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Effect of inactivation of *ccpA* and aerobic growth in *Lactobacillus plantarum*: A proteomic perspective

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ABSTRACT

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium widely used in the production of most fermented food due to its ability to thrive in several environmental niches, including the human gut. In order to cope with different growth conditions, it has developed complex molecular response mechanisms, characterized by the induction of a large set of proteins mainly regulated by HrcA and CtsR repressors as well as by global regulators such as carbon catabolite control protein A (CcpA).

In this study, the role of CcpA in the regulation of growth under anaerobiosis and aerobiosis, and the adaptation to aeration in *L. plantarum* WCFS1 were comprehensively investigated by differential proteomics. The inactivation of *ccpA*, in both growth conditions, significantly changed the expression level of 76 proteins, mainly associated with carbohydrate and energy metabolism, membrane transport, nucleotide metabolism, protein biosynthesis and folding. The role of CcpA as pleiotropic regulator was particularly evident at the shift from homolactic fermentation to mixed fermentation. Proteomic results also indicated that the mutant strain was more responsive to aerobic growth condition.

1. Introduction

Lactobacillus plantarum belongs to lactic acid bacteria (LAB), a heterogeneous group of bacteria widely used as starter microorganisms in food-technology, playing an essential role in the improvement of organoleptic and hygienic quality of fermented food. Moreover, *L. plantarum* is part of the human gastrointestinal microflora and some strains have probiotic features positively affecting human health [1]. It is a facultative heterofermentative LAB found in various environmental niches from several kinds of vegetable, meat and dairy products to fermentation plants where it faces several stress conditions (oxidation, heating and cooling, acid/basic, high osmolarity/dehydration, starvation) [2]. Therefore *L. plantarum* has developed complex molecular response mechanisms in order to cope with environmental stresses and to survive.

In general, adaptation to different growth conditions and stress response mechanisms leads to a coordinated expression of a set of genes which alters different cellular processes (cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.), mainly regulated by a network of transcriptional regulators. It is well known that in LAB the stress response is characterized by the induction of proteins such as Heat Shock Proteins (HSP), Cold Shock Proteins (CSP)

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etc., and it is essentially mediated by HrcA and CtsR repressors [3,4]. However, some common regulatory mechanisms for stress response are triggered by different environmental conditions, thus suggesting a key role for global regulators. Among those, the catabolite control protein A (CcpA) is of utmost importance since it is involved in the control of carbon catabolite repression and of various metabolic pathways in several industrially relevant LAB species including *Lactococcus lactis* [5], *Lactobacillus casei* [6,7], *Lactobacillus pentosus* [8] and *Streptococcus thermophilus* [9].

CcpA is a DNA-binding protein belonging to the LacI/GalR-family of transcriptional regulators. CcpA, linked to the seryl-phosphorylated form of phosphocarrier protein HPr (HPr-Ser-P), binds to a catabolite response element (*cre*) that is commonly located in the proximity of promoters, thereby either repressing or enhancing the transcription of downstream operons [10]. CcpA has a key role in the regulation of the aerobic metabolism in *L. lactis* [11,12] and in *L. plantarum* [13,14]. Moreover, CcpA has been recently shown to be also involved in stress response mechanisms in *L. plantarum* by exerting positive control on the *dnaK* and *groESL* operons [15,16].

As the genomes of three strains of *L. plantarum* have been completely sequenced and published [17–19], proteomics has been widely applied to gain insight into the mechanisms used by *L. plantarum* to respond to heat [20], alkaline [21] and bile exposure [22], and into the adhesion process underlying probiotic properties [23].

In the present work the role of the master regulator CcpA in controlling growth under anaerobiosis and aerobiosis, and adaptation to aeration in *L. plantarum*, were comprehensively investigated by a differential proteomic study performed on *L. plantarum* WCFS1 and on its isogenic mutant strain WCFS1-2 that carried a null mutation in the *ccpA* gene. A microbiological and metabolic study performed by Zotta et al. on the same experimental system, aptly integrated the proteomic perspective [24].

Results clearly highlighted that the inactivation of *ccpA*, in both growth conditions, significantly changed the protein expression pattern. In particular, it negatively affected the expression of enzymes belonging to glycolysis and to pyrimidine metabolism, while it enhanced the expression of proteins involved in the pyruvate and carbohydrate metabolism. For the first time, the role of CcpA in modulating aerobic metabolism, and in affecting the shift from homolactic fermentation to mixed fermentation, has been thoroughly studied at proteomic level.

2. Material and methods

2.1. Bacterial growth

L. plantarum WCFS1 and its isogenic mutant strain WCFS1-2 (Δ*ccpA ery*) were grown as reported by Zotta et al. [24]. Fermentations were carried out in a complex medium (WMB) containing 20 g/L glucose, at controlled pH (6.5) and temperature (30 °C), under air or nitrogen sparging. Bacterial samples were collected during the exponential growth phase, when the absorbance at 650 nm was approximately 1 and immediately frozen at −80 °C [24]. Two replicate fermentations were carried out for each strain and growth condition. Since growth parameters were not significantly different between the two series of fermentations, biomass samples were pooled prior to protein extraction.

2.2. Proteome extraction

Proteome extraction was carried out according to a protocol already reported and slightly modified [25]. In brief, cell pellets were submitted to enzymatic lysis with mutanolysin (134 units for 1.8×10^{10} cells, Sigma, St. Louise, MO, USA) in 20 mM Tris–HCl, 10 mM $MgCl_2$, 0.5 M sucrose pH 7.4 containing a cocktail of protease inhibitors (1/100 v/v, Sigma, St. Louise, MO, USA) for 2 h at 37 °C. DNase I and Rnase A (enzyme to substrate ratio of 1:100 v/v) were added and the reaction was carried out for 30 min at 37 °C. Finally, protein precipitation was performed using 10 volumes of acidic acetone (final concentration of HCl 1 mM) at −20 °C for 18 h.

Protein pellets were dissolved in buffer solution (8 M Urea, 4% Chaps, 40 mM Tris–HCl, 65 mM DTT) and protein concentration was determined using the Bradford assay [26].

2.3. 2-DE and image analysis

2-DE was performed as described by O'Farrell [27] and according to experimental conditions already reported [25]. IEF was performed using the Ettan IPGphor, while the SDS-PAGE was carried out using the Ettan DALT twelve System (GE Healthcare, Amersham Biosciences AB, Uppsala, Sweden). 700 μg protein samples were applied by in-gel rehydratation (according to the manufacturer's instructions) in 18-cm IPG strip, pH 4–7. Preliminary experiments performed using pH 3–10 linear range strips showed that almost all the spots migrated in the acidic region of the gel (data not shown), so that, IEF was performed using the pH 4–7 strips in order to increase resolution.

Protein spots were visualized by staining with Coomassie Brilliant Blue G-250. Samples were run in triplicate for a total of 12 gel maps/experiment.

2-DE protein patterns were recorded as digitalized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, BioRad, Hercules, CA, USA). Spot detection, quantization, and analysis were performed using the PDQuest™ 2-D Analysis Software, Version 6.2, by BioRad. The "total quantity in valid spots" normalization method was used in order to correct differences in sample loading or staining intensity among gels. Replicate gels of each sample were grouped together using the "replicate groups" function that allows to determine the average quantity of protein spots, and to perform statistical analysis on spot intensities using the Student's *t*-test function (confidence level 0.05). Variations in the mean spot intensity were considered significant according to the following criteria: a twofold or higher change when comparing the 2-DE maps of the same strain grown under the two different growth conditions; a threefold or higher change when comparing the 2-DE maps from the two different strains grown under the same condition. 400 spots consistently present in the replicate gels were included in the image analysis.