International Journal of Food Microbiology 155 (2012) 51–59

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inactivation of *ccpA* and aeration affect growth, metabolite production and stress tolerance in *Lactobacillus plantarum* WCFS1

Teresa Zotta [a], Annamaria Ricciardi [a], Angela Guidone [a], Margherita Sacco [b], Lidia Muscariello [b], Maria Fiorella Mazzeo [c], Giuseppina Cacace [c], Eugenio Parente [a,*]

[a] Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali, Università degli Studi della Basilicata, Potenza, Italy
[b] Dipartimento di Scienze Ambientali, Seconda Università di Napoli, Caserta, Italy
[c] Centro di Spettrometria di Massa Proteomica e Biomolecolare, Istituto di Scienze dell'Alimentazione, CNR, Avellino, Italy

ARTICLE INFO

Article history:
Received 22 August 2011
Received in revised form 30 November 2011
Accepted 18 January 2012
Available online 28 January 2012

Keywords:
Lactobacillus plantarum
CcpA
Carbon catabolite repression
Stress response
Aerobic growth

ABSTRACT

The growth of *Lactobacillus plantarum* WCFS1 and of its *ΔccpA ery* mutant, WCFS1-2, was compared in batch fermentations in a complex medium at controlled pH (6.5) and temperature (30 °C) with or without aeration, in order to evaluate the effect of *ccpA* inactivation and aeration on growth, metabolism and stress resistance. Inactivation of *ccpA* and, to a lesser extent, aeration, significantly affected growth, expression of proteins related to pyruvate metabolism and stress, and tolerance to heat, oxidative and cold/starvation stresses. The specific growth rate of the mutant was ca. 60% of that of the wild type strain. Inactivation of *ccpA* and aerobic growth significantly affected yield and production of lactic and acetic acid. Stationary phase cells were more stress tolerant than exponential phase cells with little or no effect of inactivation of *ccpA* or aeration. On the other hand, for exponential phase cells inactivation of *ccpA* impaired both heat stress and cold/starvation stress, but increased oxidative stress tolerance. For both strains, aerobically grown cells were more tolerant of stresses. Evidence for entry in a viable but non-culturable status upon prolonged exposure to cold and starvation was found. Preliminary results of a differential proteomic study further confirmed the role of *ccpA* in the regulation of carbohydrate catabolism and class I stress response genes and allow to gain further insight on the role of this pleiotropic regulator in metabolism and stress.
This is the first study in which the impact of aerobic growth on stress tolerance of *L. plantarum* is evaluated. Although aerobic cultivation in batch fermentations does not improve growth it does improve stress tolerance, and may have significant technological relevance for the preservation of starter and probiotic cultures.

1. Introduction

The protein CcpA (Catabolite control protein A) is a pleiotropic regulator of carbon and nitrogen metabolism in Gram positive bacteria (Fujita, 2009; Titgemeyer and Hillen, 2002). When bound to its co-repressor, Hpr-Ser-P, CcpA binds to catabolite responsive elements (*cre*, with consensus sequence WWTGNAARCGNWWWCAWW; Fujita, 2009) in the promoter or after the transcriptional start site, and represses the transcription. However, the Hpr-Ser-P/CcpA complex is also involved in activation of gene expression (Fujita, 2009; Zomer et al., 2007). CcpA directly or indirectly controls the transcription of a large number of genes (>300 in *Bacillus subtilis*, ≥237 in *Lactococcus lactis*) involved in both energy and nitrogen metabolism.

The role of CcpA in the control of metabolism has been studied in several lactic acid bacteria (LAB), including *Lact. lactis* (Gaudu et al., 2003; Lopez de Felipe and Gaudu, 2009; Zomer et al., 2007), *Lactobacillus casei* (Gosalbes et al., 1999), *L. pentosus* (Mahr et al., 2000) and *Lactobacillus plantarum* (Castaldo et al., 2006; Lorquet et al., 2004; Muscariello et al., 2001). The latter is a species widely distributed in fermented foods, beverages and silage (Siezen et al., 2010), and includes strains which are being used as probiotics (Shah, 2007). The genome of 4 strains of *L. plantarum* subsp. *plantarum* has been completely or partially sequenced (WCFS1, GenBank AL935263, Kleerebezem et al., 2003; JDM1, GenBank CP001617; ST-III, GenBank CP002222; ATCC14917, GenBank ACGZ00000000). Moreover, significant genotypic and phenotypic diversity has been found in this species, probably as a result of adaptation to different ecological niches (Parente et al., 2010; Siezen et al., 2010). As other LAB (Gaudu et al., 2002; Lechardeur et al., 2011) *L. plantarum* is capable of aerobic metabolism (Murphy and Condon, 1984a, 1984b; Quatravaux et al., 2006) and in the presence of heme and menaquinone it can synthesize a limited respiratory chain (Brooijmans et al., 2009). Pyruvate oxidase (POX) is a key enzyme in oxygen metabolism of *L. plantarum* (Goffin et al., 2006; Lorquet et al., 2004; Murphy and Condon, 1984a; Quatravaux et al., 2006): it is involved in the oxidation of pyruvate (produced from lactate by lactate dehydrogenase, LDH),

* Corresponding author at: Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano, 10, 85100 Potenza, Italy. Tel.: +39 0971 205561; fax: +39 0971 205503.
E-mail address: eugenio.parente@unibas.it (E. Parente).

0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2012.01.017

with production of hydrogen peroxide and acetyl phosphate, followed ATP generation by acetate kinase (ACK). Five different pyruvate oxidases are encoded in the genome of *L. plantarum* (Goffin et al., 2006) although only two (PoxB and PoxF) are important in acetate production. Both *poxB* and *poxF* are induced by oxygen or H_2O_2, and, since their expression is controlled by carbon catabolite repression (Goffin et al., 2006; Lorquet et al., 2004), they are expressed at the end of the exponential phase and during the stationary phase in cultures grown on glucose and throughout growth phases in cultures grown on non-PTS sugars, as maltose (Goffin et al., 2006; Lorquet et al., 2004; Murphy and Condon, 1984a, 1984b; Quatravaux et al., 2006). Accumulation of H_2O_2 (0.1–9.9 mmol/L; Murphy and Condon, 1984a, 1984b; Quatravaux et al., 2006) in aerobiosis may reduce vitality and activity of stationary phase cells. In fact, inactivation of *poxB* and *poxF* increases the survival of stationary phase cells, although the reasons are not completely clear (Goffin et al., 2006). H_2O_2 can be detoxified by NADH peroxidase (NPR), and some strains of *L. plantarum* can produce a Mn-dependent pseudocatalase (Kono and Fridovich, 1983; Yousten et al., 1975), while a heme-dependent catalase is produced in the presence of exogenous heme (Abriouel et al., 2004). Accumulation of manganese (Archibald and Fridovich, 1981) further contributes to the protection against reactive oxygen species.

In addition to being involved in the regulation of aerobic metabolism in both *L. plantarum* (Lorquet et al., 2004) and *Lact. lactis* (Gaudu et al., 2003; Lopez de Felipe and Gaudu, 2009), CcpA also regulates the expression of the class I heat shock response operons in *L. plantarum*: in a mutant carrying a null mutation in the *ccpA* gene both the expression of the *groESL* and *hrcA* operons were reduced and so was the heat stress tolerance (Castaldo et al., 2006).

Aerobic growth under respiration condition has been found to improve the tolerance of several technologically relevant stresses (heat and oxidative stress, starvation) in LAB (Lechardeur et al. 2011) and given the role of CcpA in both the control of stress response and aerobic metabolism, in this work we have investigated the impact of the inactivation of *ccpA* and aerobic growth on the growth and stress tolerance of *L. plantarum* WCFS1.

2. Materials and methods

2.1. Microbial strains and inactivation of the ccpA gene

L. plantarum WCFS1 (Kleerebezem et al., 2003) was obtained from Dipartimento di Scienze Ambientali, Seconda Università di Napoli. Inactivation of the *ccpA* gene was obtained by homologous recombination. Two fragments of 1040 (UP) and 1053 (DOWN) bp, localized upstream and downstream the *ccpA* gene, respectively, were amplified from the *L. plantarum* chromosomal DNA by PCR and cloned into BamHI-EcoRI and HindIII-PstI sites of pUC18Ery, respectively (van Kranenburg et al., 1997). UP includes the *pepQ* gene, and DOWN corresponds to the *lp_2254* locus. In the PCR reactions the oligonucleotides UP1 (5′-CCGGAATTCGCCTTCCTTAGTAACGACCCC-3′) and UP2 (5′-CGCGGATCCGGACCCAAGACAATCACGTTGACG-3′) were used to amplify the UP fragment; DOWN1 (5′-CGCGGATCCGCCATTCCGTCCACCAAA TCAAGTCC-3′) and DOWN2 (5′-CCCAAGCTTGGCCGTTGCGACCTTAGCCGGC-3′) were used to amplify the DOWN fragment. The resultant pLM13 plasmid was used to transform *L. plantarum* cells as described previously (Muscariello et al., 2001). Antibiotic-resistant transformants were selected on MRS agar containing erythromycin. About 15% of the recombinant clones, analyzed by PCR, showed that a double homologous recombination event had occurred between chromosomal regions flanking the gene of interest and their homologous regions present in pLM13, leading to replacement of the *ccpA* gene with the *ery* cassette. One of these clones, named WCFS1-2 (*ΔccpA ery*) was chosen for further analysis.

Both strains were maintained frozen (−75 °C) in 25% glycerol. Active cultures were obtained by inoculating (1% v/v) MRS broth followed by incubation for 16 h at 30 °C. For WCFS1-2 erythromycin (5 μg/mL) was used in all media.

2.2. Fermentation conditions

Fermentations were carried out in WMB (tryptone, 5 g/L; KH_2PO_4, 1 g/L; yeast extract, 10 g/L; glucose, 20 g/L = 0.11 mol/L; sodium acetate, 5 g/L; sodium citrate, 5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L, $MnSO_4 \cdot H_2O$, 0.05 g/L, Tween 80 0.5 mL). One liter fermentation vessels (Applikon, Schiedam, NL) were inoculated (5% v/v) using an overnight culture in the same medium. Two parallel fermentations (one in aerobiosis and one in anaerobiosis) were carried out using the same inoculum. Agitation was achieved by a magnetic stirrer (250 rpm), and pH (6.5, by addition of 1:1 Na_2CO_3/NaOH solution, 4 eq/L) and temperature (30 °C) were controlled using ezControl controllers (Applikon, Schiedam, NL). Foam was controlled by adding at the beginning of the fermentation 5 mL of a 1% v/v Antifoam A solution. Anaerobic or aerobic conditions were obtained by flushing with sterile nitrogen or sterile air (0.1 vol/vol min), respectively. Dissolved oxygen concentration (DO,%) was measured by polarographic electrodes (Applisens Applikon). In-line variables (temperature, pH, level, DO, ml base) were monitored using BioXpert 2. Two replicate fermentations were carried out for each treatment.

2.3. Growth, chemical, and biochemical analyses

Growth was monitored by measuring the absorbance of the culture at 650 nm (A_{650}). Cell dry weight (measured by separating the biomass by centrifugation at 10,000 *g*, 5 min, 4 °C, washing twice in distilled water and drying at 105 °C for 24 h) and plate counts (pour plating in WMB with 1.2% Agar bacteriological, WMA, followed by incubation at 30 °C for 48 h) were carried out on exponential phase cultures (A_{650} approx. 1) and at the end of fermentation (24 h) in order to calculate calibration curves to relate absorbance to cell dry weight or plate counts. Growth was modeled using the dynamic model of Baranyi and Roberts (1994) with DMFit v. 2.0 (Baranyi and Le Marc 1996). Biomass yield ($Y_{X/S}$, g/g) was calculated by dividing the net biomass production by the glucose consumed.

Spectrophotometric methods were used for the measurement of glucose (Miller, 1959), H_2O_2 (Risse et al., 1992), DL-lactic acid and acetate concentration (enzymatic kits, R-Biopharm AG, Darmstadt, Germany) in culture supernatants. Lactic acid ($Y_{L/S}$, mol/mol) and acetic acid yields ($Y_{A/S}$, mol/mol) were calculated by dividing the net acid production by the substrate (glucose) consumed. Catalase activity was measured on whole cells using the method described by Quatravaux et al. (2006).

2.4. Stress tolerance

The tolerance to heat, oxidative, acid and starvation/cold stress was evaluated on exponential and stationary phase cells, which were recovered by centrifugation (10,000 × *g*, 5 min at 4 °C), washed twice in 20 mmol/L potassium phosphate buffer pH 7 (PB7) and resuspended in PB7 to obtain a final $A_{650} = 1$.

For heat stress, cells were rapidly added to pre-warmed (55 °C) PB7 in screw cap tubes in a circulation water bath. Survival was measured by plate counts in WMA at 0, 15 and 30 min.

Oxidative stress tolerance was tested by spotting 5 μL of a 1.5% H_2O_2 on the top layer (7 mL) of WMA (containing 0.6% w/v Agar bacteriological) inoculated with 100 μL of standardized cell suspension, on a 10 mL bottom layer of sterile WMA. Two replicate plates were used for each suspension with two replicate spots. The area of the inhibition zone was measured with a calliper after incubation at 30 °C, 48 h.

Tolerance to starvation and cold storage was evaluated by storing cell suspensions in PB7 at 4 °C and by counting survivors on WMA or

WMA + 0.05% v/v cysteine (48 h, 30 °C). The proportion of metabolically active cells was evaluated by epifluorescence microscopy using propidium iodide and 5-(6)-carboxyfluorescein diacetate (PI and cFDA, respectively, both from Molecular Probes Inc., Eugene, OR, USA) as described in Zotta et al. (2012).

2.5. Proteome analysis

Proteome extraction from pooled exponential phase biomass samples (two replicate fermentations) was carried out by lysis with mutanolysin (134 units of approx. 2×10^{10} cells in 500 μL 20 mmol/L Tris–HCl, 10 mmol/L $MgCl_2$, 0.5 mol/L sucrose, pH 7.4) followed by protein precipitation with acidic acetone. 2-DE electrophoresis, image analysis and protein identification were carried out as already described (Tosco et al., 2005).

After protein identification the standardized volume (obtained by dividing spot volume by the sum of volume of all spots in a given gel) of spots containing the same protein was summed and, for each protein the volumes were standardized to *z*-scores by subtracting the mean and dividing by the standard deviation.

2.6. Statistical analysis

Univariate statistical analysis (two-way ANOVA) and graphing on the fermentation, stress tolerance and proteome data were carried out using Systat 13 (Systat Inc.). Permutation analysis on the proteome data was carried out using PermutMatrix (Caraux and Pinloche, 2005).

2.7. Reagents, culture media and ingredients

Unless otherwise stated all reagents were obtained from Sigma-Aldrich (Milan, Italy) while culture media and ingredients were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK).

3. Results

3.1. Growth and metabolite production

The growth in aerobiosis and anaerobiosis of *L. plantarum* WCFS1 and of its mutant WCFS1-2 (*ΔccpA ery*) was compared in fermentations at controlled pH. The kinetics of growth is shown in Fig. 1, while kinetic parameters and metabolite concentrations are reported in Table 1.

Inactivation of *ccpA* severely impaired growth. On the other hand, in the wild type strain a significant decrease in A_{650} was found at the end of growth. Therefore, growth kinetics was compared using the D model using the exponential phase data only. Estimated μ_{max} values and biomass concentration are shown in Table 1. Only differences between μ_{max} of WCFS1 and its mutant were significant ($p<0.05$) while no significant difference was found in final biomass concentration. Biomass yield was significantly higher in aerobiosis for WCFS1 but not for the mutant. For both strains and conditions the viable cell count at the end of growth was close to 2×10^9 cfu/mL. After base consumption ended, pH increased by 0.1–0.2 units both in aerobiosis and anaerobiosis, possibly due to lactate consumption. For WCFS1-2, but not for WCFS1, oxygen consumption continued in the stationary phase. Total lactic acid concentration was significantly lower in aerobiosis in both the exponential phase and after 24 h (stationary phase) and was lower for the mutant strain. Lactic acid yield was always lower than that predicted for homolactic fermentation (2 mol lactic acid/mol glucose) and was lower in aerobiosis for both strains and for the mutant compared to WCFS1, and was lower in the exponential phase when net acetic acid production was observed for both strains. Net acetic acid production was higher in aerobiosis compared to anaerobiosis and for the mutant strain. H_2O_2 production was always very low: higher concentrations were found for the mutant and in aerobiosis. Catalase activity was always below the sensitivity of the method used.

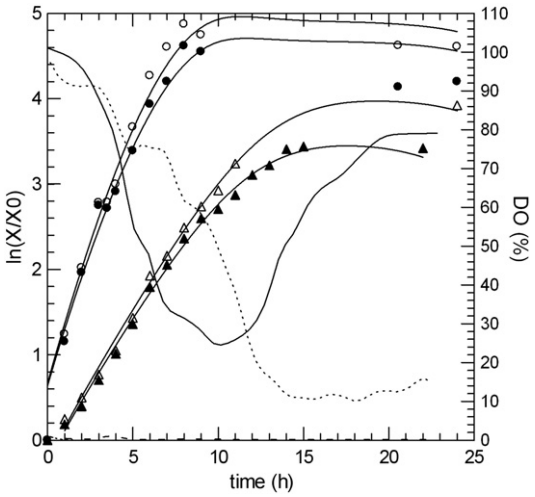

Fig. 1. Kinetics of growth and dissolved oxygen concentration (DO,% saturation) of representative fermentations for *L. plantarum* WCFS1 and its *ΔccpA ery* mutant (WCFS1-2) in batch fermentations in a complex medium at controlled pH (6.5) and temperature (30 °C) with nitrogen (N_2) or air sparging. Growth: O WCFS1 △ WCFS1-2; empty symbols air; closed symbols N_2; DO is shown only for fermentations with air sparging: continuous line WCFS1; dotted line WCFS1-2.

Cell size was measured in bright field images for both exponential phase and stationary phase cells. The results are shown in Table 1. Exponential phase cells of WCFS1-2 were significantly smaller ($p<0.01$) than those of WCFS1. For the latter, stationary phase cells were significantly smaller than exponential phase cells, while no significant difference was found for either strain between aerobically and anaerobically grown cells.

3.2. Stress tolerance

The tolerance of both exponential phase and stationary phase cells to heat, oxidative and starvation stresses was assayed.

The kinetics of heat inactivation is shown in Fig. 2. Inactivation of *ccpA* impaired heat tolerance ($p<0.001$). For both strains cells grown in aerobiosis were more tolerant of heat ($p<0.01$).

Stationary phase cells of both the wild type and the mutant were significantly more heat tolerant ($\log N/N_0$ at 30 min $-3.5/-4$) and the differences between the two strains were negligible (data not shown).

Oxidative stress tolerance was assayed using a spot on lawn method: the diameters of the zone of inhibition caused by 5 μL of a 1.5% H_2O_2 solution for exponential phase cells were respectively 0.96 ± 0.02 and 0.78 ± 0.05 for WCFS1 in anaerobiosis and aerobiosis, respectively; 0.82 ± 0.10 and 0.79 ± 0.01 for WCFS1-2 in anaerobiosis and aerobiosis, respectively. Cells grown in aerobiosis were significantly more resistant with no difference between strains. For cells grown in anaerobiosis WCFS1 was significantly more sensitive than WCFS1-2. For stationary phase cells the diameters of the zone of inhibition caused by 5 μL of a 1.5% H_2O_2 solution were respectively 1.13 ± 0.02 and 0.75 ± 0.02 for WCFS1 in anaerobiosis and aerobiosis, respectively; 0.72 ± 0.02 and 0.59 ± 0.02 for WCFS1-2 in anaerobiosis and aerobiosis, respectively. Differences between exponential and stationary phase cells were not significant for WCFS1, while they were significant for WCFS1-2 ($p<0.01$). Again, for each growth condition the mutant was more tolerant than the wild type, while for both strains aerobically grown cells were significantly more tolerant.

The survival of both exponential phase and stationary cells after cold storage in a nutrient depleted buffer (PB7) was evaluated.

Table 1
Maximum specific growth rate (μ_{max}), metabolite concentrations and yields per unit glucose consumed ($Y_{X/S}$, biomass; $Y_{L/S}$, DL lactic acid; $Y_{A/S}$ acetic acid) and average cell size for *L. plantarum* WCFS1 and its mutant WCFS1-2 (*ΔccpA ery*) grown in a complex medium at controlled pH (6.5) and temperature (30 °C) in anaerobiosis (N_2 sparging) or aerobiosis (air sparging). Mean ± standard error for two replicate fermentations. For acetic acid the net production (the medium contained 5 g/L sodium acetate) is shown.

Strain	Treatment	Growth phase	μ_{max} (h^{-1})	Biomass (g/L)	Glucose mmol/L	DL-lactic acid mmol/L	Acetic acid mmol/L	H_2O_2 μmol/L	$Y_{X/S}$ g/g	$Y_{L/S}$ mol/mol	$Y_{A/S}$ mol/mol	Cell size μ^2
WCFS1	N_2	E	0.69 ± 0.01	0.54 ± 0.05	84.4 ± 1.8	46.0 ± 1.6	10 ± 2	62 ± 2	0.10 ± 0.02	1.58 ± 0.05	0.37 ± 0.08	0.58 ± 0.08
		S	–	3.1 ± 0.4	6.5 ± 0.3	202.2 ± 2.1	12 ± 1	73 ± 2	0.14 ± 0.01	1.88 ± 0.01	0.11 ± 0.01	0.33 ± 0.04
	Air	E	0.68 ± 0.01	0.51 ± 0.05	81.7 ± 1.7	9.1 ± 0.2	17 ± 2	127 ± 6	0.09 ± 0.01	0.24 ± 0.02	0.57 ± 0.11	0.56 ± 0.06
		S	–	3.4 ± 0.3	4.8 ± 0.1	174.5 ± 2.5	35 ± 1	188 ± 16	0.16 ± 0.01	1.60 ± 0.02	0.32 ± 0.01	0.40 ± 0.05
WCFS1-2	N_2	E	0.37 ± 0.02	0.44 ± 0.01	87.5 ± 2.7	26.2 ± 1.1	13 ± 1	83 ± 5	0.09 ± 0.01	1.04 ± 0.13	0.55 ± 0.12	0.30 ± 0.03
		S	–	3.1 ± 0.7	11.7 ± 0.6	174.4 ± 1.3	12 ± 1	69 ± 2	0.17 ± 0.02	1.71 ± 0.01	0.11 ± 0.01	0.28 ± 0.01
	Air	E	0.37 ± 0.04	0.46 ± 0.03	93.0 ± 1.2	1.1 ± 0.7	36 ± 1	142 ± 2	0.12 ± 0.01	0	1.63 ± 0.01	0.32 ± 0.05
		S	–	3.4 ± 0.1	9.0 ± 0.4	114.4 ± 1.8	82 ± 1	306 ± 7	0.18 ± 0.01	1.09 ± 0.01	0.79 ± 0.01	0.27 ± 0.01

Three methods were compared to establish if the cells entered a viable but non-culturable (VNBC) state: plate counts on WMA and WMA + 0.05% cysteine and measurement of live/metabolically active cells by direct counts in epifluorescence microscopy images stained with cFDA/PI. The results of plate counts on WMA for exponential and stationary phase cells are shown in Fig. 3. Exponential phase cells of WCFS1-2 were significantly more sensitive than the parental strain and cells grown in aerobiosis were significantly more resistant. Stationary phase cells were more tolerant of cold and starvation stresses for both strains and the difference in survival for aerobic grown cells was negligible, while WCFS1 cells grown in anaerobiosis were significantly more tolerant than WCFS1-2 cells grown in the same conditions. The sensitivity of direct counts by epifluorescence microscopy was low: the number of cells per field ranged from 3 to 181 (median 27) and, even summing all the cells over replicate fields, the minimum survival fraction which could be reliably estimated ranged between 0.2 and 1%. After 30 days only red (dead) cells were detected by microscopy for 50% of the treatments. However, a comparison of the three methods was possible at 15 d. The results for exponential and stationary phase cells are shown in Fig. 4. Survival measured by direct counts was significantly higher than that measured by plate counts, and counts on WMA + cys were significantly higher than counts on WMA, especially for the mutant. At 30 d the differences between WMA and WMA + cys increased: counts on WMA were 50–60% of those on WMA + cys. At least for exponential phase cells, a significant portion of the population is sub-lethally damaged and enters a VBNC stage. This proportion was higher for WCFS1-2 and for cells grown in anaerobiosis.

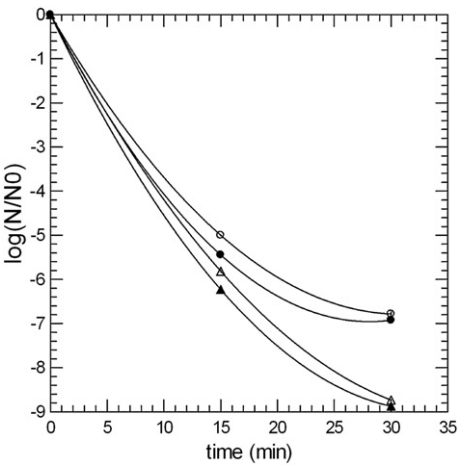

Fig. 2. Survival of exponential phase cells of *L. plantarum* WCFS1 and its *ΔccpA ery* mutant (WCFS1-2) in batch fermentations in a complex medium at controlled pH (6.5) and temperature (30 °C) with nitrogen (N_2) or air sparging following exposure to 55 °C in 20 mmol/L potassium phosphate buffer, pH 7. O WCFS1 △ WCFS1-2; empty symbols air; closed symbols nitrogen. Average of two replicates for a single fermentation with standard error bars.

3.3. Impact of inactivation of ccpA and aerobic growth on the expression of stress related proteins

Preliminary proteomic results showed that the inactivation of *ccpA* and aerobic growth significantly affected the expression of a large number of proteins (data not shown). Proteins in spots whose intensity was significantly affected (as shown by univariate and multivariate tests) by *ccpA* inactivation or by growth conditions were identified by mass spectrometry. A complete analysis of the effects on the proteome is beyond the scope of this paper and preliminary results have been published elsewhere (Mazzeo et al., 2011): here we will focus on the impact on 20 proteins whose function may be directly or indirectly related to stress. A list of these proteins is shown in Table 2. The standardized spot volumes are compared in Fig. 5. Although differences in protein expression levels are readily visible in Fig. 5, a comparison of expression patterns is made difficult by the differences in scale for the average spot volume for different proteins, with large volumes for pyruvate oxidase and very small volumes for some oxidoreductases.

The effect of inactivation of *ccpA* or growth conditions is indicated in Table 2. Two-way ANOVA was used to evaluate the two effects (strain and aeration) and of their interaction, and $p<0.01$ was used as a conservative limit for significance but for all proteins listed in the table either effect, or both, were highly significant ($p<0.001$). The expression of 4 proteins in Table 2 was significantly affected by *ccpA* inactivation, 4 by aeration only, while for 12 more than one effect was significant.

The first group includes CcpA, DnaK, GroEL, and a putative oxidase (lp_1776). CcpA, as expected, was absent in WCFS1-2, in which this gene had been inactivated, and as a consequence, expression of DnaK and GroEL, which are both in operons which are known to be activated by CcpA (Castaldo et al., 2006), was reduced. The second group included two putative alkaline shock proteins (Asp1 and Asp2) whose expression was higher in anaerobiosis in both strains, catalase (whose expression was increased in both strains aerobiosis), and two protein-methionine-S-oxide reductases, MsrA2 MsrA3 (whose expression was increased in the WCFS1-2 in aerobiosis). Several other proteins were affected by either *ccpA* inactivation (ClpE and the putative oxidoreductase gi|28379735, encoded by locus lp_3403) or aeration (the cell division protein DivIVA) but the effect of aeration appeared to be different in intensity or direction in the mutant and parent strain, while NADH oxidase (Nox5), NAD peroxidase (Npr2) and the ClpL ATP-ase were affected by both mutation and aeration. In fact, Nox5 was found in WCFS1-2 only and its expression was higher in anaerobiosis, while Npr2 expression was higher in WCFS1-2 than in WCFS1 in both aerobiosis and anaerobiosis, but was higher for both strains in aerobiosis. The small heat shock protein Hsp1 (which was present in aerobiosis in WCFS1-2 only), pyruvate

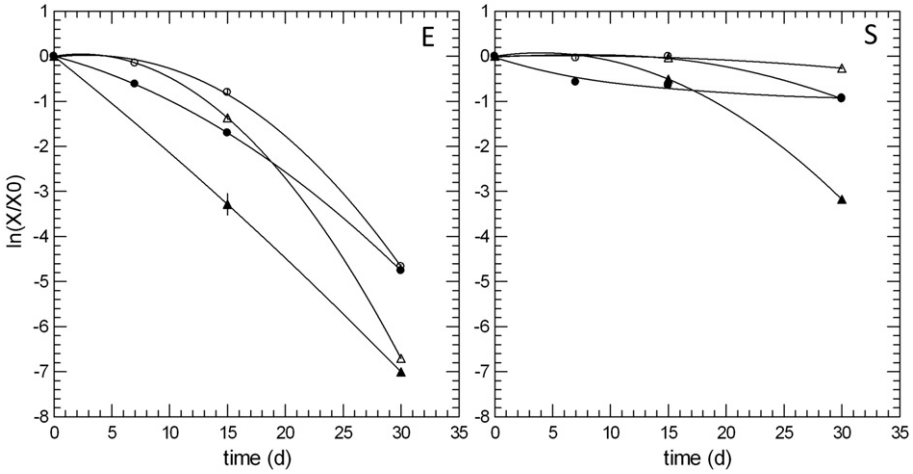

Fig. 3. Effect of cold storage (4 °C) in a nutrient free medium on the survival of exponential (E) and stationary (S) phase cells of *L. plantarum* WCFS1 and its *ΔccpA ery* mutant (WCFS1-2) grown in batch fermentations in a complex medium at controlled pH (6.5) and temperature (30 °C) with nitrogen (N_2) or air sparging. Number of survivors enumerated on WMA. A. Exponential phase cells. B. Stationary phase cells. O WCFS1 △ WCFS1-2; empty symbols air; closed symbols nitrogen. Average of two replicates for a single fermentation with standard error bars.

oxidase PoxB (which dramatically increased in the mutant in both fermentation conditions and in aerobiosis for both strains), the nitroreductase encoded by lp_2968 (gi|28379407) which was present in anaerobiosis in WCFS1-2 only, the chaperonin GrpE (which was little affected by the mutation in anaerobiosis, but whose expression was lower in WCFS1 in aerobiosis) and the putative oxidoreductase encoded by locus lp_0244 (gi|28377171) which was increased in WCFS1 only by aeration.

To readily visualize groups of proteins which responded to inactivation of *ccpA* or to growth conditions a permutation analysis was carried out on the results: the four combination strain/fermentation conditions were sharply separated and groups of proteins for which *ccpA* inactivation and/or aeration had a similar effect were clearly evident (Supplementary Fig. 1).

4. Discussion

Inactivation of *ccpA* and, to a lesser extent, growth in aerobiosis significantly affected growth and fermentation end products in *L. plantarum* WCFS1, in agreement with what has been found for other microorganisms (Esteban et al., 2004; Gaudu et al., 2003; Mahr et al., 2000; Muscariello et al., 2001; Titgemeyer and Hillen, 2002), although for some species no effect on growth has been observed in *ccpA*-disrupted mutants (Asanuma et al., 2004). Aeration did not significantly affect the growth rate nor the biomass yield in spite of increased ATP production which would be expected in the mutant grown in aerobiosis because of pyruvate oxidase and acetate kinase activity (Quatravaux et al., 2006). On the other hand, aeration did not reduce the growth rate in the exponential phase (as found for *L. plantarum* by Murphy and Condon, 1984a; and for *Lact. lactis* in aerobic heterolactic fermentation by Lan et al., 2006). No evidence for diauxic growth due to CO_2 depletion (Stevens et al., 2008) was observed, probably due to the presence of Na_2CO_3 in the neutralizing agent. The expression of glycolytic enzymes was reduced in WCFS1-2 in both aerobic and anaerobic growth (Mazzeo et al., 2011, data not shown) and this might account for the reduced growth rate of the mutant in agreement with what has been found for other species (Ludwig et al., 2001; Luesink et al., 1998).

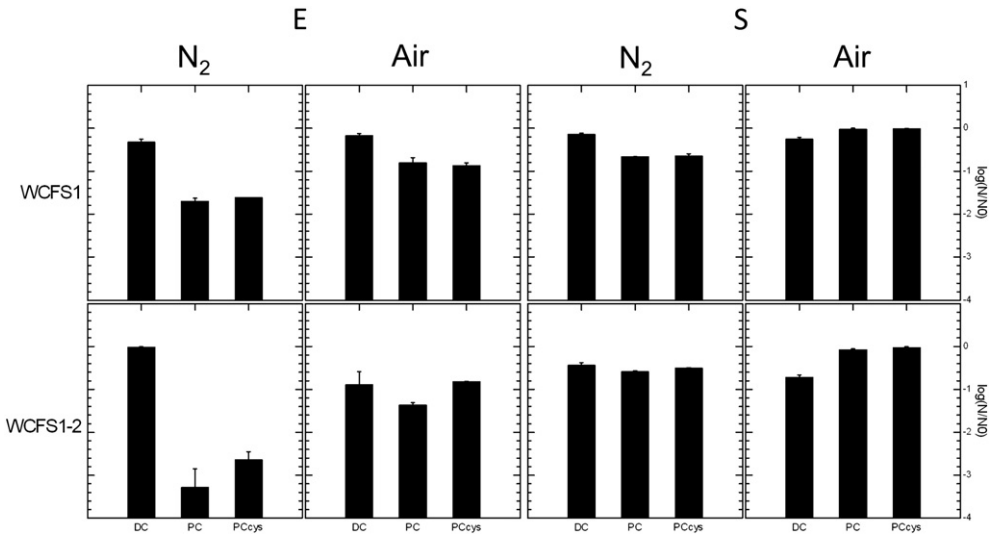

Fig. 4. Bar plot showing the logarithm of the proportion of survivors after exposure to cold and starvation stress (4 °C in 20 mmol/L potassium phosphate buffer, pH 7) of exponential (E) and stationary phase (S) cells of *L. plantarum* WCFS1 and its *ΔccpA ery* mutant (WCFS1-2) in batch fermentations in a complex medium at controlled pH (6.5) and temperature (30 °C) with nitrogen (N_2) or air sparging, as determined by direct epifluorescence microscopy counts (DC), plate counts on WMA (PC) and on WMA + 0.05% cysteine (PCC). Mean results with standard error are shown.

Table 2
A partial list of *Lactobacillus plantarum* WCFS1 proteins whose expression was affected by inactivation of ccpA (strain WCFS1-2) or by aerobic (air)/anaerobic (N_2) growth at controlled pH and temperature in a complex medium. Role categories, locus and genes are extracted from the *L. plantarum* WCFS1 genome data in the Comprehensive Microbial Resources database (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). — indicates a change in expression <1.5 fold; ↓ and ↑ indicate a change in expression ≥1.5 fold; ↓↓ and ↑↑ indicate a change in expression ≥3 fold; * indicates proteins present in the mutant only; WT indicates proteins present in the wild type strain only; and + indicates proteins present in aerobiosis only. For all proteins shown in the table the effect of either *ccpA* inactivation or aeration, or both, was highly significant ($p<0.001$).

NCBI accession number	Locus	Gene	Protein	JCVI cellular role category	Regulation			
					Air vs. N_2		WCFS1-2 vs WCFS1	
					WCFS1	WCFS1-2	Air	N_2
gi|28377757	lp_0929	*asp1*	Alkaline shock protein	Cellular processes:	↓↓	↓↓	–	–
gi|28377758	lp_0930	*asp2*	Alkaline shock protein	Adaptations to atypical conditions	↓	↓	–	–
gi|28378793	lp_2189	*divIVA*	Cell division initiation protein DivIVA	Cellular processes: Cell division	↓	–	↑	–
gi|28379886	lp_3578	*kat*	Catalase	Cellular processes: Detoxification	↑	–	–	–
gi|28379083	lp_2544	*npr2*	NADH peroxidase	Energy metabolism: Electron transport	↑	↑	↑	↑↑
gi|28379772	lp_3449	*nox5*	NADH oxidase			↓	*	*
gi|28379896	lp_3589	*pox5*	Pyruvate oxidase	Energy metabolism: Sugars	↑	↑	↑↑	↑↑
gi|28378034	lp_1269	*clpE*	ATP-dependent Clp protease, ATP-binding subunit ClpE	Protein fate: Degradation of proteins, peptides, and glycopeptides	↑	–	–	↑
gi|28379892	lp_3583	*clpL*	ATP-dependent Clp protease, ATP-binding subunit ClpL		–	↓	–	↑
gi|28377077	lp_0129	*hsp1*	Small heat shock protein	Protein fate: Protein folding and stabilization		+	*	
gi|28377591	lp_0728	*groEL*	GroEL chaperonin		–	–	↓	↓
gi|28378660	lp_2027	*dnaK*	Heat shock protein DnaK		–	–	↓	↓↓
gi|28378661	lp_2028	*grpE*	Heat shock protein GrpE		↓	–	–	–
gi|28378496	lp_1835	*msrA2*	Protein-methionine-S-oxide reductase	Protein fate:	–	↑	–	–
gi|28378497	lp_1836	*msrA3*	Protein-methionine-S-oxide reductase	Protein modification and repair	–	↑↑	–	–
gi|28378851	lp_2256	*ccpA*	Catabolite control protein A	Regulatory functions: DNA interactions			WT	WT
gi|28377171	lp_0244		Oxidoreductase (putative)	Unknown function:	↑	–	–	–
gi|28378450	lp_1776		Oxidoreductase	Enzymes of unknown specificity	–	↑	–	↓↓
gi|28379407	lp_2968		Nitroreductase			↓↓	*	*
gi|28379735	lp_3403		Oxidoreductase		–	–	↓↓	↓↓

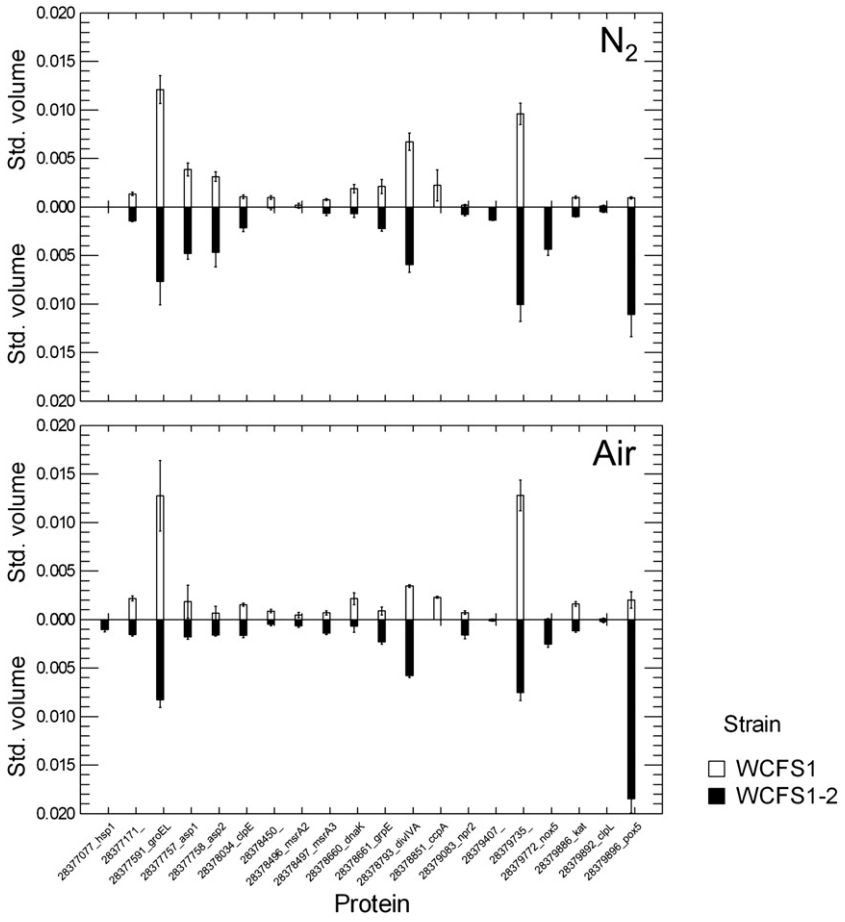

Fig. 5. Differences in the expression patterns of proteins related to stress and pyruvate metabolism for *L. plantarum* WCFS1 and its *ΔccpA ery* mutant (WCFS1-2) grown in batch fermentations in a complex medium at controlled pH (6.5) and temperature (30 °C) under nitrogen (N_2) or air sparging. For each protein the average standardized spot volume and standard deviation are shown. Proteins are identified by NCBI accession number and gene symbol (when available). A list of the proteins is shown in Table 2.

The major pathway for acetate production in aerobiosis in *L. plantarum* involves LDH, POX and ACK, with NOX and NPR involved in NAD/NADH cycling (Quatravaux et al., 2006). When grown in anaerobiosis both WCFS1 and WCFS1-2 carried out a prevalently homolactic fermentation, with final lactic/acetic acid ratios between 14 and 20 mol/mol, and with a lactic acid yield between 1.68 and 1.88 mol/mol. However, in the exponential phase acetic acid production was relatively higher and lactic acid yield was lower. The contribution of citrate, which was present in WMB, as a source of pyruvate and acetate cannot be excluded. The largest differences between the two strains were found in aerobiosis. The wild type strain showed a behavior similar to that described by other authors (Goffin et al., 2006; Murphy and Condon, 1984a, 1984b; Quatravaux et al., 2006) with O_2 consumption increasing during the exponential phase, peaking at the beginning of the stationary phase and then decreasing. At 24 h the lactic acid yield was lower than in anaerobiosis and more acetic acid was produced, as expected by the role of *ccpA* in the control of the expression of *pox* genes (Goffin et al., 2006; Lorquet et al., 2004), which is confirmed by the increased and anticipated acetic acid production in the mutant. Low H_2O_2 concentrations, even in the presence of active acetic acid production from pyruvate or lactate, cannot be due to catalase activity, which was undetectable. This depends on the lack of heme in the medium, but the presence of catalase (Table 2) may led to speculate that catalase production is regulated in a way independent from the availability of heme. Small amounts of NADH peroxidase (which is known to contribute to the detoxification of H_2O_2, Quatravaux et al., 2006) were found for WCFS1-2 in both anaerobiosis and aerobiosis, with increased expression in aerobiosis for both strains. The effect of carbon catabolite repression (CCR) and oxygen on the presence of POX, NOX and NPR is in agreement with what has been found by other authors (Lorquet et al., 2004; Murphy and Condon, 1984a, 1984b; Quatravaux et al., 2006) although our data suggest that the role of CcpA is more important. However, several other enzymes which may be involved in NADH/NAD balance were affected by *ccpA* inactivation (Mazzeo et al., 2011; data not shown).

Inactivation of *ccpA* significantly affected the size of exponential phase cells. DivIVA, which is involved in cell division and cell shape (Zapun et al., 2008) was differentially expressed in the two strains, but the effect of growth rate on cell size may be more important.

The reduced heat tolerance of the mutant is in agreement with the role of CcpA in the activation of the expression of class I heat stress response operons in *L. plantarum* (Castaldo et al. 2006) and is confirmed by the proteomic data. Interestingly, although DnaK expression was reduced as expected in WCFS1-2, GrpE, whose gene belongs to the same operon (Kleerebezem et al., 2003), was overexpressed in WCFS1-2 compared to WCFS1 at least in aerobiosis. Further investigations are needed to clarify the mechanism of GrpE expression in the mutant strain. Clp proteases and ATPases play several important roles in low G+C% Gram positive bacteria, in both stressed and non stressed cells (Frees et al., 2007). The ATP-binding subunits ClpL and ClpE were both overexpressed in WCFS1-2 compared to the parent strain at least in anaerobiosis, and in both strains a significant, although different, effect of aeration was found. The reasons for this are unclear. A further heat shock protein, Hsp1, was found only in WCFS1-2 cells grown in aerobiosis. Hsp1 is a 16 kDa protein which belongs to the conserved HSP20 family of *L. plantarum* (Fiocco et al., 2010). A BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul et al., 1997) for *hsp1* resulted in 99–100% identity with the same gene in two other sequenced *L. plantarum* genomes (ST-III and JDM1) and in significant identities with small heat shock proteins of members of the genera *Lactobacillus* and *Pediococcus* (data not shown). Both *clpL* and *hsp1* belong to the CtsR regulon in *L. plantarum* WCFS1 (Fiocco et al., 2010). Given the complex interplay of the three regulators (CcpA, HrcA and CtsR) further study is needed to clarify if the expression of these proteins may compensate the lower expression of DnaK and GroESL.

The higher stress tolerance of stationary phase cells is in agreement with data from the literature for *L. plantarum* (De Angelis et al., 2004; Parente et al., 2010) and for other LAB (van de Guchte et al., 2002) and is probably due to a generalized stress response.

No data are available in the literature on the effect of aerobic growth of *L. plantarum* on stress tolerance. Increased heat stress resistance of exponential phase cells grown in aerobiosis is in contrast with the decreased expression of some heat stress related proteins (GrpE, ClpL) but may be indirectly due to increased tolerance to oxidative stress, which may improve recovery of sub-lethally damaged cells. In *Lact. lactis* growth in aerobiosis under conditions which promote the formation of an electron transport chain has been found to significantly increase tolerance to oxidative stress (Gaudu et al., 2002).

Although *L. plantarum* is relatively resistant to oxidative stress (Murphy and Condon 1984a), a large variability has been found for tolerance to H_2O_2 by Parente et al. (2010), with stationary phase cells usually, but not always, more tolerant than exponential phase cells. *L. plantarum* is known to accumulate manganese as a response mechanism to oxygen stress (Archibald and Fridovich, 1981) and thioredoxin reductase is known to play a fundamental role in oxidative stress tolerance in *L. plantarum* WCFS1 (Serrano et al., 2007). A H_2O_2 (3.5 mmol/L) pulse during continuous cultivation of this strain has been found to affect the transcription of a large number of genes (Serrano et al., 2007). However, this sort of stress may be quite different from the effect of aerobic growth in batch culture. In fact, although *kat*, *pox5* and *npr2* are up-regulated in both situations, we found that aerobic growth did significantly affect the expression of a number of proteins which were not included among those affected by H_2O_2 pulse. Two notable examples are the protein-methionine-S-oxide reductases encoded by *msrA2* and *msrA3*, which may play a role in protein repair. Two oxidoreductases, encoded in the loci lp_0244 and lp_1776, were overexpressed in aerobiosis in the wild type and/or the mutant strain. A BLASTN search showed that lp_0244 is highly conserved in the genomes of the three sequenced *L. plantarum* strains, and shows high identity (74–99%) with NADPH-dependent FMN reductases in several other LAB, including *L. acidophilus* 30SC and several *Leuconostoc* strains (data not shown). On the other hand, lp_1766 sequence shows high identity only with *L. plantarum* JDM1 and ST-III. It is unclear if these proteins play a role in oxidative metabolism or stress tolerance.

Two further stress related proteins, Asp1 and Asp2, were overexpressed in anaerobiosis in both the parent and the mutant strains, while Serrano et al. (2007) found that their transcription increased following a H_2O_2 pulse. Both proteins are annotated as alkaline shock proteins and are highly conserved in *L. plantarum* genomes. However, in a proteomic study of the effect of exposure of *L. plantarum* ATCC8014 cells to alkaline shock (pH 7.7–9.7) Asp1 and Asp2 were not among the proteins for which a change in expression was reported (Lee et al., 2011), thus suggesting that the role of these proteins may not be related to alkaline stress.

Starvation and temperature downshift are among the factors that cause entry of bacteria, including LAB, in VBNC status (Keer and Birch, 2003). Viability of exponential phase cells of both the WCFS1 and the WCFS1-2 slowly decreased during storage, while stationary phase cells showed a much better survival. Evidence of entry in a VBNC status has been found for *L. plantarum* (Golod et al., 2009) with formation of cyst like dormant structures in some conditions. In our experimental set-up a temperature downshift to 4 °C (close to the minimum temperature for growth for *L. plantarum*, Zwietering et al., 1991) and storage in a nutrient depleted buffer provide both a cold and starvation stress. Survival during starvation is usually attributed to the capacity of maintaining an active metabolic state and avoiding energy depletion (van de Guchte et al., 2002). The higher survival of stationary phase cells may be due to a generalized stress response and to lower initial metabolic activity, while higher survival of aerobically grown exponential phase cells, at least after 15 d may

be due to higher oxidative stress tolerance as in *Lact. lactis* (Gaudu et al., 2002).

5. Conclusions

It is now well known that growth under conditions that favor the presence of a respiratory chain has important consequences on biomass yield and stress tolerance of some industrially important lactic acid bacteria (Lechardeur et al., 2011), but this is the first report showing that aerobic growth can significantly enhance heat and oxidative stress tolerance of *L. plantarum*. This may have important technological consequences for the production of probiotic and adjunct cultures containing this species, since spray drying, which is far more economic than freeze drying, does impose both oxidative and heat stresses to cells (Peighambardoust et al., 2011). Furthermore, we have confirmed the role of the pleiotropic regulator CcpA in stress resistance and aerobic metabolism of *L. plantarum*. Apart from known stress response proteins (DnaK, GroEL, GrpE, ClpL, ClpE) and proteins involved in production or detoxification of reactive oxygen species (POX, NOX, NPR, catalase), we found that a number of other oxidoreductases and proteins related to stress (Hsp1, Asp1, Asp2) or protein repair, were affected by aerobic/anaerobic growth and by *ccpA* inactivation. Further work is needed to clarify the regulation of the expression of these proteins and their role in stress response.

Supplementary materials related to this article can be found online at doi:10.1016/j.ijfoodmicro.2012.01.017.

Acknowledgments

This work was partly funded by Ministero dell'Istruzione, dell'Università e della Ricerca, Rome, Italy, PRIN n. 20088SZB9B. Dr. Teresa Zotta PostDoc grant was funded by Università degli Studi della Basilicata, Italy.

References

Abriouel, H., Herrmann, A., Stärke, J., Yousif, N.M.K., Wijaya, A., Tauscher, B., Holzapfel, W., Franz, C.M.A.P., 2004. Cloning and heterologous expression of hematin-dependent catalase produced by *Lactobacillus plantarum* CNRZ 1228. Applied and Environmental Microbiology 70, 603–606.

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W., Lipman, D., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Archibald, F.S., Fridovich, I., 1981. Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. Journal of Bacteriology 145, 442–451.

Asanuma, N., Yoshii, T., Hino, T., 2004. Molecular characterization of CcpA and involvement of this protein in transcriptional regulation of lactate dehydrogenase and pyruvate formate-lyase in the ruminal bacterium *Streptococcus bovis*. Applied and Environmental Microbiology 70, 5244–5251.

Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology 23, 277–294.

Baranyi, J., Le Marc, Y., 1996. Dmfit Manual, Version 2.0. Institute of Food Research, Norwich, UK.

Brooijmans, R.J.W., de Vos, W.M., Hugenholtz, J., 2009. *Lactobacillus plantarum* WCFS1 electron transport chains. Applied and Environmental Microbiology 75, 3580–3585.

Caraux, G., Pinloche, S., 2005. Permutmatrix: a graphical environment to arrange gene expression profiles in optimal linear order. Bioinformatics 21, 1280–1281.

Castaldo, C., Siciliano, R.A., Muscariello, L., Marasco, R., Sacco, M., 2006. CcpA affects expression of the *groESL* and *dnaK* operons in *Lactobacillus plantarum*. Microbial Cell Factories 5, 35. doi:10.1186/1475-2859-5-35.

De Angelis, M., Di Cagno, R., Huet, C., Crecchio, C., Fox, P.F., Gobbetti, M., 2004. Heat shock response in *Lactobacillus plantarum*. Applied and Environmental Microbiology 70, 1336–1346.

Esteban, C.D., Mahr, K., Monedero, V., Hillen, W., Pérez-Martínez, G., Titgemeyer, F., 2004. Complementation of a ΔccpA mutant of *Lactobacillus casei* with CcpA mutants affected in the DNA- and cofactor-binding domains. Microbiology 150, 613–620.

Fiocco, D., Capozzi, V., Collins, M., Gallone, A., Hols, P., Guzzo, J., Weidmann, S., Rieu, A., Msadek, T., Spano, G., 2010. Characterization of the CtsR stress response regulon in *Lactobacillus plantarum*. Journal of Bacteriology 192, 896–900.

Frees, D., Savijoki, K., Varmanen, P., Ingmer, H., 2007. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. Molecular Microbiology 63, 1285–1295.

Fujita, Y., 2009. Carbon catabolite control of the metabolic network in *Bacillus subtilis*. Bioscience, Biotechnology, and Biochemistry 73, 245–259.

Gaudu, P., Lamberet, G., Poncet, S., Gruss, A., 2003. CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*. Molecular Microbiology 50, 183–192.

Gaudu, P., Vido, K., Cesselin, B., Kulakauskas, S., Tremblay, J., Rezaïki, L., Lamberet, G., Sourice, S., Duwat, D., Gruss, A., 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek 82, 263–269.

Goffin, P., Muscariello, L., Lorquet, F., Stukkens, A., Prozzi, D., Sacco, M., Kleerebezem, M., Hols, P., 2006. Involvement of pyruvate oxidase activity and acetate production in the survival of *Lactobacillus plantarum* during the stationary phase of aerobic growth. Applied and Environmental Microbiology 72, 7933–7940.

Golod, N.A., Loiko, N.G., Mulyukin, A.L., Neiymatov, A.L., Vorobjeva, L.I., Suzina, N.E., Shanenko, E.F., Gal'chenko, V.F., El-Registan, G.I., 2009. Adaptation of lactic acid bacteria to unfavorable growth conditions. Microbiology 78, 280–289.

Gosalbes, M.J., Monedero, V., Pérez-Martínez, G., 1999. Elements involved in catabolite repression and substrate induction of the lactose operon in *Lactobacillus casei*. Journal of Bacteriology 181, 3928–3934.

Keer, J.T., Birch, L., 2003. Molecular methods for the assessment of bacterial viability. Journal of Microbiological Methods 53, 175–183.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W.E.J., Stiekema, W., Klein Lankhorst, R.M., Bron, P.A., Hoffer, S.M., Nierop Groot, M.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W.M., Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proceedings of the National Academy of Sciences 100, 1990–1995.

Kono, Y., Fridovich, I., 1983. Functional significance of manganese catalase in *Lactobacillus plantarum*. Journal of Bacteriology 155, 742–746.

Lan, C.Q., Oddone, G., Mills, D.A., Block, D.E., 2006. Kinetics of *Lactococcus lactis* growth and metabolite formation under aerobic and anaerobic conditions in the presence or absence of hemin. Biotechnology and Bioengineering 95, 1070–1080.

Lechardeur, D., Cesselin, B., Fernandez, A., Lamberet, G., Garrigues, C., Pedersen, M., Gaudu, P., Gruss, A., 2011. Using heme as an energy boost for lactic acid bacteria. Current Opinion in Biotechnology 22, 143–149.

Lee, K., Rho, B.-S., Pi, K., Kim, H.-J., Choi, Y.-J., 2011. Proteomic analysis of protein expression in *Lactobacillus plantarum* in response to alkaline stress. Journal of Biotechnology 153, 1–7.

Lopez de Felipe, F., Gaudu, P., 2009. Multiple control of the acetate pathway in *Lactococcus lactis* under aeration by catabolite repression and metabolites. Applied Microbiology and Biotechnology 82, 1115–1122.

Lorquet, F., Goffin, P., Muscariello, L., Baudry, J.-B., Ladero, V., Sacco, M., Kleerebezem, M., Hols, P., 2004. Characterization and functional analysis of the *poxB* gene, which encodes pyruvate oxidase in *Lactobacillus plantarum*. Journal of Bacteriology 186, 3749–3759.

Ludwig, H., Homuth, G., Schmalisch, M., Dyka, F.M., Hecker, M., Stülke, J., 2001. Transcription of glycolytic genes and operons in *Bacillus subtilis*: evidence for the presence of multiple levels of control of the *gapA* operon. Molecular Microbiology 41, 409–422.

Luesink, E.J., van Herpen, R.E.M.A., Grossiord, B.P., Kuipers, O.P., de Vos, W.M., 1998. Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Molecular Microbiology 30, 789–798.

Mahr, K., Hillen, W., Titgemeyer, F., 2000. Carbon catabolite repression in *Lactobacillus pentosus*: analysis of the *ccpA* region. Applied and Environmental Microbiology 66, 277–283.

Mazzeo, M.F., Cacace, G., Peluso, A., Zotta, T., Musariello, L., Sacco, M., Parente, E., Siciliano, R.A., 2011. Proteomics for the elucidation of oxidative stress response mechanism in *Lactobacillus plantarum*. In: Casella, S., Daffonchio, D., Gobbetti, M., Parente, E. (Eds.), Proceedings of the First International Conference on Microbial Diversity 2011 — Environmental Stress and Adaptation, MD 2011, 26–28 October 2011, Milan, Italy, pp. 205–206.

Miller, G.L., 1959. Use of dinitrosalycilic acid reagent for determination of reducing sugar. Analytical Chemistry 31, 426–428.

Murphy, M.G., Condon, S., 1984a. Comparison of aerobic and anaerobic growth of *Lactobacillus plantarum* in a glucose medium. Archives of Microbiology 138, 49–53.

Murphy, M.G., Condon, S., 1984b. Correlation of oxygen utilisation and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. Archives of Microbiology 138, 44–48.

Muscariello, L., Marasco, R., De Felice, M., Sacco, M., 2001. The functional *ccpA* gene is required for carbon catabolite repression in *Lactobacillus plantarum*. Applied and Environmental Microbiology 67, 2903–2907.

Parente, E., Ciocia, F., Ricciardi, A., Zotta, T., Felis, G.E., Torriani, S., 2010. Diversity of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: a multivariate screening study. International Journal of Food Microbiology 144, 270–279.

Peighambardoust, S.H., Golshan Taftia, A., Hesaria, J., 2011. Application of spray drying for preservation of lactic acid starter cultures: a review. Trends in Food Science & Technology 22, 215–224.

Quatravaux, S., Remize, F., Bryckaert, E., Colavizza, D., Guzzo, J., 2006. Examination of *Lactobacillus plantarum* lactate metabolism side effects in relation to the modulation of aeration parameters. Journal of Applied Microbiology 101, 903–912.

Risse, B., Stempfer, G., Rudolph, R., Mollering, H., Jaenicke, R., 1992. Stability and reconstitution of pyruvate oxidase from *Lactobacillus plantarum*: dissection of the stabilizing effects of coenzyme binding and subunit interaction. Protein Science 1, 1699–1709.

Serrano, L.M., Molenaar, D., Wels, M., Teusink, B., Bron, P.A., de Vos, W.M., Smid, E.J., 2007. Thioredoxin reductase is a key factor in the oxidative stress response of

Lactobacillus plantarum WCFS1. Microbial Cell Factories 6, 29. doi:10.1186/1475-2859-6-29.

Shah, N.P., 2007. Functional cultures and health benefits. International Dairy Journal 17, 1262–1277.

Siezen, R.J., Tzeneva, V.A., Castioni, A., Wels, M., Phan, H.T.K., Rademaker, J.L.W., Starrenburg, M.J.C., Kleerebezem, M., Molenaar, D., van Hylckama Vlieg, J.E.T., 2010. Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. Environmental Microbiology 12, 758–773.

Stevens, M.J.A., Wiersma, A., de Vos, W.M., Kuipers, O.P., Smid, E.J., Molenaar, D., Kleerebezem, M., 2008. Improvement of *Lactobacillus plantarum* aerobic growth as directed by comprehensive transcriptome analysis. Applied and Environmental Microbiology 74, 4776–4778.

Titgemeyer, F., Hillen, W., 2002. Global control of sugar metabolism: a Gram-positive solution. Antonie Van Leeuwenhoek 82, 59–71.

Tosco, A., Siciliano, R.A., Cacace, G., Mazzeo, M.F., Caponea, R., Malorni, A., Leone, A., Marzullo, L., 2005. Dietary effects of copper and iron deficiency on rat intestine: a differential display proteome analysis. Journal of Proteome Research 4, 1781–1788.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., Maguin, E., 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 82, 187–216.

van Kranenburg, R., Marugg, J.D., van Swam, I.I., Willem, N.J., de Vos, W.M., 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Molecular Microbiology 24, 387–397.

Yousten, A.A., Johnson, J.L., Salin, M., 1975. Oxygen metabolism of catalase-negative and catalase-positive strains of *Lactobacillus plantarum*. Journal of Bacteriology 123, 242–247.

Zapun, A., Vernet, T., Pinho, M.G., 2008. The different shapes of cocci. FEMS Microbiology Reviews 32, 345–360.

Zomer, A.L., Buist, G., Larsen, R., Kok, J., Kuipers, O.P., 2007. Time-resolved determination of the CcpA regulon of *Lactococcus lactis* subsp. *cremoris* MG1363. Journal of Bacteriology 189, 1366–1381.

Zotta, T., Guidone, A., Tremonte, P., Parente, E., Ricciardi, A., 2012. A comparison of fluorescent stains for the assessment of viability and metabolic activity of lactic acid bacteria. World Journal of Microbiology and Biotechnology. doi:10.1007/s11274-011-0889-x.

Zwietering, M.H., De Koos, J.T., Hasenack, B.E., De Wit, J.C., van 'T Riet, K., 1991. Modeling of bacterial growth as a function of temperature. Applied and Environmental Microbiology 57, 1094–1101.