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Research Article

Omics: a novel tool for safety assessment of probiotic strains

J.B. Prajapati and Suja Senan

SMC College of Dairy Science, Anand Agricultural University, Anand 388110 Gujarat, India.

Email: jbprajapati@aau.in

Abstract

In the field of functional genomics, the derivatives of gene transcription and translation are referred to with the suffix 'ome'. Omics (comprehensive analysis of biochemical molecular species like genes, transcripts, proteins and metabolites) have been applied a great deal in probiotic studies. Together with the numerous records validating the health benefits of probiotics, sporadic cases of risk posed by such strains are also reported. Data mining of the complete genome sequences reveals the maximum potential risk of a probiotic organism and whole-genome sequencing of bacteria has recently emerged as a cost-effective and convenient approach for identifying virulent strains, testing resistance to antibiotics and monitoring the spread of bacterial pathogens. Hence a laboratory study was undertaken on the established probiotic strain *Lactobacillus helveticus* MTCC 5463 to re-assure its safety using metagenomic approaches. In total MTCC 5463 showed presence of 4 resistance genes associated with beta lactam, 6 for flouroquinolone and 22 resistance determinants. The genome does not pose the threat of transferability of such resistance as it lacks the presence of a CRISPRs, plasmids and complete prophages, implying the mode as intrinsic resistance. Ten structural and regulatory genes associated with prophages and integrases, six uncategorised mobile genetic elements, 154 homologs of transposases and 13 genes related to R/M systems were established. For heavy metal effluxing MTCC 5463 carried a total of 16 genes involved in copper homeostasis, cobalt, cadmium, nickel and iron uptake system with 8 adverse metabolism genes associated with formation of putatively genotoxic metabolites. Falling into the category of host-interaction factors rather than virulence factors, the presence of 44 fitness related genes have been observed in the genome viz., adhesion, biofilm, surface proteins interacting with immunity, lipopolysaccharides formation and 15 stress related proteins that provide gastrointestinal robustness of the strain. Three homologs of classical virulence gene, hemolysin have been found revealing a theoretical risk but it needs to be experimentally validated as the detection of homologues thereof does not show whether they are intact and functional. Further, on the lines of EFSA proposed Quality Presumption of Safety guidelines, the above results provide safety assurance for the use of *L. helveticus* MTCC 5463.

Keywords: genetics, bioinformatics, *Lactobacillus helveticus*, hemolysin, food safety, India.

Introduction

To understand the activities taking place in the cell, the scientific community followed the logical channel from DNA to mRNA to proteins. With the recent availability of many complete genome sequences in the public domain, the scientists are close to their mission. To comprehensively establish the link between the genotype and phenotype, the science of omics is developing with terminologies having ome as suffix. Omics means a comprehensive analysis of biochemical molecular species like the genes, transcripts, proteins and metabolites [1]. Multiple omic analysis is necessary as more than one biomolecule is interacting together in response to a total cellular system. As a consequence, a number of omics branched out and are defined in Table 1 [2].

Table 1. Partial list of various omic terminologies.

Omics	Definition
Bionomics	Study of the interaction of the organisms among themselves and the environment.
Fluxomics	Study of the genes involved in the regulation of flux in metabolic pathways occurring in a cell in a given time
Functomics	Study of the various functional entities of a cell like enzymes in action in a cell
Genomics	Study of the totality of all the functional genes of an organism
Interactomics	Study of the totality of all the molecular functions in the cell
ORFeomics	Study of the totality of all cDNA or clone based proteomics in cell
Phenomics	Study of the physical and biochemical traits responding to genetic and environmental factors
Proteomics	Study of the totality of all the proteins expressed in an organism
Ribonomics	Study of RNAs associated with RNA-binding proteins
Transcriptomics	Study of the entire RNA transcripts produced by the genome
Metabolomics	Study of the whole metabolome (intracellular concentration of metabolites) under a given set of conditions
Metabonomics	Study of the measurement of the dynamic response of a living system to pathophysiological stimuli or genetic modification.

Probiotics

Consumers now demand dietary supplements to maintain gastrointestinal health or recommendations in diet regulation to maintain a healthy balance of microorganisms in the gut. In this pursuit of a panacea between drug and diet lies the candidate "probiotics". The probiotic concept has been in vogue since 1900 and is now well defined by FAO/WHO [3] as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host'. In the last few decades probiotics were established for preventive-therapeutic use, particularly for some gastroenterological diseases, to restore and/or rebalance the functionality of microbiota, the intestinal mucosa and the immunological aspects. Well-studied probiotic applications are in antibiotic, *Clostridium difficile* and traveller's diarrhea, infection caused by *Helicobacter pylori,* necrotizing enterocolitis, bacterial vaginosis, irritable bowel syndrome, Crohn's disease, ulcerative colitis, atopic dermatitis and dental caries. Advances have been made from the traditional use to the modern applications in designing novel synbiotic formulations both in therapeutic field and in the functional food sector.

Application of omics in probiotics

Recent advances in omics tools and sequencing techniques have furthered our understanding of probiotic functionality and the specific interactions between probiotics and their human hosts. Although it is known that not all probiotics use the same mechanisms to confer benefits to hosts, some specific mechanisms of action have been revealed through omic investigations. The ability to examine fully sequenced and annotated genomes has greatly accelerated the application of genetic approaches to elucidate many important functional roles of probiotic microbes [4].

Martin *et al*. [5] used NMR-based metabolomic approach to study the metabolite profile of mice fed with probiotics. They analyzed several metabolite pools simultaneously from different biofluids and tissues throwing light on organ specificity and mode of action of probiotics. H NMR spectroscopy was applied for studying the changes of the metabolic profiles of human faecal slurries on consumption of synbiotic foods [6]. Omics were again adopted for the metabolic profiling of the fecal extracts of mice to assess the effects of probiotics on colonic inflammation [7]. In order to elucidate interactions in microorganisms at all levels of organisation, it is necessary to structure the data and generate appropriate linkages. Bioinformatics is a crucial discipline to that end [8]. Its primary purpose is to store and facilitate interpretation of omics data obtained at the cellular and sub-cellular level. These data include, but are not limited to, genome sequence information as well as molecular stress responses at the transcriptomic, proteomic and metabolomic level. Large scale analysis of gene expression and protein expression using transcriptomics and proteomics, respectively, in parallel to the use of metabolomics has been advocated as an approach to expand significantly the range of metabolites that can be measured to assess more stringently the potential for any unintended effects. Safety assessment can be greatly benefitted using mechanistic omics-based data in measuring phenotypic response and data integration for quantitative microbiological risk assessment in food [9].

Probability of risk with probiotics

Peer-reviewed papers on evaluation of commercial probiotic-containing products have identified the following problems; (1) The number of live microbes of each strain delivered through the end of shelf life is often not accurately reflected on the label, (2) microbes contained in the product are not always named in accordance with scientifically valid nomenclature, (3) claims of efficacy are not always adequately substantiated and (4) use of the term ‘probiotic’ on labels of products with no established record of a physiological (health) benefit in humans [10]. Although they have been used since ancient times in fermentation processes without any identified major concern, recent discovery of rare events of adverse effects caused by microorganisms in fermented food raise uncertainty about the level of risk, depending either on the food matrix or the susceptibility of the host [11], including rare cases of bacteraemia, endocarditis, pneumoniae, septic arthritis and meningitis, associated with certain *Lactobacillus* strains in immunocompromised patients.

The risk that probiotic bacteria carrying acquired antimicrobial resistance traits may be horizontally transferable to autochthonous gut microbiota [12] and issues such as virulence and transfer of antibiotic resistance [13] have eaten into the confidence of consumers and the sales of manufacturers. Lactobacillus infections occur at a very low rate in the generally healthy population, estimated 0.5/1 million per year [14, 15]. A specific risk assessment should be conducted on strains presenting these undesirable properties, even if they belong to a species with a long history of use [16].

Benefit risk analysis

The scientific approach for the estimation and management of food safety risks posed by undesirable bacteria in the "farm to fork" spectrum is the Microbiological Risk Assessment (MRA), adopted globally under the auspices of Codex Alimentarius. There is also a growing documentation of desirable bacteria used in the production of fermented food with beneficial effects on food nutrition and consumer health that fall under the umbrella term Microbial Food Cultures (MFCs) [17].

In this light, benefit-risk analysis (BRA) of food and food ingredients could be a valuable methodology to support evaluations and decision making regarding microbiological food safety management [18]. For setting priorities within the risk assessment of those microorganisms used in food/feed production European Food Safety Authority proposed a pre-market safety assessment of selected groups of microorganisms leading to a Qualified Presumption of Safety (QPS). In essence this proposed that a safety assessment of a defined taxonomic group (e.g. genus or group of related species) could be made based on four pillars (establishing identity, body of knowledge, possible pathogenicity and end use). If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded, the grouping could be granted QPS status. QPS group would be freed from the need for further safety assessment while microorganisms not considered suitable for QPS would remain subject to a full safety assessment (Figure 1).

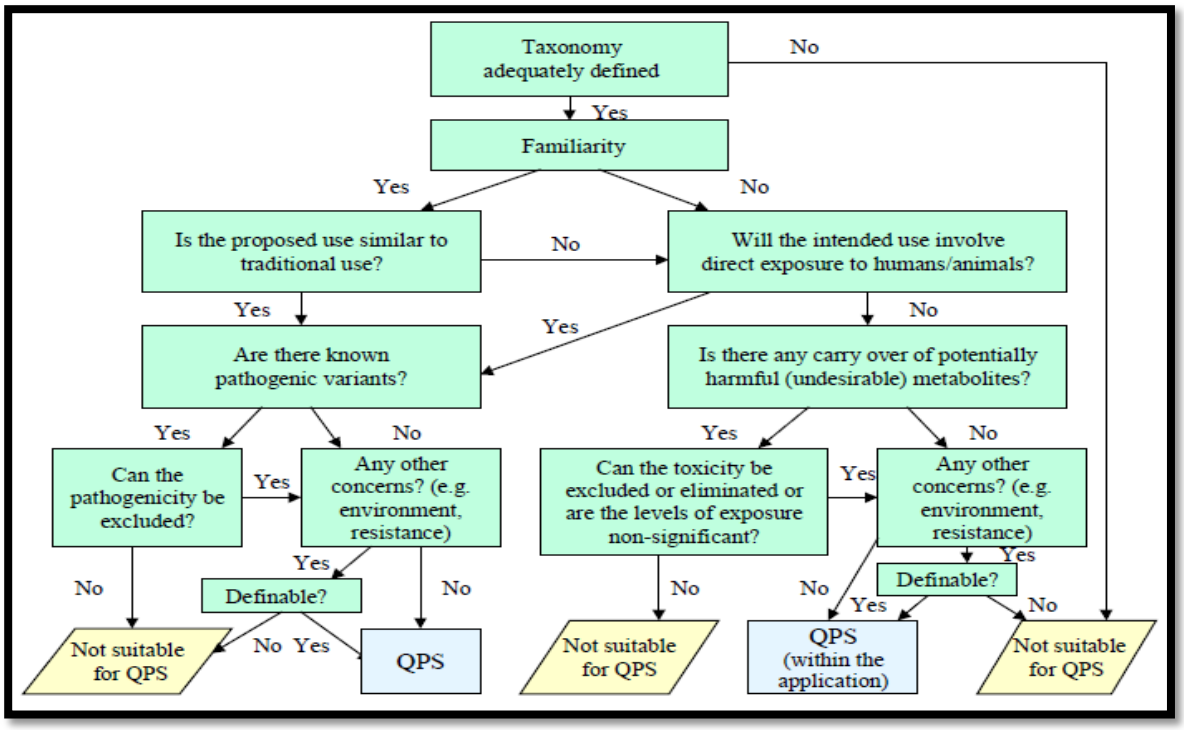

Figure 1. A pre-market safety assessment for food microbes proposed by European Food Safety Authority.

Data mining

Data collection, analysis and interpretation are crucial for the implementation of food safety controls. Data Mining (DM), also known as Knowledge Discovering in Databases (KDD) has emerged as an important analyses method for the identification of relationships between various elements of the collected information in order to discover useful knowledge and support strategic decision making systems. A systems-level understanding of the global physiology of the host-microbiota super-organism in health and disease will provide a platform for the identification and development of new therapeutic strategies. In addition, integrative bioinformatics analysis will further our understanding of the microbial biotransformation of exogenous compounds or xenobiotics, which could lead to safer and more efficacious drugs [19].

To analyze strain differences at the level of primary genome sequence, it is currently possible to use low cost shotgun sequencing techniques. Sequencing microbial genomes is now fully in reach of the scientific community as costs have fallen tremendously and faster than in some other technical fields over the past few years [20].

Whole genome vide data mining of *L. helveticus* MTCC 5463 strain for biosafety assessment

Data mining of the complete genome sequences reveals the maximum potential risk of a probiotic organism [21] and whole-genome sequencing of bacteria has recently emerged as a cost-effective and convenient approach for identifying virulent from non virulent strains, testing resistance to antibiotics, virulence and monitoring the spread of bacterial pathogens. Hence a study was undertaken in our lab on the established probiotic strain *L. helveticus* MTCC 5463 strain to reassure its safety using metagenomic approaches.

Whole genome sequence of *L. helveticus* MTCC 5463 was determined by 454 GS-FLX (Roche). Resultant sequencing reads were generated by GS Run Browser and assembled using GS De Novo Assembler V.2.6. Reads were simultaneously subjected to GS Reference Mapper software V.2.3 for aligning it to the reference sequence (*Lactobacillus helveticus* DPC4571) and generate a consensus sequence of sample DNA library.

The genome annotation of MTCC 5463 and its comparative analysis against the published complete genome of DPC 4571 was performed using RAST-Rapid Annotation using Subsystem Technology V. 4.0. Putative virulence factors and putative antibiotic resistance genes were identified by blast with virulence factors database (VFDB) and antibiotic resistance genes database (ARDB) respectively. Safety genes were searched based on published literature [17, 21, 22] and insilico genomes [23].

Taxonomy

Guidelines for probiotics have been set by ICMR and DBT and the primary requirement is the precise identification of the stain. Culture dependent tests have low discriminatory power leading to ambiguous identification of probiotic isolates. The probiotic isolate in this study was initially identified as *Lactobacillus acidophilus* V3 using biochemical tests. Applying 16S ribosomal RNA sequence analysis the strain was identified as *Lactobacillus helveticus* MTCC 5463 (Accession number GQ253959). The partial 16S rRNA 1053 bp sequence of the *L.acidophilus* V3 showed similarities of above 97%, which is the generally accepted limit, with DNA sequences from other species too. Hence the rational next step was to proceed for whole-genome sequencing resulting in

mega bases genomic data to identify unique genomic variations leading to precise identification. The strain in question was finally re-identified as *Lactobacillus helveticus* MTCC 5463.

Table 2. *In silico* analysis of safety genes in the genome of *L. helveticus* MTCC 5463.

CATEGORY	GENE NAME	GENE ID AAULH_.....
RESISTOME		
Beta Lactam	Class B metallo-beta-lactamase superfamily protein	05014
	Class A beta-lactamase	10045
	Ribonuclease Z	05881
	Class C beta-lactamase putative esterase	05881
	Penicillin binding proteins	05204, 00407, 06271, 06776, 11461
Flouroquinolones	Aminoglycoside N3'-acetyltransferase	03446
	major facilitator superfamily efflux pump	13121
Others	small multidrug efflux protein	00277
	multidrug resistance protein	00978
	multidrug ABC transporter	03236, 09950
	bacteriocin ABC transporter	06336
	ABC-type multidrug transport system	03286, 03291, 06511, 06341, 06536, 06531 00075, 06546 08778, 08773 09718, 09518, 10117, 10582, 10587, 11546: 13036, 13141 13416, 13821
	streptothricine-acetyl-transferase	06431
	ribosomal protection proteins elongation factor Tu	05109
	phospho-beta-glycosidase	00460
	elongation factor Ts	07866
	elongation factor P	08266, 10152
	rRNA methylase	02043, 09158
	aminoglycoside acetyltransferases	13106, 03193, 06801, 07006
	D-ala, D-ala ligase	10747
	CheY-like receiver domain and a winged-helix DNA-binding domain	04400, 08643, 09183, 10577
	LytR/AlgR family two-component response regulator	14541
	D-lactate dehydrogenase	00367
	L-lactate dehydrogenase	05541
	Signal transduction histidine kinase	04405 00589, 12656, 12661
	undecaprenyl pyrophosphate synthase	07851
	Dihydropteroate synthase	06851
	dihydrofolate reductase	05431

HEAVY METALS		
Copper	copper-transporting ATPase	11711
	copper chaperone	13076
Cobalt	ABC-type cobalt transport system, permease component CbiQ	05671, 00628, 00633, 11092, 01878, 03296
	cbiO	01873
	cobalt transport ATP-binding protein	11097
	ABC-type cobalt transport system, ATPase	11152, 11157
Cadmium	cadmium efflux ATPase	11521
	cadmium-translocating P-type ATPase	13126
Iron	Siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin synthetase	14001
Nickel	nickel transport system permease protein	01205, 01200
ADVERSE METABOLIC GENES		
Biogenic amines	carbamate kinase	07591, 07596
	ornithine decarboxylase	10242
	Spermidine/putrescine ABC transporter permease components	04155, 04160
	ermidine/putrescine import ATP-binding protein PotA	04150
	beta-glucuronidase	05339
	Lac L	08993
	Lac M	08998
	conjugated bile salt hydrolase	13111
MOBILOME		
prophage	putative prophage repressor	00520,
	prophage DNA packaging protein NU1	12306
	Phage associated protein	01093
	group II intron-encoded maturase	06181
	integrase-recombinase	06106, 06131
	Integrase	11866, 12081, 12406, 12411, 12796
	integrase catalytic region encoding genes	06921 00643
	uncategorised mobile genetic elements	05641, 08036, 08543, 09663, 10882, 03391
Insertion Sequences	IS 1201	05791, 00559, 07096, 07111, 10887, AULH_03683
	ISLhe15	05601
	IS*4* family	00287, 04689
	Type I restriction-modification system, S subunit	08548
R/M systems	type I site-specific deoxyribonuclease	08553
	Type I restriction enzyme EcoKI subunit R	08568
	Type II restriction enzyme, methylase subunit	11871, 13216
	Type III restriction-modification system	12356, 12361, 00172, 14601, 12906 13491, 00167, 14601

VIRULENCE RELATED GENES		
	hemolysin III	06041
	hemolysin a,	08201
	hemolysin-type calcium-binding repeat protein VCBS	14191
	Putative sortase gene	07706
	GroEL	02328
	Putative aggregation promoting protein	10742, 09428, 02328
	fibronectin-binding protein	07186, 01148
	S-layer protein	01325, 12271
	Mucus binding protein	05511 05516
	bactoprenol glucosyl transferase	04749, 00465, 00470, 00480, 00773, 11811, 11816
	putative hexosyltransferase YtcC	13501
	TagF/TagB/EpsJ/RodC	03006
	Membrane protein involved in the export of O-antigen and teichoic acid	11486, 11921
	6-diaminopimelate-D-alanyl-D-alanine ligase	01552
	D-alanyl-alanine synthetase A	00828
	D-alanyl-D-alanine Carboxypeptidase	09853
	UDP-N-acetyl-D-mannosamine transferase	02956
	polysaccharide transporters specific to O-antigen and teichoic acid	09930 11431, 14176
	teichoic acid glycosylation protein	03611
	D-alanine esterification of lipoteichoic acid and wall teichoic acid.	11466
	lipopolysaccharide biosynthesis glycosyltransferase	00470
	capsular polysaccharide synthesis PROTEIN	03011
	exopolysaccharide biosynthesis protein	10282, 10287, 10292, 10297
	Phosphoglucosyltrasnferase	10277
	Glycosyltransferases	02538, 02543, 00465, 00480, 04749, 13501, AULH_13506, 13536 11816, 11811
	Galactosyltransferase	02961
STRESS RELATED GENES		
	universal stress protein UspA	00893
	co-chaperonin GroES,	02323
	chaperonin GroEL,	02328
	ATP-dependent Clp protease	01657
	low molecular weight heat shock protein	01235
	heat shock protein HtpX	00653
	heat shock protein GrpE,	07746
	dnaK,	07741
	signal recognition particle-docking protein FtsY	07996
	chaperone protein DnaJ	07736
	HtrA-like serine protease.	00609
	F_0F_1 ATP synthase subunit C	04505
	F_0F_1 ATP synthase subunit B	04510
	F_0F_1 ATP synthase subunit delta	04515
	F_0F_1 ATP synthase subunit alpha	04520

Mobilome: Representing the genomic stability

Mobile and accessory genetic elements (mobilome), such as bacteriophages, plasmids, transposons, IS, etc. are important for adaptation to special growth conditions, e.g., colonization of new ecological niches, symbiosis, host-cell interaction, and most importantly the pathogenicity [24].

Plasmids

Among probiotics that are intentionally introduced in the food chain, a growing biosafety concern is the transfer of antibiotic resistance genes from bacteria that normally reside in the human intestinal tract to bacteria that cause human disease. Plasmid-encoded antibiotic resistance encompasses most, if not all classes of antibiotics. MTCC 5463 has a 1.91 Mb long single chromosome genome. Similar to AB-1 and *L. acidophilus* NCFM [25], MTCC 5463 show no plasmids. The absence of detectable plasmids further suggest that a transfer of or the acquisition of traits especially antibiotic resistance is highly improbable in this strain which makes it an excellent probiotic candidate.

Prophages and integrases

Prophages are a common feature among prokaryotic genomes and several phages have been reported in LAB [26]. MTCC 5463 lack the presence of a complete prophage but genome analysis reveal the presence of structural and regulatory genes associated with prophages. As shown in Table 2, they include a putative prophage repressor, DNA packaging protein NU1 and a phage associated protein. MTCC 5463 further showed the presence of group II intron-encoded maturase, integrase-recombinase, putative integrases with integrase catalytic region encoding genes. Presence of phage-related proteins suggests a history of inactivation or elimination of integrated prophages and development of highly stable genomic integration systems.

Transposases and Insertion Sequence (IS) elements

Annotation shows presence of six uncategorised mobile genetic elements and a large number of putative transposase genes (154 copies). The annotation documented six copies of IS 1201, one of IS he15 and two coding sequences for IS 4 family in MTCC 5463. IS elements are generally viewed as facilitators of increased genomic rearrangement, conferring an advantage in variant generation. Similar to the IS-loaded DPC 4571 genome [27], this probiotic strain demonstrates exceptional stability concluding that IS elements appear to be particularly unobtrusive in the genome. Further analysis revealed that no core gene was clustered with ISs restricting its transferability.

CRISPRs and R/M systems

Clustered regularly interspaced short palindromic repeats (CRISPR) represent a family of DNA repeats shown to provide acquired immunity against foreign genetic elements. The probiotic bacteria does not exhibit the presence of CRISPRs and CRISPR-associated proteins. A range of R/M systems like the Type I, Type II restriction enzyme and Type III restriction-modification system (Table 2) have been observed in the genome. Natural immune systems against phage infections (e.g., Restriction Modification Systems) would be crucial in order to devise suitable strategies that avoid or limit the negative effects of phage infections and to counteract phage predation upon administration of probiotics.

Resistome: Antibiotic and heavy metal resistance

A growing biosafety concern is of the transfer of antibiotic resistance genes from commensals to pathogens in the gut (reservoir hypothesis). Antibiotic resistance profile of MTCC 5463 anlaysed by disc diffusion assay showed resistance to the beta lactams cloxacillin ((10 µg/disc), ampicillin (30 µg/disc) and flouroquinolone norfloxacin (10 µg/disc) (data not shown). To link the above mentioned phenotype to its genotype, the genome was mined for the presence of resistance genes.

Beta lactam: The most common and important mechanism through which bacteria can become resistant against β-lactams is by expressing β-lactamases. It was observed that MTCC 5463

possessed three homologs -beta-lactamases of various classes as shown in Table 2. In addition to the production of β-lactamases, resistance can also be due to possession of altered penicillin binding proteins (PBPs). Although 5 homologs of PBPs are present in the genome, BLAST analysis show no mutation in the sequence implying that altered PBPs are not the resistance conferring agents.

Flouroquinolone: The molecular targets in the genome, DNA gyrase and topoisomerase IV shows no mutation and aminoglycoside N3'-acetyltransferase is present as a hypothetical protein, hence it is unclear whether they encode actual proteins. Thus the probable determinant of resistance against quinolones are the ABC transporter, ATPase and permease components and efflux pumps found in the genome which are in large numbers as shown in Table 2.

Other antibiotic resistance genes: Tetracycline resistance determinants like the NADP-requiring oxidoreductase and Xanthine-guanine phosphoribosyltransferase are absent in the genome while the other tetracycline resistance mechanisms like ribosomal protection proteins having homology to elongation factors were determined. Additionally the presence of ABC transporter, ATPase and permease components and efflux pumps mentioned above confer the theoretical tetracycline resistance. Other antibiotic genes mined in the MTCC 5463 genome include Macrolide-Lincosamide-Streptogramin B (MLS_B) resistance conferring rRNA methylases, aminoglycoside acetyltransferases and members of the GCN5 superfamily of proteins that include the histone acetyltransferases find their presence in the genome (Table 2).

Acquisition of and tolerance to heavy metals

MTCC 5463 genome carries genes involved in copper homeostasis like the copper-transporting ATPase and the copper chaperone. It has also developed systems for removal of excess cobalt from cells by efflux system genes to avoid toxicity. Cadmium efflux mechanisms are also exhibited by the probiotic strain as evidenced by the presence of cadmium efflux ATPase and cadmium-translocating P-type ATPase. Genes to acquire iron and nickel transport system permease protein predicted to transport dipeptides, oligopeptides and nickel are exhibited in the genome.

A carpet search of all the established resistance genes as given in (antibiotic resistance genes database (ARDB). ARDB gave us an idea of the maximum theoretical risks posed by the strain. Using analysis of genome sequences, we found no known transferable determinants for antibiotic resistance. This study demonstrated that the antibiotic resistance observed *in L. helveticus* MTCC 5463 was due not to dedicated mechanisms but to intrinsic resistance. According to the QPS criteria, these results provide safety assurance for the ongoing use of strain as a probiotic. This data needs to be validated with studies on the transferability of the resistance traits in conjugation experiments. Lactic acid bacteria can add further functionality by binding and effluxing heavy metals from food and water [28]. Apart from the toxic effects, the concern is the co occurrence of genes conferring metal-resistance with antibiotic resistance genes which could lead to the selection of antibiotic-resistant organisms in human gut. This concern is mitigated due to the absence of mobilome associated resistant determinants. The suggested roles of heavy-metal-transporting ATPases and copper homeostasis in the acid tolerance of *L. bulgaricus* ATCC 11842 [29] further supports the role of heavy metal tolerance mechanisms as a survival factor for the strain in gut.

Adverse metabolic genes

A criteria for evaluating the safety and functional characteristic of probiotic strain includes its aminogenic potential and bacterial synthesis of enzymes involved in the formation of putatively genotoxic metabolites, including beta-glucosidase (GS), arylsulphatase (AS), beta-glucuronidase (GN), nitroreductase (NR) and azoreductase (AR). The potential of MTCC 5463 to produce the biogenic amines agmatine and putrescine from arginine, and its amino acid derivative, ornithine, was investigated.

It was found that among the three arginine deiminase pathway enzymes, except carbamate kinase the others exist as pseudogenes (arginine deiminase, ornithine transcarbamylase) rendering the pathway non functional. The strain encodes ornithine decarboxylase which catalyzes the conversion of ornithine to putrescine, while Spermidine/putrescine ABC transporter permease components and

spermidine/putrescine import ATP-binding protein PotA may accommodate ornithine uptake. The presence of such genes do not directly pose a health risk until qualitatively and quantitatively analysed. Putrescine itself does not seem to possess a directly harmful biologic activity; instead, it enhances the toxic effects of histamine and tyramine, which in this case is not produced by the strain. Another explanation to their presence could be that this pathway generates proton-motive force and alkalinizes the cytoplasm [30] which can be exploited for acid stress resistance and/or the production of metabolic energy in the form of ATP. We could not detect any orthologs for AS, NR and AR. The bacterium MTCC 5463 exhibits the orthologs for beta-glucuronidase, Lac L and Lac M. The genome under study harbours conjugated bile salt hydrolase that proves to be more of a fitness factor than an adverse gene determinant.

Virulence genes

Factors required to survive the harsh conditions of the stomach and bile, and to interact with the host may be termed as mechanisms of invasion and pathogenesis in case of undesirable bacteria and a more symbiotic interaction in case of probiotics. Although the result of probiotic host interactions is generally beneficial for the host, many "classic" virulence factors, such as adhesions, were also encoded in the genomes of commensal bacteria and hence such virulence factors are referred to as "host-interaction factors" [31].

We used the set of virulence factors in the virulence factors database (VFDB), a well-established, published set based on experimentally demonstrated virulence factors extracted from the literature to search for the presence of such classical and defensive virulence factors in the MTCC 5463 genome. We took the liberty of classifying genes associated with carbohydrate synthesis and modification, protein synthesis and modification, transcriptional regulator genes, genes involved in DNA metabolism, cell surface carbohydrates as fitness factors rather than virulence factors. Virulence factors are proteins having more "offensive" functions, e.g. active invasion of the host, mucin degradation, cytotoxicity, hemolysis, adhesion to the epithelium, biofilm production and antimicrobial resistance.

It is generally assumed that a good adherence capacity is a desirable trait for probiotic lactobacilli, as it can promote the gut residence time, pathogen exclusion, and interaction with host cells for the protection of epithelial cells or immune modulation. Data mining the probiotic strain's genome, such adhesion factors include a putative sortase gene, GroEL, aggregation promoting protein, two copies of fibronectin-binding protein, S-layer protein and a mucus binding protein. Exopolysaccharide (EPS) are probiotic effector molecules interacting with pattern recognition receptors to circumvent enzymatic breakdown by competing microbiota or host enzymes, implying an important role in the competitive environment of the GIT. Biofilm forming ability that help them

colonize in the gut include the glycosyltransferases that are important for the biosynthesis of exopolysaccharide (EPS) including bactoprenol glucosyl transferase and putative hexosyltransferase YtcC. Probiotic strain displays the lipopolysaccharide biosynthesis glycosyltransferase, protein for capsular polysaccharide synthesis and four exopolysaccharide biosynthesis protein. The epsE gene encoding phosphoglucosyltrasnferase is present in a single copy. The central portion of the locus is occupied by encoding ten potential uncharacterized glycosyltransferases and one galactosyltransferase. In order to survive in the human body, probiotics must be able to adapt to new nutrient sources and environmental stresses, probiotics have also evolved a range of specific factors that facilitate direct interaction with host tissues which should not be considered to be a virulence factor.

Stress resistance is an adaptive factor for probiotcs to counteract changes in intestinal barrier function, gut motility and ability to cope with digestive (acid and bile) stresses. General stress adaptation genes, exhibited by the strain includes the universal stress protein UspA, chaperonins GroES and GroEL, endopeptidase *clp*P Clp protease, heat shock proteins HtpX, GrpE, dnaK, FtsY, DnaJ, HtrA-like serine protease and F0F1 ATP synthase subunits. As the roles of these genes cannot be comprehensively slotted as an offensive or defensive virulence trait, it can be suggested that inherent differences in gene expression shall explain the infectivity patterns of the strains that can help characterize a strain as virulent or non virulent.

Conclusion

As the host's health status, dosage and food matrix of the intentionally added probiotics differs, a general assumption of safety *or history of safe use* does not suffice for its safety assessment. The present strain had been subjected to Phase I and Phase II human clinical studies to validate its probiotic characteristics like hypocholesterolemic effect, intestinal well being and immunological parameters in healthy subjects, modulation of immune status in chick model and inhibition of growth of pathogenic microorganisms. We are the first in India to make patent deposit of indigenous probiotic culture and carry out its whole genome sequencing.

The availability of the complete genome sequence comparisons was put to use to resolve questions related to the gene set that makes the strain harmless or the probability to become an opportunistic pathogen. Furthermore, studies on gene regulation in different hosts and environments give further insight into their pathogenicity.

The study findings support our view that MTCC 5463 exhibits a specific pattern of fitness factors which might contribute to its colonization efficiency and survival in the host body. Such fitness factors can neither be categorically stated to be pathogenicity factors not its presence confirm its pathogenicity as it still remains to be proved if the genes are functional in the genome. Following the QPS approach, the strain is taxonomically adequately defined, its use is similar to the traditional use, no pathogenic variants are known and the antibiotic resistance, virulence and environment resistance factors are definable. The lack of plasmids and absence of core genes on mobile elements justify the non transferability of resistance genes further proving the resistance are intrinsic and not acquired in nature. These results provide safety assurance and QPS status to *L. helvetiucs* MTCC 5463 as a probiotic.

References

1. Lee, S.Y. (2009). Systems Biology and Biotechnology of *Escherichia coli*, Springer, Berlin. Korea Advanced Institute of Science and Technology, Daejeon, Korea (Ed.) XXII, 466 p.

2. Senan, S. and Prajapati, J.B. (2011). Metagenomics – the concept and prospects in probiotic foods. ***Nutritional Therapy and Metabolism,*** 29: 22-29.

3. FAO/WHO (2001). Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Co´rdoba, Argentina: Food and Agriculture Organization of the United Nations and World Health Organization, 5-7.

4. Baugher, J.L. and Klaenhammer, T.R. (2011). Application of omics tools to understanding probiotic functionality. ***Journal of Dairy Science***, 94:4753-65.

5. Martin, F.P., Wang, Y., Sprenger, N. *et al* (2008). Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. ***Molecular Systems Biology,*** 4: 157.

6. Ndagijimana, M., Laghi, L., Vitali, B., Placucc, G., Brigidi, P. and Guerzoni, E. (2009). Effect of a synbiotic food consumption on human gut metabolic profiles evaluated by H Nuclear Magnetic Resonance spectroscopy. ***International Journal of Food Microbiology,*** 134: 147-153.

7. Hong, Y.S., Ahn, Y.T., Park, J.C*., et al* (2010). H NMR-based metabonomic assessment of probiotic effects in a colitis mouse model. ***Archives of Pharmacal Research,*** 33:1091.

8. Schneider, M.V. and Orchard, S. (2011). Omics technologies, data and bioinformatics principles. ***Methods in Molecular Biology,*** 719, 3- 30.

9. Brul, S., Bassett, J.P., Cook, *et al* (2012). Omics' technologies in quantitative microbial risk assessment. ***Trends in Food Science and Technology,*** 27:12–24.

10. Huff, B.A. (2004). "Probiotics" might not be what they seem. ***Canadian Family Physician,*** 50:583-7.

11. Miceli, M.H., Diaz, J.A., Lee, S.A. (2011). Emerging opportunistic yeast infections. ***The Lancet: Infectious Diseases.*** 11, 142–151.

12. Danielsen, M. and Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. ***International Journal of Food Microbiology,*** 82: 1-11.

13. Agostoni, C., Axelsson, I., Braegger, C. *et al* (2004). Probiotic bacteria in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. ***Journal of Pediatric Gastroenterology and Nutrition,*** 38:365-374.

14. Borriello, S.P., Hammes, W.P., Holzapfel, W. *et al* (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. ***Clinical Infectious Diseases,*** 36:775–780.

15. Bernardeau, M., Guguen, M. and Vernoux, .J (2006). Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. ***FEMS Microbiology Reviews,*** 30:487–513.

16. Semedo, T., Santos, M.A., Lopes, M.F., *et al* (2003). Virulence factors in food, clinical and reference Enterococci: a common trait in the genus? ***Systematic and Applied Microbiology,*** 26:13–22.

17. Bourdichon, F., Serge Casaregola, Choreh Farrokh, *et al* (2012). Food fermentations: microorganisms with technological beneficial use. ***International Journal of Food Microbiology,*** 154:87-97.

18. Magnússon, S.H., Gunnlaugsdóttir, H., van Loveren, H. *et al* (2012). State of the art in benefit-risk analysis: Food microbiology. ***Food and Chemical Toxicology,*** 50: 33-39.

19. Collison, M., Hirt, R.P., Wipat, A., *et al* (2012) Data mining the human gut microbiota for therapeutic targets. ***Briefings in Bioinformatics,*** 24: 1-6

20. Delmont, T., Malandain, O., Prestat, E., Larose, C. *et al*. (2011). Metagenomic mining for microbiologists. ***ISME Journal of Multidisciplinary Microbial Ecology,*** 5: 1837-1854.

21. Zhang, Z.Y., Chang Liu and Yong-Zhang Zhu (2012). Safety assessment of *Lactobacillus plantarum* JDM1 based on the complete genome. ***International Journal of Food Microbiology,*** 153:166-70.

22. Wei, Y.X., Zhang, Z.Y., Liu, C., *et al* (2012). Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences. ***World Journal of Gastroenterology,*** 18: 479-488.

23. Bennedsen, M., Birgitte Stuer-Lauridsen, Morten Danielsen, and Eric Johansen (2011). Screening for Antimicrobial Resistance Genes and Virulence Factors via Genome Sequencing. ***Applied Environmental Microbiology,*** 77: 2785–2787.

24. Hacker, J. and Kaper, J.B. (1999). The concept of pathogenicity islands. *In* Pathogenicity islands and other mobile virulence elements, JB Kaper and J Hacker (ed), ASM Press, Washington DC, pp 1-11.

25. Altermann, E., Russell, W.M., Azcarate-Peril, M.A. *et al* (2005). Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. ***Proceedings of the National Academy of Sciences,*** 15, 102:3906-12.

26. Desiere, F., Lucchini, S., Canchaya, C. *et al* (2002) Comparative genomics of phages and prophages in lactic acid bacteria. ***Antonie Leeuwenhoek,*** 82**:**73–91.

27. Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O. *et al*. (2008) Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. ***Journal of Bacteriology,*** 190: 727–735.

28. Halttunen, T., Collado, M.C., El-Nezami, H. *et al.* (2008). Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution. ***Letters in Applied Microbiology,*** 46:160-5.

29. Penaud, S., Fernandez, A., Boudebbouze, S., *et al.* (2006). Induction of heavy-metal-transporting CPX-type ATPases during acid adaptation in *Lactobacillus bulgaricus*. ***Applied Environmental Microbiology,*** 72:7445-54.

30. Sobczak, I. and Lolkema, J.S. (2005) Structural and mechanistic diversity of secondary transporters. ***Current Opinion in Microbiology,*** 8(2):161-7.

31. Sui, S.J., Fedynak, A., Hsiao, W.W.L., *et al*. (2009) The Association of Virulence Factors with Genomic Islands. ***PLoS ONE,*** 4(12): e8094.