequenced with an average base redundancy of 4×. A preliminary analysis of the genome content was carried out with the CDS prediction software SHOW (Nicolas et al. 2002) revealing 1792 putative protein-encoding genes for 1772 kp sequenced. Automated annotation followed by manual curation will be done with a improved version of Artemis software from the Sanger Center, UK.

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Lactobacillus casei ATCC 334 (contributed by Jeffrey Broadbent)

Lactobacillus casei are rod-shaped LAB with a G + C content of 45–47% (Kandler & Weiss, 1986). Within the genus *Lactobacillus*, *L. casei* is clustered with facultatively heterofermentative ('Group II') species which produce lactic acid from hexose sugars via the Embden–Meyerhof pathway and from pentoses by the 6-phosphogluconate/phosphoketolase pathway (Axelsson, 1998). Growth occurs at 15 °C but not 45 °C, and requires riboflavin, folic acid, calcium pantothenate, and niacin (Kandler & Weiss 1986). Previously, four subspecies of *L. casei* were recognized: *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplantarum*, *L. casei* subsp. *rhamnosus*, and *L. casei* subsp. *tolerans* (Kandler & Weiss 1986). However, recent phylogenetic studies have led to proposals that members of the *L. casei* group be divided into three species: *L. rhamnosus*, *L. zeae* and *L. casei*, with *L. casei* ATCC 334 as the neotype strain for the latter species (Collins et al. 1989; Dellaglio et al. 1991; Dicks et al. 1996; Mori et al. 1997; Chen et al. 2000).

Members of the *L. casei* cluster are remarkably adaptive, and may be isolated from raw and fermented dairy products, fresh and fermented plant products, and the reproductive and intestinal tracts of humans and other animals (Kandler & Weiss 1986). Industrially, *L. casei* have application as human probiotics (health-promoting live cultures), as acid-producing starter cultures for milk fermentation, and as specialty cultures for the intensification and acceleration of flavor development in certain bacterial-ripened cheese varieties (Kosikowski 1982; Fox et al. 1998; Fonden et al. 2000).

L. casei ATCC 334 was originally isolated as an adventitious contaminant from Emmental cheese. This bacterium, which has an estimated genome size of

2.2 Mb (Ferrero et al. 1996; Tynkkynen et al. 1999), was selected for genome sequencing because: (a) it was isolated from ripening cheese; (b) it is amenable to transformation and genetic manipulation; and (c) it is the proposed type strain for *L. casei*. Sequence analysis of the *L. casei* ATCC 334 genome is expected to provide a comprehensive view of the enzymes and metabolic pathways that are potential contributors to cheese flavor development. In addition, improved knowledge of global gene regulation and integrative metabolism in *L. casei* will help answer long-standing questions regarding mechanisms for the health-promoting benefits of LAB, identify means by which LAB species grow in harsh environments , highlight the most rational strategies for metabolic and genetic improvements to industrial strains, and improve molecular biology resources for genetic manipulation of dairy lactobacilli.

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Lactobacillus casei BL23 (contributed by Josef Deutscher and Axel Hartke)

Lactobacillus casei is a heterofermentative organism widely used in milk fermentation and is therefore of great biotechnological importance. In addition, it belongs to the few bacteria for which probiotic effects have been unequivocally established (for a critical review on probiotics see Marchand & Vandenplas 2000). 'Healthy' effects of this organism have been observed in numerous studies with men and animals. For example, *Lb. casei*, which survives transit through the gastrointestinal tract (Yuki et al. 1999), has been shown to positively affect the immune response (Matsuzaki & Chin 2000; Yasui et al. 1999), to enhance the resistance towards certain forms of diarrhea and to prevent infections by several pathogenic organisms (Wagner et al. 2000; Alvarez et al. 2001). Genetic tools, which allow the transformation of many *Lb. casei* strains and the construction of predetermined chromosomal mutants carrying deletions or point mutations (Dossonnet et al. 2000; Viana et al. 2000), are available, which together with its probiotic features makes this organism a very interesting subject for further genetic and functional genomic studies.

Genome sequencing status and results

The genome size of *Lb. casei* BL23 has previously been estimated to be 2.3 Mb, but recent pulsed-field gel electrophoresis analysis (in collaboration with the laboratory of M. Zagorec) revealed a genome size between 2.5 and 2.7 Mb. In order to sequence the genome of *Lb. casei* BL23 we used a shotgun approach (collaboration between the Laboratoire de Génétique des Microorganismes, INRA-CNRS, Thiverval-Grignon and the Laboratoire Microbiologie de l'Environnement, Université de CAEN). Sequencing *Lb. casei* DNA fragments with a mean size of 1.3 kb from both ends, present in about 8000 isolated clones, allowed us to determine about 90% of the complete genome. We presently attempt to close the about 560 remaining gaps. *Lb. casei* BL23 has been cured of plasmid pLZ15 and therefore contains only a single circular chromosome.

Lb. casei seems to be a versatile organism capable of adapting to variable growth conditions. Most intriguingly it seems to be able to utilize a large variety of different carbon sources. This is reflected by the relatively large number of predicted phosphoenolpyruvate:sugar phosphotransferase systems (PTS) (see Table 3). In *Lb. casei*, the coordinate action of the various carbon utilization systems was found to be controlled by a protein kinase/phosphatase (HprK/P) implicated in carbon catabolite repression and inducer exclusion (Dossonnet et al. 2000). Inactivation of only the phosphatase activity of this bifunctional enzyme had a drastic effect on the growth behavior by preventing the utilization of almost any carbohydrate (Monedero et al. 2001).

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Lactobacillus brevis ATCC 367 (contributed by Milton Saier)

Lactobacillus brevis is a heterofermentative bacterium that can be isolated from many different environments. It is frequently used as a starter culture in silage fermentation, sourdough production and LAB-type beer fermentation. In beverages obtained by alcoholic fermentation, lactobacilli may contribute to the quality of the product but may also cause spoilage. Certain *L. brevis* strains are resistant to hop bittering substances such as isohumulone and are able to grow in beer. Their growth changes the turbidity, flavor and aroma of the beer (Richards & Macrae 1964). *L. brevis* strains involved in wine fermentation may produce biogenic amines by decarboxylation of precursor amino acids through the action of substrate-specific enzymes (Moreno-Arribas & Lonvaud-Funel 2001). The ingestion of foods containing high levels of such amines, particularly histamine and tyramine, can lead to several toxicological disturbances (ten Brink et al. 1990; Mariné-Font et al. 1995).

We have studied the involvement of HPr(ser-P) in the regulation of non-PTS permeases in *L. brevis* (Ye et al. 1994, 1995). When provided with an exogenous energy source such as arginine, galactose grown cells of *L. brevis* transport galactose and the non-metabolizable galactose analogue, thiomethyl-β-galactoside (TMG), by a sugar:H^+ symport mechanism (Romano et al. 1987; Djordjevic et al. 2001). *L. brevis* shows very low transformation efficiency by electroporation rendering this bacteria difficult to manipulate. The cryptic plasmid of 14 kb from *L. brevis* has recently been sequenced and will be used in the development of novel vectors and essential genetic tools.

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Pediococcus pentosaceus ATCC 25745 (contributed by Jeffery Broadbent and James Steele)

Phylogenetically *Pediococcus* and *Lactobacillus* form a super-cluster that can be divided in to two sub-clusters, all species of *Pediococcus* fall within the *Lactobacillus casei–Pediococcus* sub-cluster. Morphologically, pediococci and lactobacilli (rods) are distinct. The formation of tetrads via cell division in two perpendicular directions in a single plane is a distinctive characteristic of pediococci. *Pediococcus* can be described as 'the only acidophilic, homofermentative, LAB that divide alternatively in two perpendicular directions to form tetrads' (Simpson & Taguchi 1995). Lactic acid is produced from hexose sugars via the Embden–Meyerhof pathway and from pentoses by the 6-phosphogluconate/phosphoketolase pathway (Axelsson, 1998).

Strains of *P. pentosaceus* have been reported to contain between three and five resident plasmids (Graham & McKay 1985). Plasmid-linked traits include the ability to ferment raffinose, melibiose, and sucrose, as well as, the production of bacteriocins (Daeschel & Klaenhammer 1985; Gonzalez & Kunka 1986). Plasmids can be conjugally transferred between *Pediococcus* and *Enterococcus*, *Streptococcus*, or *Lactococcus* (Gonzalez & Kunka 1983). Electroporation has been utilized to introduce plasmids into pediococci, including *P. pentosaceus* (Kim et al. 1992; Caldwell 1996).

P. pentosaceus can be isolated from a variety of plant materials and bacterial-ripened cheeses. This organism is used as an acid-producing starter culture in sausage fermentations, cucumber and green bean fermentations, soya milk fermentations, and silage (Simpson & Taguchi 1995). *P. pentosaceus* are also a typical component of the adventitious or non-starter microflora of most cheese varieties during ripening (Beresford et al. 2001). In addition, it has been suggested that this organism may have value as an acid-producing starter culture in the dairy fermentations (Caldwell et al. 1996, 1998).

Genetic studies of *P. pentosaceus* have generated a limited quantity of information on plasmid and chromosomal encoded genes. With only one plasmid and eight unique chromosomal regions sequenced, the vast majority of genes encoding industrially important attributes have yet to be described. Genomic sequence analysis of *P. pentosaceus* genome will help fill key knowledge gaps by providing a comprehensive view

of the enzymes and metabolic pathways related to: (1) acid and flavor production in fermented meat and vegetable foods; (2) mechanisms by which by *P. pentosaceus* and other non-starter LAB grow and direct flavor development in ripening cheese; and (3) mechanisms by which *P. pentosaceus* and related LAB spoil wine and other alcoholic beverages.

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Streptococcus thermophilus LMD-9 (contributed by Robert Hutkins)

Research during the past two decades has revealed that *Streptococcus thermophilus* has properties that make it one of the most commercially important of all LAB. *S. thermophilus* is used, along with *Lactobacillus* spp., as a starter culture for the manufacture of several important fermented dairy foods, including yogurt and Mozzarella cheese. It's use has increased significantly during the past two decades as a result of the tremendous increase in consumption of these products. According to USDA statistics, in 1998, more than 2.24 billion pounds Mozzarella cheese and 1.37 billion pounds of yogurt were produced, respectively, with a combined economic value of nearly $5 billion. This increase has led to new demands on the performance and production requirements of such starter cultures. Industrial strains, for example, should be insensitive to bacteriophage, have stable fermentation characteristics, and produce products having consistent flavor and texture properties. Although research on the physiology of *S. thermophilus* has revealed important information on some of these properties, including sugar and protein metabolism, polysaccharide production, and flavor generation, only recently has the genetic basis for many of these traits been determined.

Currently, several traits in *S. thermophilus* have been targeted for strain improvement programs (Delcour et al. 2000). Since bacteriophage are responsible for considerable economic losses during cheese manufacture, efforts are underway to engineer restriction and other phage-resistance systems into commercial strains. Enhancing stability and expression of exopolysaccharides that act as natural thickening agents has also attracted significant attention. Finally, *S. thermophilus* has an important role as a probiotic, alleviating symptoms of lactose intolerance and other gastrointestinal disorders.

The genome of *S. thermophilus* is 1.8 Mb, making it among the smallest genomes of all LAB. Although a moderate thermophile, it is phylogenetically related to the more mesophilic lactococci and has a comparable low G+C ratio (40%). Genes coding for metabolic pathways involved in sugar catabolism (Poolman et al. 1989; Vaughan et al. 2001), protein and peptide utilization (Fernandez-Espla et al. 2000; Garault et al. 2002), polysaccharide production (Almirón-Roig et al. 2000), the stress response system (Perrin et al. 1999), and phage resistance mechanisms (Solow & Somkuti 2000; Burrus 2001) have been sequenced and characterized. Although most strains do not harbor plasmids, other mobile elements have been reported (Guedon et. al. 1995), and techniques for gene transfer and mutagenesis have been developed (Coderre & Somkuti 1999; Baccigalupi et al. 2000).

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Streptococcus thermophilus LMG18311 and CNRZ1066 (contributed by Alexander Bolotin, Ross Overbeek and Pascal Hols)

Streptococcus thermophilus is one of the most economically important LAB used in the manufacture of yogurt and some Swiss- or Italian-type hard cooked cheeses. This Gram-positive, non-sporulating, catalase-negative, facultative-anaerobe coccus produces remarkable quantities of polysaccharides contributing to the rheological properties of fermented products. The comprehensive knowledge of *S. thermophilus* biology is important for the dairy industry but is rather limited (Delcour et al. 2000). The availability of the whole genome sequence of this species would strongly improve the knowledge of its metabolism and pave the way for engineering of new starter cultures and better control of existing fermentation processes.

The Life Science Institute at the Catholic University of Louvain (Belgium), the Laboratory of Genetic Microbiology, Jouy en Josas, INRA (France) and Integrated Genomics Inc, Chicago (USA) joined their efforts in determining the genome sequences of two strains of *S. thermophilus*.

Genome sequencing status and results

The complete sequences were determined by the random shotgun sequencing strategy followed by multiplex PCR described earlier (Bolotin et al. 2001). Sequences of the two strains were determined by construction of two independent sequence datasets containing 20 000 random and 1500 primer directed reads for LMG18311 strain and 28 000 random and 2000 primer directed reads for CNRZ1066 strain. The assembled genomes were analysed using the ERGO bioinformatics suite. Both genomes contain ~1.8 Mb encoding six rRNA, a compete set of tRNAs, several types of IS, and about 1800 open reading frames. They are organized as a single circular chromosome with 39% GC content, and show 95% nucleotide identity. Although the *S. thermophilus* species is characterized as GRAS (Generally Recognized As Safe), several features of its metabolism are similar to that of pathogenic Streptococci. Analysis of the genome has revealed several key aspects of the pathways of carbohydrates, amino acids, nucleotides, polysacharides and lipids metabolism. More than 100 genes involved in the transport of a variety of substrates such as peptides, sugars, metal ions, and cofactors have been identified. The presence of several phage and plasmid related genes indicates the importance of horizontal gene transfer for evolution of *S.thermophilus*. More information can be found at web sites: http://www.biol.ucl.ac.be/gene/genome/, http://spooky.jouy.inra.fr (restricted access), http://www.integratedgenomics.com (restricted access)

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Leuconostoc mesenteroides subsp. *mesenteroides* LA81 (contributed by Fred Breidt)

Leuconostoc species are epiphytic bacteria that are wide spread in the natural environment and play an important role in several industrial and food fermentations. *Leuconostoc mesenteroides* is a facultative anaerobe requiring complex growth factors and amino acids (Reiter & Oram 1982; Garvie 1986). Most strains in liquid culture appear as cocci, occuring singly or in pairs and short chains; however, morphology can vary with growth conditions; cells grown in glucose or on solid media may have an elongated or rod-shaped morphology. Cells are Gram-positive, asporogenous and non-motile. A variety of LAB, including *Leuconostoc* species are commonly found on crop plants (Mundt et al. 1967; Mundt 1970). *L. mesenteroides* is perhaps the most predominant LAB species found on fruits and vegetables and is responsible for initiating the sauerkraut and other vegetable fermentations (Pederson & Albury 1969). *L. mesenteroides* starter cultures also used in some dairy and bread dough fermentations (Server-Busson et al. 1999). Under microaerophilic conditions, a heterolactic fermentation is carried out. Glucose and other hexose sugars are converted to equimolar amount of D-lactate, ethanol and CO_2 via a combination of the hexose monophosphate and pentose phosphate pathways (Demoss et al. 1951; Garvie 1986; Gottschalk 1986). Other metabolic pathways include conversion of citrate to diacetyl and acetoin (Cogan et al 1981) and production of dextrans and levan from sucrose (Alsop 1983; Broker 1977). Viscous polysaccharides produced by *L. mesenteroides* are widely recognized as causing product losses and processing problems in the production of sucrose from sugar cane and sugar beets (Tallgren et al. 1999). The first observation of the production of polysaccharide 'slime' from sugar, dates to the earliest days of the science of microbiology; Pasteur (1861) attributed this activity to small cocci, presumably *Leuconostoc* species. Commercial production dextrans and levans by *L. mesenteroides*, for use in the biochemical and pharmaceutical industry, has been carried out for more than 50 years (Alsop 1983; Sutherland 1996). Dextrans are used in the manufacture of blood plasma extenders, hep-

arin substitutes for anticoagulant therapy, cosmetics, and other products (A;sp[1983; Kim & Day 1994; Leathers et al 1995; Sutherland 1996). Another use of dextrans is the manufacture of Sephadex gels or beads, which are widely used for industrial and laboratory protein separations (Sutherland 1996). We have selected *L. mesenteroides* subsp. *mesenteroides* LA81 for sequencing. The strain is widely available from a variety of culture collections listed as: ATCC 8293, DSM 20343, NCIB 8023, CCM 1803, NCDO 523, and NRRL B-1118. The strain is generally accepted as a neotype strain for this species; it produces dextran, and was originally isolated from fermenting olives.

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Oenococcus oeni PSU-1 (ATCC BAA-331) (contributed by David Mills)

Oenococcus oeni (formerly called *Leuconostoc oenos*) is a lactic acid bacterium that occurs naturally in fruit mashes and related habitats (VanVuuren & Dicks 1993). *O. oeni* is employed commercially to carry out the malolactic conversion, an important secondary fermentation in the production of wine (Kunkee 1991). *O. oeni* is a facultative anaerobe and one of the most acid- and alcohol-tolerant LAB. *O. oeni* shares relatively little DNA homology with the other genera in the *Leuconostoc* branch of the LAB (Dellaglio et al. 1995).

Perhaps the most studied aspect of *O. oeni* is its ability to carry out the malolactic conversion. This involves uptake of malate, decarboxylation to L-lactic acid and CO_2, and subsequent export of end products. The malolactic conversion generates energy for the cell in the form of a proton motive force (Salema et al. 1996). Recently the genes encoding the malate decarboxylase (*mleA*) and malate permease (*mleP*) have been cloned and characterized (Labarre et al. 1996a,b). Other chromosomal genes from *O. oeni* that have been characterized include: histidine decarboxylase (Coton et al. 1998), α-acetolactic acid decarboxylase (Garmyn et al. 1996), stress-related genes (Jobin et al. 1997, 1999), genes involved in arginine metabolism (Tonon et al. 2001) and rDNA genes (Martinez-Murcia & Collins 1990). Several genetic elements from *O. oeni* have been characterized including bacteriophages (Gindreau et al. 1997; Gindreau & Lonvaud-Funel 1999), and plasmids (Fremaux et al. 1993). In addition, the conjugative transposon Tn916 has been mobilized into the *O. oeni* genome (Zuniga et al. 1996b).

Many researchers have examined the diversity of *O. oeni* strains within and around wineries. Studies have employed various molecular typing methods (protein profiling, plasmid profiling, RAPD, PFGE, rDNA RFLP, etc.) to discern regional differences in strains. An outcome of this analysis is the general view that *O. oeni* is a genetically homogenous species (Zapparoli et al. 2000).

The strain sequenced in this project, *O. oeni* PSU-1 (ATCC BAA-331), was originally isolated at Penn State University from red wine undergoing a spontaneous malolactic fermentation (Beelman et al. 1977). PSU-1 was shown to reliably induce the malolactic fermentation in wines and is currently employed commercially (Beelman et al. 1980). While the *O. oeni* is considered homogenous species, the strain PSU-1 was previously shown to be representative of the larger of two divergent groupings (Tenreiro et al. 1994). An extensive physical map of PSU-1 generated by Ze-Ze et al. 1998, 2000) has tremendously aided sequence compilation and genome scaffolding operations.

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Oenococcus oeni IOEB 8413 (contributed by Jean Guzzo)

Oenococcus oeni is a LAB most often responsible for an important step in the winemaking process, the malolactic fermentation (MLF). The main transformation is decarboxylation of malic acid to lactic acid. MLF leads to a natural decrease of acidity, together with an enhancement of stability and quality of wine. Normally, it occurs spontaneously after alcoholic fer-

mentation, but in some wines the environment is so hostile to bacterial growth that MLF is delayed or even totally prevented. To solve this problem, winemakers use malolactic starters prepared by the industrial culture of selected strains. The laboratory of microbiology, UMR INRA, from Dijon University and the laboratory of biotechnology and applied microbiology, UA INRA, from Bordeaux University, in collaboration with GENOME Express, have determined the complete nucleotide sequence of the chromosome of *O.oeni* IOEB8413, a strain isolated from Bordeaux wine. The knowledge of the *O. oeni* genome represents a considerable value for developing new tools for the control of malolactic fermentation in wine.

Genome sequencing status and results

We first sequenced two random small insert and large insert libraries to favour the scaffold formation between sequence contigs during the assembly stage. Subsequently primers were designed for gap closure sequencing by gene walking and multiplex PCR approaches. The sequenced contigs were assembled using the Phred and Phrap software packages. The current assembly represents 1 753 879 nucleotides and a G+C content of 37.9%. At this stage, early annotation has been undertaken in order to improve the finishing step. The annotation was performed using the GenoAnnot^{tm} software (Geno^{*tm} platform), an integrated computer environment specialised in large-scale sequence annotation. A first pass in an automatic mode with well-dedicated annotation strategies revealed a total of 1784 putative coding sequences, 43 tRNA and 588 predicted rho-independant terminators. Functional annotation was then based on similarity searches with the Blast programs family. Preliminary Blast analysis of the current assembly revealed two ribosomal RNA operons, the presence of three different IS elements in several copies, and protein-coding genes belonging to prophages. Some functional categories of proteins are being studied in detail: stress proteins, transcriptional regulators, central carbon metabolism enzymes and transporters, proteins involved in nitrogen assimilation and the membrane anabolic pathway.

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***Brevibacterium linens* BL2 Bio**
(contributed by Bart Weimer)

The *Brevibacterium* genus is a heterogeneous mixture of coryneform organisms that have particular application to industrial production of vitamins, amino acids for fine chemical production, and are commonly used in cheese production (Amador et al. 1999; Rattray & Fox 1999). This genus contains nine species from diverse habitats, such as soil, poultry, fish, human skin, and food. While *Brevibacterium linens* is phenotypically similar to *Arthrobacter globiformis,* cellular pigmentation, cell wall composition, DNA/DNA hybridization and 5s RNA analysis show that *Brevibacterium* is distinctly different (Park et al. 1987). PFGE analysis indicates that diversity within the species is related to polymorphisms in the 16S rRNA genes with genome sizes that range from 3.2 and 3.9 Mbp (Lima & Correia 2000).

B. linens is a non-motile, non-spore forming, non-acid fast, Gram-positive coryneform that tolerates high salt concentrations (8–20%) and is capable of growing in a broad pH range (5.5–9.5), with an optimum of pH 7.0. They also survive carbohydrate starvation and drying for extended periods (Boyaval et al. 1985). *B. linens* is unusual as they produce base as they grow, raising the pH to ~9.5 within 24–36 h. Recent interest in *B. linens* has focused around their ability to produce a self-processing extracellular protease (Rattray et al. 1997; Weimer et al. 2000), their ability to produce high levels of volatile sulfur compounds (Ferchichi et al. 1985; Dias & Weimer 1998a,b), bacteriocin production (Valdes-Stauber & Scherer 1996), cell-membrane-associated carotenoid pigment production (Arrach et al. 2001), and aromatic amino acid metabolism (Leuschner & Hammes 1998; Ummadi & Weimer 2001). These organisms are also noted for their metabolize heterocyclic and polycyclic ring structures–a trait that is not associated with other bacteria but is common in fungi. Of particular note is the degradation of insecticides (including DTT and DDE). These organisms also metabolize amino acids, particularly aromatic amino acids, to produce plant growth hormones.

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wagen, P. Richardson, and K. Kadner are gratefully acknowledged.

Propionibacterium freudenreichii ATCC 6207 (contributed by Herman Pel)

Propionibacteria are high G+C Gram-positive bacteria belonging to the class of Actinobacteria that prefer anaerobic growth conditions and have a peculiar physiology. They produce propionate as their major fermentation product. Propionate fermentation yields more energy and, consequently, biomass than any other anaerobic microbial fermentation. Furthermore, propionibacteria utilize polyphosphate and pyrophosphate instead of ATP for several energy-dependent reactions and their metabolism is tuned to synthesize high levels of porphyrins, in particular B12.

DSM Food Specialties uses a classical dairy microorganism *Propionibacterium freudenreichii* to produce vitamin B12 for feed applications. Vitamin B12 is a highly complex but essential vitamin that can only be produced economically through the fermentation of microorganisms. Friesland Coberco Dairy Foods (FCDF) is a world player in cheese production. Propionibacteria have long been employed in the production process of Swiss-type cheeses for which they are indispensable for the typical eye-formation and production of characteristic taste components.

Genome sequencing status and results

The lack of fundamental physiological knowledge of propionibacteria hampers their efficient industrial exploitation. DSM Food Specialties and Friesland Coberco Dairy Foods have therefore in a combined effort sequenced and annotated the genome of the *P. freudenreichii* type strain ATCC 6207. A total of 2 641 522 base pairs were sequenced using a shotgun approach resulting in 12 contigs. The genome sequence revealed a GC content of 67% and was found to contain at least 2552 open reading frames. The annotated genome, including the resulting blueprint of the metabolic capabilities of the cell, forms a solid basis for the use of powerful tools such as transcriptomics and metabolomics to analyze the metabolic response of *P. freudenreichii* to genetic and environmental changes.

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Bifidobacterium breve NCIMB 8807 (contributed by: Douwe van Sinderen)

Species of the genus *Bifidobacterium* are Gram-positive bacteria, strictly anaerobic, fermentative rods, often Y-shaped or clubbed at the end and contain DNA with a relatively high G+C content. They represent a major element in the microflora of the human gastrointestinal tract. They are considered to have a significant role in maintaining the good health of the human host, while there is mounting evidence pointing to the activity of these bacteria in mediating other positive health attributes such as the alleviation of lactose tolerance, stimulation/potentiation of the immune system, and production of vitamins and antimicrobials. Bifidobacteria have been shown to be the predominant species in the gastrointestinal tract of infants, and represent the third most numerous species encountered in the colon of adult humans, considerably outnumbering other groups such as *Lactobacillus* species. The role of these bacteria in human health has stimulated significant interest in the health care and food industries, and has highlighted the position of these bacteria in the development of functional and pharma foods, which would contain these bacteria as active ingredients.

Despite growing consumer interest, key aspects regarding *Bifidobacterium* species, such as metabolic activities (particularly relating to catabolism of prebiotics) and physiology are still poorly understood. The determination of the complete genome of the *B. breve* strain NCIM 8807 (National Collection of Industrial and Marine bacteria, Aberdeen, Scotland), an isolate from nursling stools, was undertaken as a first step towards the molecular analysis of a probiotic *Bifidobacterium* species. This plasmid-free strain was selected because it is reasonably easy to transform ($\pm 10^4$ transformants per μg of plasmid DNA), shows good adherence properties to epithelial cells, and exhibits reproducible growth properties and moderate tolerance to oxidative, osmotic and acid stresses.

Genome sequencing status and results

Random sequences were obtained using a small-insert (2–4 kb) plasmid bank and a large insert (20–35 kb) cosmid library generating a total of roughly 10.5 million of raw sequence data (approximately 4.4-fold redundancy). These sequences have each been checked for quality and contaminating cloning vector sequences, and subsequently been used for an overall assembly by means of a combination of the Staden software package, and PHRED/PHRAP. This resulted in an initial assembly into 376 contigs, which were then examined individually for internal joints to reduce the total number of contigs to 250. Gap closure by primer walking is now ongoing and has reduced the number of contigs to 140, representing a total DNA sequence of 2.43 Mb. Preliminary analysis of the obtained sequence has revealed quite a high number of IS elements, and a large number of genes predicted to be involved in the degradation of poly- and oligosaccharides, e.g., arabinogalactan endo-1,4-β-galactosidase and α-mannosidase. Four rRNA-encoding operons have sofar been identified, while the calculated G+C content of the genome is roughly 53%.

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Bifidobacterium longum DJO10A (contributed by Daniel O'Sullivan)

Bifidobacteria are anaerobic, Gram-positive, irregular or branched rod-shaped bacteria that are commonly found in the intestines of humans and most animals and insects. They were first isolated and described over 100 years ago from human feces and were quickly associated with a healthy GI tract due to their numerical dominance in breast-fed infants compared to bottle-fed infants (Tissier 1899, 1906). While they were first grouped in the genus *Bacillus,* the genus *Bifidobacterium* was proposed in the 1920s (Orla-Jensen 1924). However, there was not a taxonomic consensus for this new genus and for much of the 20th century, they were classified in the genus *Lactobacillus*, due to their rod-like shapes and obligate fermentative characteristics. However, the accumulation of studies detailing DNA hybridizations, G+C content and unique metabolic capabilities resulted in the resurrection of the *Bifidobacterium* genus. They are characterized by a unique hexose metabolism that occurs via a phosphoketolase pathway often termed the 'bifid shunt'. Fructose-6-phosphate phosphoketolase (F6PPK) is a key enzyme of the 'bifid shunt' and its presence is the most common diagnostic test for this genus, as it is not present in other Gram-positive intestinal bacteria.

The genus is comprised of 31 characterized species, 11 of which have been detected in human feces (Tannock 1999). *B. longum* is often the dominant species detected in humans and is the only species to regularly harbor plasmids. It is a leading member of the probiotic bacteria due to numerous studies that have provided a growing body of evidence for its role in a myriad of potential health benefits. These include diarrhea prevention in antibiotic treated patients (Black et al. 1991), cholesterol reduction (Dambekodi & Gilliland 1998), alleviation of lactose intolerance symptoms (Jiang et al. 1996), immune stimulation (Takahashi et al. 1998), and cancer prevention (Reddy & Rivenson 1993). This myriad of potential health benefits attributed to the *B. longum* species clearly illustrates that this species possesses many very interesting characteristics. It is anticipated that identification and functional analysis of the genetic determinants involved in these activities will strengthen the evidence for the involvement of *B. longum* in these significant health benefits. Selection of suitable strains for probiotic purposes is very difficult as inherent characteristics of strains of *B. longum* that are necessary for its survival and competition in the human large intestine are currently very poorly understood (O'Sullivan 2001). The use of the sequenced genome in microarray analysis should reveal the pertinent traits that are important for these bacteria to attain dominance in these complex ecosystems.

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Bifidobacterium longum NCC2705 (contributed by Fabrizio Arigoni)

Bifidobacteria are obligate anaerobes in the *Actinomycetales* branch of the high G+C Gram-positive bacteria. There are at least 32 species of bifidobacteria, largely isolated from the GITs of many mammals as well as chickens and honeybees (Biavati & Mattarelli 2001). They are among the first colonizers of the sterile GITs of newborns and predominate until weaning when they are surpassed by other groups (Harmsen et al. 2000; Favier et al. 2002). Although the bifidobacteria represent <6% of the adult fecal flora, their presence has been associated with beneficial health effects (Biavati & Mattarelli 2001). For example, some studies showed that infant formula containing bifidobacteria reduces incidence of diarrhea (Saavedra et al. 1994). These types of studies have led to widespread use of bifidobacteria as components of health-promoting foods (probiotics). Although foods containing bifidobacteria are widely consumed probiotics, there is only fragmentary information about the physiology, ecology, and genetics of any one species. To rapidly increase knowledge and understanding of bifidobacterial biology and their complex interactions with their human hosts and GIT microflora, we determined and extensively analyzed the genome sequence of a *B. longum* strain isolated from infant feces.

Genome sequencing status and results

The genome sequence of *B. longum* NCC2705 was determined by shotgun-sequencing of randomly-cloned, 1.5- and 3-kb fragments. ORFs were identified with ORPHEUS (Pedant-Pro; Biomax Informatics). Total genome sequence analysis of *B. longum* revealed a large 2 256 646-bp chromosomal replicon and a small 3626-bp plasmid replicon that is 89% identical to pKJ36 (Genbank AF139129). The average G+C content was 60.1%, although six regions had much lower G+C content. The chromosome contains four nearly identical *rrn* operons and 57 tRNAs. We identified 1730 probable coding regions, representing 86% of the genome. The average gene length was 1100 bp, and a specific or general function was assigned to approx. 70% of them. We were able to attribute 78% of the predicted proteins to a COG family. The percentages of predicted proteins assigned to COGs in each of the 18 defined functional categories (Tatusov et al. 2001) were similar to those for other prokaryotes, except for an unusual number (>8.5%) assigned to the carbohydrate transport-metabolism category. This suggests that *B. longum* is well adapted to take advantage of the wide diversity and fluctuations in the nutrient composition in the colon. In addition, we observed that gene duplication and horizontal gene transfer have played an important part in this physiological adaptation. Using SignalP (Nielsen et al. 1997), we identified approximately 200 proteins with probable Sec-type signal peptides. Of these 59 were predicted as surface-associated lipoproteins (PROSITE accession PS00013) and 26 as solute-binding proteins of ABC transporter systems.

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