Genomics update

Probiotics genomics

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We were sitting in the Irish pub on quiz night, dumb-founded by trivia questions about ingredients of Mornay sauce and best-selling Boy Bands, when the following question came up: What are ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host?’ At long last, we had a correct answer: PROBIOTICS! The quizmaster was totally disinterested in our extensive elaboration on this topic, so we offer it to the readers of this Genomics Update.

The Russian Noble Prize winner Elie Metchinkoff first suggested that certain bacteria could modify the composition of the gut flora (Metchnikoff, 1907). He suggested that the longevity of Bulgarians and Russians of the Steppes was due to their consumption of ‘sour milk’ containing beneficial microbes, which in fact probably were lactic acid bacteria (LAB) such as Lactobacillus bulgarius. Henry Tissier of the Pasteur Institute isolated bacteria (now called Bifidobacterium bifidum) from the faeces of healthy breast-fed infants and recommended giving it to babies suffering from diarrhoea (Tissier, 1900). In 1935, Minoru Shirota in Japan developed the first commercial probiotic drink called Yakult, which contains Lactobacillus casei Shirota that can survive the passage through the stomach and colonize the intestine. The probiotic market is now estimated to be worth about $6 000 000 000 a year and is growing at around 10% annually (UBIC-Consulting, 2008). Since 1981 there have been over 2000 patent applications on probiotics filed (with ‘probiotic’ mentioned in patent somewhere) and some 524 granted (in the USA and Europe). The two most commonly used probiotics in commercial products are lactobacilli, members of the LAB, and bifidobacteria, but some yeasts and other bacteria have been claimed to have probiotic potential.

See Table 1 and Ouwehand and colleagues (2002) for an overview of commercially used strains and their claimed probiotic effects.

Probiotic mechanisms

What do probiotics actually do? What is the meaning of ‘confer a health benefit’? Probiotics are most commonly known as yoghurts or yoghurt-type drinks that people ingest. The consumption of probiotics by humans is intended to improve or maintain a healthy intestine. The claimed modes of action of probiotics include strengthening of the intestinal barrier function, modulation of immune responses, supply of vitamins, and antagonism of pathogens (or other commensals) either by producing antimicrobials or by binding to the mucosa (so called competitive exclusion). (For recent reviews see Marco et al., 2006; Ventura et al., 2007; Kalliomaki et al., 2008; Lebeer et al., 2008; Kleerebezem and Vaughan, 2009.) In general, desired attributes of probiotic strains include adequate survival of the stomach passage (i.e. low pH stability), and adaptation to the host gut environment, including stress response, active and synergistic metabolism, and adherence to the intestinal mucosa and mucus. Probiotics are presumed to have an ecological advantage owing to their capacity to metabolize complex sugars that are derived from the diet as well as from the host. Sugar metabolism enzymes include various glycosyl hydrolases (GHS) which can degrade plant-derived dietary fibres or complex host carbohydrate structures. Bacteriocin production may enhance their competitiveness in the gut. From an industrial perspective, crucial attributes of probiotic strains are good technological properties for production and storage and low health risk to consumers.

Probiotics need not be restricted to food applications or oral delivery. Some can be applied to the skin as lotions or cream (Krutmann, 2009) and have been used to treat vaginal infections (Reid, 2008). Probiotics are also added to animal and fish feed to enhance growth, replacing the banned additive antibiotics or growth hormones (Gatesoupe, 2008; Higuchi et al., 2008; Wynn, 2009). They appear to work by inhibiting/reducing the pathogenic bacterial load that some animals or fish carry. There is evidence for all of these probiotic modes,

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but the exact mechanisms of action are still not very clear. Genome-scale analyses of health-promoting bacteria, also coined ‘probiogenomics’ (Ventura et al., 2009), should provide clues for probiotic mechanisms and potential. Here, we provide an update of recent genomics studies in this field.

### Genome sequencing

Table 2 and Fig. 1 give an overview of genome sequencing of putative probiotic bacteria that are publicly available, and Table 3 gives examples of proprietary sequences of commercial probiotics. By far the most

| Species/strain | Brand name | Producer | Claimed effect in humans/animals |
|----------------|------------|----------|----------------------------------|
| *Bacillus coagulans* GBI-30, 6086 | GanedenBC<sup>©</sup> | Ganeden Biotech | Improves abdominal pain and bloating in IBS patients. Increases immune response to viral challenge |
| *Bifidobacterium animalis* ssp. *lactis* BB-12 | BB-12 | Chr. Hansen | Reduction in Strept. mutans in mouth; IBS amelioration in a multispecies trial |
| *Bifidobacterium animalis* ssp. *lactis* HN019 (DR10) | Howaru Bifido | Danisco | Reduced prevalence of atopy and eczema in the first 2 years of life |
| *Bifidobacterium breve* Yakult | Bifiene | Yakult | Irritable bowel syndrome treatment |
| *Bifidobacterium infantis* 35624 | Align | Procter & Gamble | Treatment of allergy, especially Japanese cedar polinosis |
| *Bifidobacterium longum* BB536 | BB536 | Morinaga | |
| *Escherichia coli* M-17 | ProBactrix | BioBalance | Irritable bowel syndrome treatment |
| *Escherichia coli* Nissle 1917 | Mutalflor | Ardeypharm | Enteroceitis, remission of ulcerative colitis |
| *Lactobacillus acidophilus* DDS-1 | LDS-1 | Nebraska Cultures | Alleviation of traveller’s diarrhoea; vitamin production |
| *Lactobacillus acidophilus* LA-5 | LA-5 | Chr. Hansen | Alleviation of acute diarrhoea |
| *Lactobacillus acidophilus* NCFM | Howaru acidophilus | Danisco | Improvement of intestinal health, treatment of vaginal/vaginal infections |
| *Lactobacillus acidophilus* GAL-2 | Ghenisson 22 | GHEN Co | Improves digestive health in poultry |
| *Lactobacillus acidophilus* brevis KB290 | LABRE | Kagome | Improvement of bowel movement, enhances NK activity and interferon-α activity |
| *Lactobacillus casei* DN114-001 | Actimel, DanActive | Danone | Acute diarrhoea treatment; infection prevention; gut development |
| *Lactobacillus casei* CRL431 | CRL431 | Chr. Hansen | Immune stimulation, Alleviation of acute diarrhoea |
| *Lactobacillus casei* F19 | Cultura | Aria Foods | Improvement in bowel function |
| *Lactococcus lactis* S11 | Lactobacillus fortis | Nestlé | Natural defense/immune system, gut health |
| *Lactobacillus johnsonii* NCC533 | LC1 range | VERUM HÅLSOFIL | Immunodenocation; pathogen inhibition |
| *Lactococcus lactis* lactis | NCF31 | Normejerier | Immune stimulation; improves digestive health; reduces antibiotic-associated diarrhoea |
| *Lactobacillus plantarum* 299v | GoodBelly, ProViva, TuZen | NextFoods, Probi, Ferring | Iron absorption |
| *Lactobacillus reuteri* ATCC 55730 | L. reuteri Protectis | BioGaia Biologics | Diarrhoea prevention and mitigation; eradication of *H. pylori* infection; amelioration of gingivitis. |
| *Lactobacillus rhamnosus* GG | Vifi and others | Valio | Immune stimulation; improves digestive health; prevents antibiotic-associated diarrhoea |
| *Lactobacillus rhamnosus* LB21 | Verum | Normejerier | Vaginal colonization and prevention of vaginitis |
| *Lactobacillus rhamnosus* GR-1 & *Lactobacillus reuteri* RC-14 | Bion, Flore, Intime, Jarrow, Fem-Dophilus | Chr. Hansen | Reduction of *C. difficile*-associated disease (CDAD) |
| *Lactobacillus acidophilus* NCFM & *Bifidobacterium bifidum* BB-12 | Florajen3 | American Lifeline, Inc | Improves digestive health; prevents *Antibiotic Associated Diarrhea* (AAD; inhibition of pathogens) |
| *Lactobacillus acidophilus* CL1285 & *Lactobacillus casei* | Bio-K<sup>®</sup> CL1285 | Bio-K<sup>®</sup> International | |
| *Lactobacillus acidophilus* MNFLM01 & *Enterococcus faecium* | LAB-MOS | Alltech | Lowers pathogen numbers in lamb intestine |
| *Lactobacillus helveticus* R0052 & *Lactobacillus rhamnosus* R0011 | A’Biotica and others | Institut Rosell | *Helicobacter pylori* inhibition |

For several other products with mixtures of probiotic bacteria see http://en.wikipedia.org/wiki/Probiotic#cite_note-48.
used probiotics and the ones which have their genomes sequenced are those associated with gut health. Details of genomes sequenced before 2009 have been summarized by Mayo and colleagues (2008) and Ventura and colleagues (2009). Infants are born with a sterile gastrointestinal (GI) tract but in breast-fed babies colonization by bifidobacteria is rapidly seen. It is thought that these bacteria confer a health benefit to the infant. The first colonizer is *Bifidobacterium longum* spp. *infantis*, which has the largest genome of any sequenced bifidobacteria at 2.83 Mb (Sela et al., 2009). The genome has complete pathways for the synthesis of some vitamins and the most commonly used probiotic in Europe and North America, has a genome size of only 1.9 Mb. These bifidobacteria lack the HMO cluster as presumably post-weaned animals no longer require this functionality. They do, however, contain the fos gene cluster necessary to produce the enzymes to break down and utilize health-promoting fructo-oligosaccharides, a well-known prebiotic and bifidogenic factor.

Several new genome sequences of probiotics have been released in 2009. *Bifidobacterium animalis* ssp. *lactis* AD011, isolated from a healthy breast-fed infant, has a high level of immunomodulatory activity (Kim et al., 2009). Its genome encodes multiple glycosylases than can degrade plant- or milk-derived oligosaccharides, and the fos gene cluster for processing of fructo-oligosaccharides (Kim et al., 2009). *Bifidobacterium infantis* strains AD011 and DSM10140, both from *Lactobacillus rhamnosus* (Zhang et al., 2009), are the probiotic strains which appears to have lost 100 kb of genomes relative to the non-commercial strain WCFS1, encoding sugar transport and metabolism, possibly due to prolonged growth of this probiotic strain in rich medium (Zhang et al., 2009). *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* ATCC53103, probiotic strains

### Table 2. Publicly available sequenced complete genomes of (putative) probiotic bacteria (adapted from the GOLD Database (http://www.genomesonline.org; October 2009).)

| Species                                      | Strain                  | Accession | Isolation source          | Reference               |
|----------------------------------------------|-------------------------|-----------|---------------------------|-------------------------|
| **ACTINOBACTERIA**                           |                         |           |                           |                         |
| *Bifidobacterium adolescentis*               | ATCC 15703              | NC_008618 | Human faeces              | Unpublished; Gifu University, Japan |
| *Bifidobacterium animalis ssp. lactis*       | AD011                   | NC_011883 | Human infant faeces       | Kim et al. (2009)        |
| *Bifidobacterium animalis ssp. lactis*       | ATCC SD5219             | NC_012814 | Human infant faeces       | Barrangou et al. (2009)  |
| *Bifidobacterium animalis ssp. lactis*       | DSM 10140               | NC_012815 | Swiss yoghurt             | Barrangou et al. (2009)  |
| *Bifidobacterium breve*                      | UCC203                  | NC_015079 | Human faeces              | Leahy et al. (2005)      |
| *Bifidobacterium longum*                     | NCC2705                 | NC_004307 | Human infant faeces       | Schell et al. (2002)     |
| *Bifidobacterium longum*                     | DJO10A                  | NC_010816 | Human adolescent faeces   | Lee et al. (2008)        |
| *Bifidobacterium longum ssp. infantis*       | ATCC 15697              | NC_011593 | Human infant faeces       | Sela et al. (2008)       |
| *Propionibacterium freudenreichii*           | ATCC9614                | NC_008556 | Emmental cheese           | Makarova et al. (2006)   |
| *Propionibacterium shermanii*                | ATCC 55721              | NC_010999 | Human ileum               | Unpublished; INRA, Rennes, France |
| *Lactobacillus acidophilus*                  | NCFM                    | NC_006814 | Human intestine            | Altermann et al. (2005)  |
| *Lactobacillus casei*                        | ATCC 334                | NC_008526 | Emmental cheese           | Makarova et al. (2006)   |
| *Lactobacillus casei*                        | BL23                    | NC_010999 | Emmental cheese           | Unpublished; INRA, Jouy-en-Josas, France |
| *Lactobacillus delbrueckii ssp. bulgaricus*  | ATCC BAA-365            | NC_008529 | French starter culture    | Makarova et al. (2006)   |
| *Lactobacillus delbrueckii ssp. bulgaricus*  | ATCC 11842              | NC_008954 | Bulgarian yoghurt         | van de Guchte et al. (2006) |
| *Lactobacillus fermentum*                    | IFO 3596                | NC_010610 | Japanese fermented plant   | Morita et al. (2008)     |
| *Lactobacillus gasseri*                      | ATCC 33323              | NC_008530 | Human intestine            | Morita et al. (2006)     |
| *Lactobacillus helveticus*                   | DPC 4571                | NC_010080 | Swiss cheese               | Callanan et al. (2008)   |
| *Lactobacillus johnsonii*                    | NCC533                  | NC_005362 | Human intestine            | Pridmore et al. (2004)   |
| *Lactobacillus johnsonii*                    | FI9785                  | FN298497  | Poultry                    | Wegmann et al. (2009)    |
| *Lactobacillus plantarum*                    | WCFS1                   | NC_004567 | Human saliva               | Kleerebezem et al. (2003) |
| *Lactobacillus plantarum*                    | JDM1                    | NC_012984 | Human adult intestine      | Zhang et al. (2009)      |
| *Lactobacillus rhamnosus*                    | GG                      | NC_013198 | Human faeces               | Kankainen et al. (2009)  |
| *Lactobacillus rhamnosus*                    | ATCC53103               | AP011548  | Human intestine            | Morita et al. (2009)     |
| *Lactobacillus salivarius*                   | UCC118                  | NC_007929 | Human small intestine      | Claesson et al. (2008)   |
| *Leuconostoc citreum*                        | KM20                    | NC_010471 | Korean fermented vegetables| Kim et al. (2005a)       |
used widely for nearly 20 years in a variety of functional foods, differ only by deletion of 5 kb in ATCC53103, and an inversion of 8.9 kb (Kankainen et al., 2009; Morita et al., 2009). Compared with other sequenced intestinal lactobacilli, both *Lb. rhamnosus* genomes have a relatively high number of proteins involved in carbohydrate and amino acid metabolism and transport, and defence mechanisms. In particular, 28 complete PTS-type transporters and 25 putative GHs are encoded, including the alpha-L-fucosidase (GH29; see Cazy database http://www.cazy.org) and alpha-mannosidase (GH38) families, which are not found in other sequenced lactobacilli. In addition, these *Lb. rhamnosus* genomes have three gene clusters encoding proteins with WxL domains which can attach to the peptidoglycan on cell surfaces (Siezen et al., 2006; Brinster et al., 2007); again, these gene clusters have not been found in other intestinal lactobacilli, but rather in plant-associated Gram-positive bacteria (Siezen et al., 2006). Most novel is the finding that *Lb. rhamnosus* GG has a gene cluster *spaCBA*, encoding three secreted pilin proteins with LPxTG-type peptidoglycan anchors, which is not present in the highly syntenous genome of *Lb. rhamnosus* LC705 (Kankainen et al., 2009). SpaA is the major scaffolding protein upon which the minor pil proteins SpaB and SpaC are attached. Using insertional inactivation of *spaC*, a truncated SpaC protein was produced which resulted in cells with a greatly reduced binding to human mucus (Kankainen et al., 2009). The authors suggest that the presence of SpaC-containing pili (Fig. 2) may possibly explain the longer persistence of this

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**Fig. 1.** Evolutionary relationships between the main gastrointestinal tract commensal lactobacilli, based on a neighbour-joining tree of 16S rRNA gene sequences. Bootstrap values above 600 are indicated. Bacterial taxa for which whole genome sequences are available are shaded in green. The outgroup is shaded in grey. Lactobacilli for which genome sequencing is ongoing/incomplete are shaded in red. Reproduced and adapted from Ventura and colleagues (2009), with permission from Macmillan Publishers Limited, 2009.
strain in the GI tract than strain LC705. Together with the high potential for sugar uptake and metabolism, this may explain probiotic effects of these Lb. rhamnosus strains.

**Experimental omics exploration of molecular mechanisms**

Ingested probiotic microbes themselves will react to the new environment of the intestine and change their gene expression accordingly. Transcriptional responses of bifidobacteria to human and formula milk have been described in *in vitro* and *in vivo* experiments, the latter from faecal samples of infants (Gonzalez *et al.*, 2008; Klaassens *et al.*, 2009). Carbohydrate metabolism genes are commonly upregulated, and include enzymes for degradation of complex plant carbohydrates, which are poorly digested by the host or other intestinal microbes (Klaassens *et al.*, 2009), and for metabolism of mucin and HMOs (Gonzalez *et al.*, 2008). In addition, putative genes were upregulated for cell-surface type 2 glycoprotein-

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**Table 3. Proprietary genome sequences of commercial (putative) probiotic bacteria.**

| Species | Strain | Genome size (Mb) | Company | Reference |
|---------|--------|-----------------|---------|-----------|
| ACTINOBACTERIA | | | | |
| *Bifidobacterium animalis* ssp. *lactis* | BB-12 | 2.0 | Chr. Hansen, Denmark | christel.garrigues@dk.chr-hansen.com |
| *Bifidobacterium breve* | Yakult | 2.35 | Yakult, Japan | yuko-shirasawa@yakult.co.jp |
| *Bifidobacterium breve* | M-16V | 2.3 | Morinaga Milk, Japan | k_nanba@morinagamilk.co.jp |
| *Bifidobacterium longum* *biot infantis* | M-63 | 2.8 | Morinaga Milk, Japan | k_nanba@morinagamilk.co.jp |
| *Bifidobacterium longum* | BB536 | 2.5 | Morinaga Milk, Japan | k_nanba@morinagamilk.co.jp |
| *Bifidobacterium lactis* | | 1.94 | Danone, France | tamara.smokvina@danone.com |
| FIRMICUTES | | | | |
| *Lactobacillus brevis* | KB290 | 2.49 | Kagome, Japan | masanori_fukao@kagome.co.jp |
| *Lactobacillus casei* | Shirota | 3.03 | Yakult, Japan | yuko-shirasawa@yakult.co.jp |
| *Lactobacillus casei* | | 3.14 | Danone, France | tamara.smokvina@danone.com |
| *Lactobacillus reuteri* | ATCC55730 | 2.0 | SLU, Sweden | klara.bath@nikrob.slu.se |

Source: Abstracts Symposium on Lactic Acid Bacteria 2005 and 2008, Egmond aan Zee, the Netherlands.

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**Fig. 2.** Identification of pili in *L. rhamnosus* GG by immunogold high-resolution electron micrography. Multiple pili are shown with gold-labelled SpaC proteins. Reproduced with permission from Kankainen and colleagues (2009).
binding fimbriae that are implicated in attachment and colonization in the intestine (Gonzalez et al., 2008).

In vitro transcriptional response of Lactobacillus reuteri ATCC55730, a strain marketed for probiotic usage, to bile stress has been described (Whitehead et al., 2008). Upregulation was seen for genes involved in multidrug transport, membrane/cell wall stress, oxidative stress, DNA damage and protein denaturation. Transcription and comparative genomics analysis of Lb. johnsonii NCC533, an isolate characterized by long gut persistence, identified three genetic loci that were specifically expressed in the jejunum of mice mono-colonized with this strain, encoding a PTS-type sugar transporter, glycosyltranseras and an IgA-type protease (Denou et al., 2008). Several years ago, a very elegant resolvase-based in vivo expression technology was developed to study specific in vivo gene expression in L. plantarum WCFS1, using the mouse GI tract as a model system (Bron et al., 2004). This has now been followed up by whole genome transcriptome profiling of strain WCFS1 during colonization of the caeca of germ-free mice fed either standard low-fat rodent diet rich in complex plant polysaccharides or a Western diet rich in simple sugars and fats (Marco et al., 2009). Numerous carbon metabolism pathways of L. plantarum were upregulated on both diets, including uptake and utilization of raffinose, cellulose, maltose, lactose/galactose, sucrose, melibiose, sugar alcohols and sialic acid. Sialic acid is a common component of (human) gut glycoproteins.

Host responses to potential probiotics have recently been described in intervention studies in healthy human volunteers. Duodenal mucosa was sampled after intraduodenal infusion (Troost et al., 2008) or oral ingestion (van Baarlen et al., 2009) of L. plantarum WCFS1. The continuous perfusion study showed that after prolonged exposure, mucosal cells switched to a more proliferative phase with upregulation of genes involved in lipid metabolism, cellular growth and development. Cell death and immune responses were triggered, but cell-death executing cells or inflammatory signals were not expressed. In the second study, consumption of live L. plantarum cells showed striking modulation of NF-κB-dependent pathways in mucosal cells, and identified cellular pathways that correlated with the establishment of immune tolerance in healthy adults (van Baarlen et al., 2009). Figure 3 summarizes some of the mechanistic events underlying probiotic effects that are beginning to be understood from these in vitro and in vivo studies.

Adaptation of probiotic strains

Bifidobacterium longum DJO10A, an intestinal isolate, was shown to lose functionality by gene loss after prolonged pure culture (Lee et al., 2008). It would appear that when growing in the competitive environment of the colon the cells retained some important functionalities predicted to be involved in diverse traits pertinent to the human intestinal environment, specifically oligosaccharide and polysaccharide utilization, arsenic resistance and bacteriocin production. The targeted loss of genomic regions was experimentally validated when growth of the intestinal B. longum in the laboratory for 1000 generations resulted in two large deletions, one in a bacteriocin-encoding region, analogous to a predicted deletion event in the commercial strain B. longum NCC2705 (O’Sullivan, 2008). This deletion strain showed a significantly reduced competitive ability against Clostridium difficile and Escherichia coli. The deleted region was between two IS30 elements which were experimentally demonstrated to be hyperactive within the genome. Hence, deletion of genomic regions, often facilitated by mobile elements, allows bifidobacteria to adapt to fermentation environments in a very rapid manner (two genome deletions per 1000 generations) and the concomitant loss of possible competitive abilities in the gut. This has implications for industry, because the claims for the use of a probiotic need to be fully substantiated.

Future

One of the most remarkable probiotic discoveries was made by the German Alfred Nissle in 1917 in World War I. Life in the trenches was dangerous and not just from the fighting. Disease was rife, especially enterocolitis (inflammation of the small and large intestine) caused by outbreaks of shigellosis. One soldier did not succumb to the disease and Nissle isolated from his faeces a bacterium with which he successfully treated other soldiers. Escherichia coli Nissle 1917 is still in use and is one of the few examples of a non-LAB probiotic (Mutaflor) (Table 1). At present, many of the commercial probiotic strains originate from the intestine of healthy infants and adults. Current research focuses on the determination of the characteristics these bacteria use to survive and compete successfully in the intestine, and with this knowledge more effective probiotic strains can be identified. To speed up this search, numerous gut metagenomic sequencing efforts are ongoing world-wide to identify potential new probiotic candidates (Gill et al., 2006; Kurokawa et al., 2007). See also the Human Gut Metagenome Initiative (http://www.international.inra.fr/press/mapping_the_human_intestinal_metagenome) and the Human Gut Microbiome Initiative (Gordon et al., 2006) (http://genomeold.wustl.edu/hgm/HGM_frontpage.cgi).

Perhaps the future will bring us health-promoting drinks containing mixtures of many probiotic strains, much like the cocktails used these days for vaccination against infectious diseases. And what will be the next hype? Memory-enhancing drinks would definitely be a commercial success on quiz night in the pub!
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