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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