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Proteomic investigation of the aggregation phenomenon in *Lactobacillus crispatus*

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Abstract

Aggregation process affects the ability of *Lactobacillus crispatus*, a probiotic, to survive into the gastro-intestinal environment and to adhere to the intestinal mucosa. To elucidate mechanisms underlying this process, a comparative proteomic study was carried out on a wild type strain M247 and its spontaneous isogenic mutant Mu5, which had lost the aggregative phenotype. Results highlighted an overall lower amount of enzymes involved in carbohydrate transport and metabolism in strain M247 compared to strain Mu5, suggesting a reduction in the general growth rate, probably caused by nutrient limitation in cell aggregates, coherently with the phenotypic traits of the strains. Moreover, the up-regulation of a putative elongation factor Tu in the wild type M247 strain could suggest a role of this particular protein in the adhesion mechanism of *L. crispatus*.

Keywords: Probiotics; Adhesion; Aggregation; Elongation factor EF-Tu; *Lactobacillus*; Proteome

1. Introduction

Probiotics are living microorganisms that confer benefits to the host when supplied in adequate amounts [1]. The mechanisms by which probiotics positively affect human health include strengthening of intestinal barrier function, modulation of the immune response and antagonism towards pathogens, either by producing antimicrobial compounds or through competition for mucosal binding sites [2–6].

In order to express their activity, probiotics should be able to survive to the harsh physical–chemical environment of the gastro-intestinal tract. Moreover, adhesion to human intestinal mucosa has been considered essential for efficient gut colonization and it is used as an important criterion to select new probiotic strains [4].

Lactobacilli, indigenous inhabitants of the human gastro-intestinal tract, are thought to be among the dominant colonists of the small intestine. Some species, including *Lactobacillus acidophilus*, *Lactobacillus johnsonii* and *Lactobacillus plantarum* are considered to have probiotic properties [7]. In recent years, the genomes of these species, as well as those of other lactobacilli, have been completely sequenced [8–13]. Comparative genomic analyses and other high-throughput post-genomic approaches led to clarify many aspects of the physiology of such organisms as well as to identify potential bacterial components, mainly cell-surface associated proteins, involved in host interaction and cell-adherence [14–17]. Overall, studies aimed at deciphering probiotic functionality demonstrated that different species of lactobacilli exhibit different colonization strategies and adhesion processes, most of them still poorly known.

Accordingly, the adhesion does not depend on a unique and ubiquitous mechanism and both small molecules and proteinaceous compounds have been described to be involved in the process. Lipotheicoic acid, a surface associated molecule, participates in the adhesion process of some *Lactobacillus* strains to intestinal cells [18]. Surface proteins acting as

Abbreviations: CID, Collision induced dissociation; EF-Tu, elongation factor Tu; etag, error tolerant sequence tag; MW, molecular weight; *m/z*, mass to charge ratio; PMF, peptide mass fingerprint; 2-DE, two dimensional electrophoresis

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mediators of the attachment to epithelial cells, mucins or extracellular matrix have also been described. A genomic-based approach led to the identification of a fibronectin-binding protein, a mucin-binding protein and a surface layer protein as adhesion factors in *L. acidophilus* [19]. In silico genotype–phenotype matching was used to identify a mannose specific adhesin gene in *L. plantarum* [20]. However, to date only a few proteins involved in the adhesion process have been directly identified and characterized [21–24].

Probiotic features of *Lactobacillus crispatus* have been reported for strain JCM 8779, which inhibited the adhesion of pathogens to human enterocyte-like CaCo-2 cells [25], and for strain JCM 5810, which inhibited the adhesion of pathogens to synthetic basement membrane used as a model of a damaged intestinal tissue site [26]. Furthermore, the role of the S-layer proteins in the adhesion and competitive exclusion of pathogens of different strains of *L. crispatus* has also been documented [27,28].

Recently, studies demonstrated that *L. crispatus* M247 exhibited a specific beneficial effect on intestinal inflammatory disorders. Surface-mediated properties such as cell-aggregation might play a pivotal role in the protective effects observed following dietary supplementation with *L. crispatus* M247 in a mouse model of colitis [29]. A spontaneous isogenic mutant strain, named *L. crispatus* Mu5, which had lost the auto-aggregation phenotype, was selected from wild-type M247 cultures and showed to have similar strain-specific genetic pattern compared to M247 [30]. Therefore the strain M247 and its mutant Mu5 can represent an ideal model to study the molecular basis of the aggregation process in *L. crispatus*.

An aggregation-promoting-factor (APF), a 32-kDa secreted protein having amino acid composition and genetic structure quite similar to those of S-layer proteins, mediates aggregation in strain M247 [31]. Notwithstanding, APF is produced by both the wild-type and mutant strain, thus demonstrating that this protein is not the unique component involved in aggregation. The specific phenotype of strain Mu5 could be due to an anomaly in the expression and synthesis of other unknown molecules, also including APF receptors [31].

Up to now no proteomic studies on *L. crispatus* have been reported. Therefore, to reveal overall changes in metabolic pathways possibly caused by aggregation process, we carried out a comparative proteomic study of strains M247 and Mu5, integrating two dimensional electrophoretic separation, image analysis of the 2D-maps and identification by mass spectrometry of differentially expressed proteins. Results herein reported could represent basic findings to gain insight into mechanisms underlying the aggregation processes in *L. crispatus*.

2. Materials and methods

2.1. Reagents

Immobiline DryStrip 18 cm, pH 4–7 L, IPG buffer, DryStrip cover fluid, protein molecular weight markers for SDS-PAGE and agarose for IEF were purchased from GE Healthcare, Amersham Biosciences AB (Uppsala, Sweden). 30% Acrylamide/bis solution (37.5:1, 2.6%C), Coomassie Brilliant Blue G-250 from Bio-Rad (Hercules, CA, USA); urea, Chaps, dithiothreitol were from Sigma (St. Louise, MO, USA.). Tris–HCl, SDS, glycine, iodoacetamide, bromophenol blue, ammonium persulfate, TEMED were purchased from ICN Biomedicals Inc.(Aurora, OH, USA). Sequencing grade modified trypsin (porcine) was from Promega (Madison, WI, USA). Trizol reagent was purchased from Invitrogen (Milan, Italy). Glycerol, glacial acetic acid, acetonitrile, and all the other solvents were from Carlo Erba (Milan, Italy).

2.2. Strains, medium and growth conditions

Strains *L. crispatus* M247 and *L. crispatus* Mu5 (LMG P-23257 and LMG P-23258, respectively) were provided by AAT Srl (Piacenza, Italy). Strains were cultured in microaerophilic conditions in MRS broth (Difco, MI, USA) at 37 °C for 24 h. Plates were anaerobically incubated for 48 h at 37 °C.

2.3. Preparation of whole cell extract

The cell pellet from 10 ml broth culture harvested in late exponential phase (OD_{600}=0.8), corresponding to 3×10^8 CFU/ml, was centrifuged at 7000×*g* for 15 min and then suspended in 100 μl cold Tris–HCl 50 mM pH 7.4. Protein extraction was carried out by using the Trizol protocol provided by the manufacturer. Two ml Trizol reagent were then added, cell suspension was sonicated on ice (3 times for 2 min) and centrifuged at 10,000×*g* for 10 min at 4 °C to remove debris and the supernatant was incubated for 5 min at RT. 200 μl chloroform were added and shaken vigorously by hand; after centrifugation at 10,000×*g* for 15 min at 4 °C, the upper aqueous phase was discarded. 300 μl ethanol were added, mixed by inversion and sample was incubated for 3 min at RT. Then the two phases phenol–ethanol supernatant, separated by centrifugation at 4000×*g* for 5 min at 4 °C, was carefully removed. Finally 1.5 ml isopropanol were added, followed by mixing by inversion and incubation for 1 h at RT. Protein pellet was recovered by centrifugation at 10,000×*g* for 10 min at 4 °C and washed first with 0.3 M Guanidine/HCl in 95% ethanol, and then with ethanol. The protein pellet was vacuum dried and dissolved in buffer solution (8 M Urea, 4% Chaps, 40 mM Tris, 65 mM DTT). About 3 mg of proteins were obtained in each sample, as determined by the Bradford method [32].

2.4. 2-D Electrophoresis

Two dimensional electrophoresis (2-DE) was performed as described by O'Farrell [33]. The equipment was purchased from Amersham Biosciences. The first-dimensional isoelectric focusing (IEF) was performed using the Ettan IPGphor, whilst the second dimensional SDS-PAGE was carried out using the Ettan DALT twelve System. Gels were cast using the Gel Caster system. 2-DE was carried out as described previously [34]. 700 μg of each protein sample were diluted in rehydration solution (8 M Urea, 2% Chaps, 18 mM DTT, 0.5% v/v IPG Buffer pH 4–7, 0.002% bromophenol blue) to a final volume of 350 μl and applied by in-gel rehydration (according to the manufacturer's instructions) in IPGStrip 18 cm, pH 4–7L. The second dimensional SDS-PAGE was carried on a gel 21 cm×25 cm, 12% polyacrylamide. Protein spots were visualized by staining with Comassie Brilliant Blue G-250. Experiments were carried out on two independent cell growths and three gels were run for each sample.

2.5. Image analysis

The 2-DE protein patterns were recorded as digitalized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA, USA). Spot detection, quantization and analysis were performed using the PDQuest™ 2-D Analysis Software, Version 6.2 by Bio-Rad. Average spot intensity was determined and the Student's *t*-test function (confidence level 0.05) was used to select spots qualitatively and quantitatively reproducible in the replicate gels. Spots whose mean intensity showed a 2-fold or higher change were chosen for further mass spectrometric analyses.

2.6. Protein tryptic digestion and identification by Peptide Mass Fingerprint (PMF)

In-gel tryptic digestion and mass spectrometric analyses were carried out as described previously [34]. Protein identification was achieved by using the

MALDI mass spectral data for database search against the NCBInr database using the Mascot search algorithm (http://www.matrixscience.com/) [35]. Parameters for all searches were as follows: no taxonomic category was specified, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues and methionine oxidation as variable modification, one missed cleavage and 30 ppm as mass tolerance for the monoisotopic peptide masses.

2.7. Protein identification by Sequence-Tag

MS/MS experiments were carried out on a QStar-Pulsar mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a static nanospray interface (Proxeon Biosystems A/S, Odense, Denmark). 1 μl of the obtained peptide mixture was mixed with 1 μl of acetonitrile and loaded into a gold-coated borosilicate capillary (Proxeon Biosystems). Collision induced dissociation (CID) was used to fragment selected triply and doubly charged ions generated from peptides. CID experiments were performed using nitrogen as collision gas and collision energies variable in the range 20–30 V, just above the ion generation threshold. Mass spectra, acquired in range of mass to charge ratio (m/z) 100–2000 Da, were manually interpreted.

Protein identification was achieved by using the obtained sequence tags for database searches against the NCBInr database using the Mascot Sequence Query algorithm (http://www.matrixscience.com/). Searches were performed also considering the Error Tolerant Sequence Tag (etag) option. Parameters for all searches were as follows: no taxonomic category was specified, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues and methionine oxidation as variable modification, up to one missed cleavage, 2+ or 3+ peptide charge, according to the charge of the precursor ion, 100 ppm peptide mass tolerance and 0.2 Da fragment ion mass tolerance.

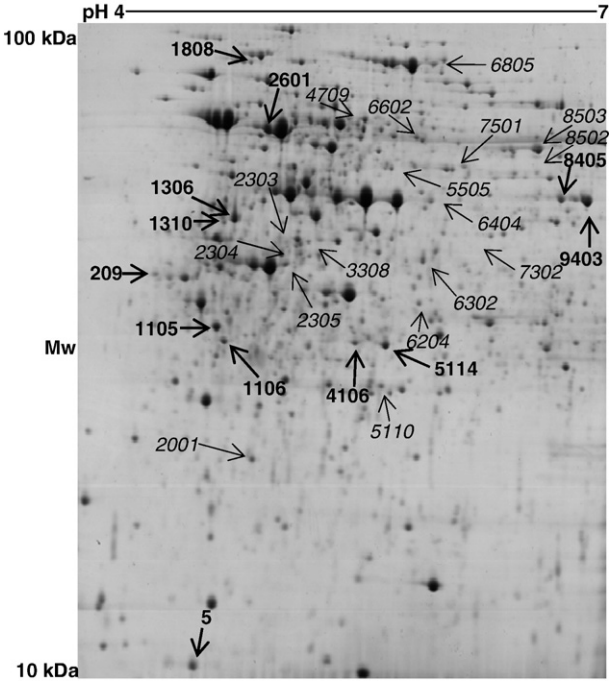

Fig. 2. 2-DE map of wild type strain M247, selected as reference map. Spots up-regulated in the mutant strain Mu5 are reported in bold, while the ones down-regulated are reported in italics.

3. Results

L. crispatus wild type strain M247 and its spontaneous isogenic mutant Mu5 cells were collected at OD_{600} 0,8 in order to assure a comparable concentration of living cells (about 3×10^8 CFU/ml) in the same growth phase (late exponential phase) for the following extraction steps. Since a delay of about 2 h in the growth curve of M247, compared to that of Mu5, was revealed, cultures were collected at different time during the incubation (Fig. 1).

The 2-DE gel image analysis of samples from the strains M247 and Mu5 showed a similar protein pattern, and, particularly, a similar number of protein spots. Therefore, the 2-DE gel of the M247 sample showing the best resolution across the whole gel was selected as a reference proteomic map (Fig. 2).

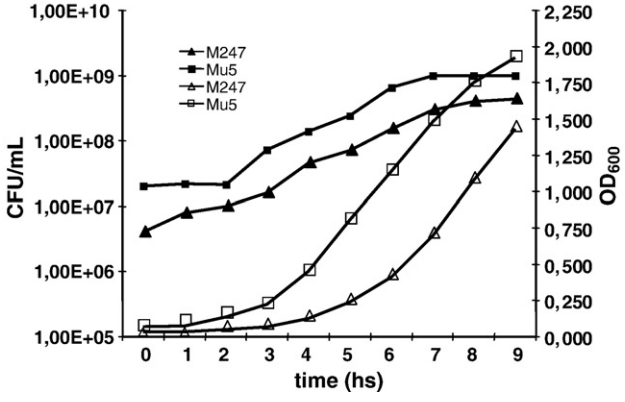

Fig. 1. Growth curves of *L. crispatus* M247 and Mu5 strains.

In total, 520 different spots were detected in the reference map and 29 of them showed a variation in their mean intensity of at least 2-fold between the two strains. In the 2-DE map of the mutant strain Mu5, twelve spots exhibited a lower intensity while for 17 of them a higher one was detected (Fig. 2). Therefore, proteins contained in the 29 spots of interest were submitted to mass spectrometric analyses; identification was successful for 23 spots, containing 19 different proteins (Table 1).

As the *L. crispatus* genome has not been completely sequenced yet, protein identification was achieved on the basis of sequences of homologous proteins from other *Lactobacillus* spp, annotated in databases, mainly from *L. acidophilus*, by combining the PMF strategy and the sequence tag-approach.

Among the identified proteins, only glyceraldehyde-3-phosphate dehydrogenase, contained in spot 6404, and putative elongation factor Tu (EF-Tu), contained in spot 2601 and 3105, were from *L. crispatus*, so that their identification was achieved with the highest Mascot scores and high protein coverages. However, the position of spot 2601 in the 2-DE map was in disagreement with the molecular weight of the identified protein. Actually, EF-Tu from *L. acidophilus*, with a molecular weight in agreement with the position in the 2-DE map (gi|58254469, MW 43.609 Da), had the second highest Mascot score (score 203), thus suggesting that also *L. crispatus* expresses a longer chain protein.

A similar discrepancy was also found for spot 6302; the identified protein was fructose-specific enzyme II of PTS system (gi|58338030, MW 70.867 Da), a multidomain protein involved in carbohydrate membrane transport. It is likely that spot 6302 contained a protein fragment, probably the two

Table 1
Identification of proteins differentially expressed in the two strains

Spot	Accession number	Protein Identification	Source	MW	pI	Strategy	Mascot Score	Matched peptdes [a]	Coverage (%)
1106	gi|58337659	hypothetical protein LBA1388	*L. acidophilus NCFM*	23.620	5.21	SeqTag	118	2	10
1306	gi|58337001	thioredoxin reductase	*L. acidophilus NCFM*	33.932	4.78	PMF	86	7/18	17
1310							83	7/24	27
1808	gi|58255530	pyruvate oxidase	*L. acidophilus NCFM*	66.629	4.85	PMF	81	11/36	14
2001	gi|42519506	two-component system histidine kinase	*L. johnsonii*	57.877	7.25	PMF	51	7/22	15
						SeqTag	136	2	5
2304	gi|58337219	citrate lyase beta chain	*L. acidophilus NCFM*	33.743	4.83	PMF	94	9/33	32
2305	gi|58337860	fructose-bisphosphate aldolase	*L. acidophilus NCFM*	33.560	4.94	PMF	95	10/38	25
2601	gi|22266014	putative elongation factor Tu	*L. crispatus*	28.208	4.53	PMF	218	18/35	68
3105						PMF	145	12/27	38
3308	gi|58335031	Fructose 1-P kinase	*L. acidophilus*	32.968	5.30	PMF	156	11/19	18
2303							147	11/20	23
4709	gi|58254582	30S ribosomal protein S1	*L. acidophilus NCFM*	44.414	5.15	PMF	146	11/18	24
4106	gi|58338147	glutamine amidotransferase	*L. acidophilus NCFM*	26.872	5.60	PMF	60	7/20	22
5114						PMF	67	7/28	33
						SeqTag	192	3	14
5505	gi|58336540	inosine-5'-monophosphate dehydrogenase	*L. acidophilus NCFM*	39.816	5.75	PMF	195	13/22	33
6204	gi|42519064	ABC transporter ATPase component	*L. johnsonii NCC 533*	29.433	5.11	PMF	109	10/28	38
6302	gi|58338030	PTS system, fructose-specific enzyme II [b]	*L. acidophilus NCFM*	70.867	5.80	SeqTag	89	1	2
6404	gi|57336902	glyceraldehyde 3-P dehydrogenase	*L. crispatus*	36.387	5.38	PMF	222	21/35	57
6602	gi|58336545	aminopeptidase E	*L. acidophilus NCFM*	50.040	5.06	PMF	60	7/22	17
						SeqTag	173	2	4
6805	gi|58337255	pyruvate kinase	*L. acidophilus NCFM*	63.136	5.23	PMF	99	11/32	25
7501	gi|58336474	transposase	*L. acidophilus NCFM*	48.703	10.18	PMF	48	6/21	16
						SeqTag	99	2	4
8502	gi|58337651	carbamoyl-phosphate synthase, small subunit	*L.acidophilus NCFM*	40.508	6.02	PMF	82	6/9	11
8503	gi|58253917	serine hydroxymethyltransferase	*L. acidophilus NCFM*	45.276	5.56	PMF	90	9/28	26

[a] For PMF strategy matched peptides means the number of matched peptides/the number of *m/z* values searched, for Sequence-tag strategy it means the number of identified peptides by sequence tags.

cytoplasmic hydrophilic domains of the protein, IIA and IIB, having a molecular weight of about 30 kDa in agreement with spot position in the 2-DE map.

In some cases, the obtained Mascot scores and protein coverages could not be considered significant for a reliable identification (as for spots 2001, 4106, 5114, 6602 and 7501) [35]. Furthermore, proteins contained in spots 1106 and 6302 could not be identified by the PMF strategy, notwithstanding the high quality of the obtained mass spectra. For all these spots, nano-ESI-MS/MS experiments were carried out to achieve identification by means of the sequence tag-approach and to partially check the sequence of selected peptides [36] (Table A in Supplementary Material).

Concerning spot 1106, MS/MS spectra were obtained from the fragmentation of the doubly charged ions at *m/z* 564.33 and 650.80 (corresponding in the MALDI spectrum to the singly charged ions at *m/z* 1127.69 and 1300.69, respectively). Their manually interpretation led to define the following sequence tags 603.34, AG[IL]VA, 1014.60 and 816.41, SG[IL], 1073.55 (data not shown) used for database search by means of Mascot Sequence Query algorithm. Protein was identified as hypothetical protein LBA1388 (gi|58337659) having a general function of NADH-flavin reductase. Furthermore, the complete amino acid sequences determined from the two MS/MS spectra exactly matched the ones of the identified peptides. It is worth noting that, in the MALDI spectrum, only the signals at *m/z* 851.47, 1127.69 and 1300.69 could be related to tryptic peptides of the identified protein, while all the other signals are probably originated from peptides containing amino acid substitutions.

Similarly, identification of the protein contained in spot 5114 was confirmed by using both the sequence-tag and the etag search mode by Mascot. In particular, the tag (185.13, EENG [IL], 727.34) deduced from the MS/MS spectrum of the doubly charged ion at *m/z* 770.90 and the etags (519.26, EEGY, 997.43) and (202.10, DVAF, 634.28) from the doubly charged ions at *m/z* 572.75 and 708.34, respectively, led to confirm the identification of the protein as glutamine amidotransferase from *L. acidophilus* (gi|58338147). Manual interpretation of the MS/MS data led to determine the amino acid variations: for instance, the signal at *m/z* 708.34 was originated from the peptide 224–235 where P_{225} is S in the protein from *L. crispatus* (Fig. 3).

Finally, some proteins could be identified in more than one spot appearing near in the 2-DE map (1306 and 1310, 5114 and 4106, 3308 and 2303), probably due to the presence of different isoforms.

In summary, the differentially expressed proteins identified in this study are listed in Table 1, together with source, Mascot score and protein coverage. However, it should be mentioned that proteins contained in spots 5, 209, 1105, 5110, 8405 and 9403 could not be identified, although good quality MALDI and MS/MS spectra were recorded, probably due to a lower sequence identity of proteins contained in these spots with the homologous proteins from other lactobacilli.

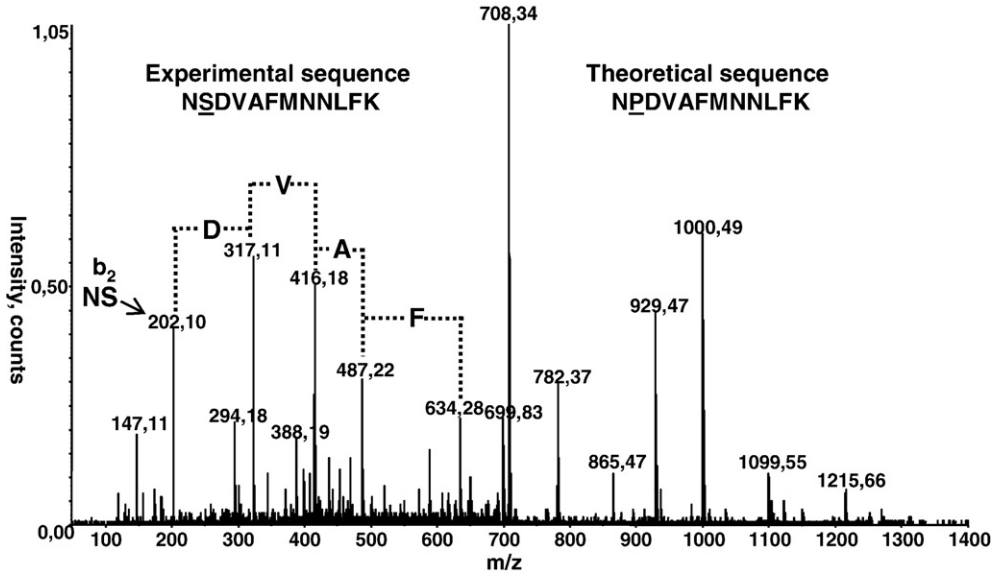

Fig. 3. Identification of protein contained in spot 5114 by error tolerant sequence tag strategy. MS/MS spectrum of the doubly charged ion at *m/z* 708.34. Sequence tag used for etag database search is reported. Peptide from *L. crispatus* contained an amino acid variation (P_{225}-S) with respect to the matched peptide from *L. acidophilus*.

All the identified proteins were grouped based on their cellular functions and metabolic pathways, using the KEGG (Kyoto Encyclopedia of Genes and Genomes) resource (http://www.genome.jp/kegg/) [37]. This classification helped to highlight a stringent correlation between proteins with similar general function and regulation in the expression pattern of the two strains (Table 2).

Table 2
Classification of the identified proteins according to their general function and pathways

General function	Pathway	Accession number	Protein	Orf	Gene	Fold-change in Mu5 [a,b]
Carbohydrate metabolism	Glycolysis — fructose and mannose metabolism	gi|58338030	PTS system, fructose-specific enzyme II [c]	LBA1777	fruA	↑↑↑↑
		gi|58335031	Fructose 1-P kinase	LBA1778	fruK	↑↑↑↑
		gi|58337860	Fructose-bisphosphate aldolase	LBA1599	fbaA	↑↑
		gi|57336902	Glyceraldehyde 3-P dehydrogenase		gap	↑↑↑
		gi|58337255	Pyruvate kinase	LBA0957	kpyK	↑↑
	Citrate cycle	gi|58337219	Citrate lyase beta chain [c]	LBA0916	citE	↑↑
	Pyruvate metabolism	gi|58255530	Pyruvate oxidase	LBA1974	poxB	↓↓
Nucleotide metabolism	Pyrimidine metabolism	gi|58337001	Thioredoxin reductase	LBA0679	trxB	↓↓↓
		gi|58337651	Carbamoyl-phosphate synthase, small subunit [c]	LBA1380	carA	↑↑↑↑
	Purine metabolism	gi|58336540	Inosine-5′-monophosphate dehydrogenase	LBA0199		↑↑
Amino acid metabolism	Gly, Ser and Thr metabolism	gi|58253917	Serine hydroxymethyltransferase	LBA0261	glyA	↑↑
	Glutamate metabolism	gi|58337651	Carbamoyl-phosphate synthase, small subunit [c]	LBA1380	carA	↑↑↑↑
Environmental information processing	Signal transduction	gi|58337219	Citrate lyase beta chain [c]	LBA0916	citE	↑↑
		gi|42519506	Two-component system histidine kinase	LJ1630		↑↑
	Membrane transport	gi|42519064	ABC transporter ATPase component	LJ1138		↑↑
		gi|58338030	PTS system, fructose-specific enzyme II [c]	LBA1777	fruA	↑↑↑↑
Genetic information processing	Translation	gi|22266014	Putative elongation factor Tu		tuf	↓↓
		gi|58254582	30S ribosomal protein S1	LBA0968		↑↑
Metabolism of cofactors and vitamins	Vitamin B6 metabolism	gi|58338147	Glutamine amidotransferase	LBA1903		↓↓↓
Miscellaneous		gi|58337659	Hypothetical protein LBA1388	LBA1388		↓↓↓
		gi|58336545	Aminopeptidase E	LBA0204	pepE	↑↑↑
		gi|58336474	Transposase	LBA0127		↑↑

Regulation of their expression pattern in Mu5.

[a] Spot intensity in the 2 DE maps of M247 strain is considered as reference, arrows indicate range of fold increase (↑) or decrease(↓) in Mu5 strain; ↑↑ from 2,0 to 3; ↑↑↑ from 3 to 4; ↑↑↑↑>4.

[b] Regulation of non-identified spots is as follows: ↑↑ spot 5110; ↓↓↓ spots 5, 1105, 8405, 9403; ↓↓ spot 209.

[c] Proteins belonging to more than one pathway.

4. Discussion

The present paper reports the first proteomic study on the probiotic *L. crispatus,* aimed to shed light on the aggregation process by comparing the proteome expressed by the wild type strain M247 and that of its isogenic mutant Mu5 which had lost the auto-aggregation phenotype.

It is worth nothing that the genome of this bacterial species has not been sequenced and the identification of proteins differentially expressed in the two strains was hampered by the limited number of protein sequences from *L. crispatus* present in the data-bases (as few as 60 proteins entries are present in NCBInr database). Therefore, a combination of PMF strategy with the sequence tag one, also taking into account the error tolerant option, was required to achieve protein identification.

The proteomic study highlighted, as major difference, a higher amount of enzymes involved in carbohydrate transport and metabolism, mainly in the glycolysis, expressed by the mutant strain Mu5. In fact, PTS system fructose specific enzyme II and fructose 1-P kinase, together with fructose bisphosphate aldolase, glyceraldehyde 3-P dehydrogenase, pyruvate kinase and citrate lyase, exhibited at least a two fold increase in the expression level of the mutant strain. Moreover, other up-regulated proteins in strain Mu5 included enzymes associated with amino acid and nucleotide biosynthesis such as: (i) inosine-5′-monophosphate dehydrogenase which catalyses the rate-limiting reaction of the *de novo* GTP biosynthesis; (ii) carbamoyl-phosphate synthase involved in both arginine and pyrimidine biosynthesis; (iii) serine hydroxymethyltransferase, an enzyme belonging to the pyridoxal-P group whose principal physiological role is the interconversion of serine and glycine and the generation of one-carbon groups in the form of 5,10-methyltetrahydrofolate, a crucial intermediate for nucleotide biosynthesis.

This finding could be correlated to the phenotypic traits of the strains. In fact, the presence of a lower amount of metabolic enzymes in strain M247 compared to the mutant strain Mu5 could be triggered by formation of cell aggregates undergoing a reduction in the general growth rate, probably due to nutrient limitation. A similar down-regulation has already been reported in *Streptococcus mutans* when comparing the proteome of cells grown in a mature biofilm state, where cell aggregation occurs, with that of a planktotic state [38,39].

Furthermore, in strain M247 a higher amount of pyruvate oxidase, responsible for the conversion of pyruvate into acetyl phosphate, could also be correlated to a reduced bioavailability of nutrients, in particular glucose. In fact, this induces ATP production through an alternative pathway with production of acetate, as reported in *L. plantarum* [40,41].

Although thioredoxin reductase is classified as belonging to the pyrimidine metabolism, this enzyme, in conjunction with thioredoxin, constitutes a ubiquitous oxidoreductase system with antioxidant and redox regulatory roles that controls the thiol-disulfide bond balance involved in structure and activity of proteins. A higher amount of this enzyme in M247 could be explained considering that Trx–TrxR system is over expressed in bacteria exposed to a variety of environmental stress factors such as nutrient limitation and accumulation of toxic bacterial metabolic products, particularly reactive oxygen species [42,43].

It is worth noting that elongation factor Tu (EF-Tu) resulted to be over expressed in the wild type strain. In the cytoplasm, this protein plays a central role in the elongation phase of protein biosynthesis by positioning the aminoacyl tRNA onto the active site of ribosomes and belongs to the large family of G proteins. In strain M247, the over expression of EF-Tu is quite in disagreement with the trend of a general lower expression of proteins involved either in metabolism or in biosynthesis, due to its aggregating phenotype, as clearly evidenced in the present study. Therefore, this finding opens perspective to speculation. EF-Tu, originally thought to be restricted to the cytoplasm, has been identified as a cell wall associated component in several bacterial species. In 1976, EF-Tu, was shown to be associated with the membrane of *Escherichia coli* where it seemed to be produced in a fourfold excess over the amount required for protein synthesis [44]. Moreover, EF-Tu is also present on the bacterial cell surface of *Listeria monocytogenes*, where it is involved in binding to human plasminogen, thus mediating the invasion process [45]. This protein is also located on cell wall of pathogens such as *Mycobacterium leprae* [46] and *Neisseria gonorrhoeae* [47]; in particular, in *Mycoplasma pneumoniae* it also directly binds fibronectin, an extra cellular matrix component [48]. More interestingly, in *L. johnsonii* EF-Tu has been recently identified as a novel surface protein possessing the characteristic of an adhesion factor directly involved in bacterial binding in the intestinal tract; in fact this protein was shown to be able to bind to CaCo-2 and HT29 cells and mucin as well as to numerous components of gram-positive bacteria [22]. The presence of EF-Tu on the cell wall of different *Lactobacillus* spp., including *L. acidophilus, L. reuteri, L. gasseri* and *L. salivarius* is also been reported [22,49].

In this contest, results obtained from the present proteomic study could be coherently interpreted by postulating that in *L. crispatus* M247, as well as for other *Lactobacillus* spp, EF-Tu could be localized on the cell wall, playing a key role in the aggregation phenomenon.

It should be mentioned that, together with EF-Tu, other cytosolic proteins, in particular DnaK, GroEL GAPDH, enolase and pyruvate kinase have been found associated with cell wall of both pathogenic [45–48] and lactic acid bacteria [21,22,49], including *L. crispatus* [50,51], and found to be involved in adhesion processes. Therefore, probiotic bacteria could mimic the adhesion mechanisms of pathogens, thus mediating competitive exclusion of enteropatogenic bacteria [52,53] and triggering immunomodulation activities [54].

In conclusion, we might suggest that EF-Tu could be considered as one of the “moonlighting proteins”, i.e. proteins having multiple, seemingly unrelated functions in different cell locations, cell types, etc. [55,56]. Future work will investigate the possible involvement of this protein in the adhesion mechanism of *L. crispatus* and in its probiotic characteristics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2007.11.007.

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