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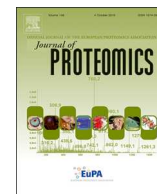
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Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*

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ABSTRACT

Propionibacterium freudenreichii is a beneficial bacterium used as a cheese starter and as a probiotic. Indeed, selected strains of *P. freudenreichii* combine both technological and health-promoting abilities. Moreover, during large-scale industrial production of dried bacteria and during consumption, *P. freudenreichii* may undergo different stressful processes. Osmotic adaptation was shown to enhance *P. freudenreichii* tolerance towards stresses, which are encountered during freeze-drying and during digestion. In this report, we compared the osmoadaptation molecular mechanisms of two *P. freudenreichii* strains. Both osmotolerance and osmoadaptation were strain-dependent and had different effects on multiple stress tolerance, depending on the presence of osmoprotectants. Availability of glycine betaine (GB) restored the growth of one of the two strains. In this strain, osmotic preadaptation enhanced heat, oxidative and acid stresses tolerance, as well as survival upon freeze-drying. However, addition of GB in the medium had deleterious effects on stress tolerance, while restoring optimal growth under hyperosmotic constraint. In the other strain, neither salt nor GB enhanced stress tolerance, which was constitutively low. Accordingly, whole cell proteomics revealed that mechanisms triggered by salt in the presence and in the absence of GB are different between strains. Osmotic adjustment may thus have deleterious effects on industrial abilities of *P. freudenreichii*.

Biological significance: Propionibacteria are found in various niches including fodder, silage, rumen, milk and cheeses. This means adaptation towards different ecological environments with different physicochemical parameters. *Propionibacterium freudenreichii*, in particular, is furthermore used both as dairy starter and as probiotic and is thus submitted to high scale industrial production. Production and subsequent stabilization still need optimization. Drying processes like freeze-drying are stressful. Osmotic adjustments may modulated tolerance towards drying. However, they are strain-dependent, medium-dependent and may either reduce or increase stress tolerance. A case-by-case study, for each strain-medium thus seems necessary. In this work, we identify key proteins involved in osmoadaptation and give new insights into adaptation mechanisms in *P. freudenreichii*. This opens new perspectives for the selections of strains and for the choice of the growth medium composition.

1. Introduction

Propionibacterium freudenreichii belongs to the Actinomycetales order, and possesses the GRAS (Generally Recognized As Safe, USA, FDA) and QPS (Qualified Presumption of Safety, EFSA, European

Union) status [1,2]. *P. freudenreichii* is consumed in high amounts, mainly in Swiss-type cheeses, but also in functional probiotic food supplements. Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [3]. Modification of the life style, including Western diet (which is high in

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fat and protein and low in fruits and vegetables), leads to increased incidence of inflammatory bowel disease (IBD), of irritable bowel syndrome (IBS) and of antibiotic-associated diarrhea (AAD), in developed countries. This constitutes a public health concern [4,5]. Probiotics in fermented functional foods, in tablets or in capsules, may reduce ailments linked to some of these troubles, in conjunction with usual treatments [6].

P. freudenreichii is a promising probiotic bacterium. Indeed, its consumption leads to increased intestinal bifidobacteria population in human volunteers [7,8]. Several *P. freudenreichii* potent beneficial effects were reported, including the anti-inflammatory modulation of the gut mucosa in the context of inflammation [1,9]. Selected strains of *P. freudenreichii* indeed modulate gut inflammation via the induction of the immunomodulatory cytokine IL-10, which is mediated by S-layer proteins [10,11]. Efficacy of probiotics depend on the amount of live probiotic bacteria within a preparation, which often has to be optimized [12]. It also depends on probiotic bacteria survival during the digestion [2]. The international Dairy Federation (IDF) recommends a minimum of 10^7 live probiotic bacterial cells per gram or milliliter of product at the time of consumption [13]. To produce tablets or capsules, the drying process and the probiotic product formulation require optimization, in order to keep alive the maximum of probiotic bacteria per gram during drying and storage. Two drying processes can be used to stabilize bacteria: freeze-drying and spray-drying. The freeze-drying process is a well-known process, the most used, and it allows drying of bacteria with a high viability [14]. However, spray-drying requires less energy, with a higher productivity, compared to freeze-drying, thus offering a promising alternative [15–17]. These two processes are stressful, freeze-drying imposing osmotic and cold stresses, whereas spray-drying imposes thermal, osmotic and oxidative stresses [18]. Ingested probiotic bacteria are submitted to the digestion process and should reach the colon alive, thus surviving acidic conditions of the stomach and presence of enzymes and bile salts in the intestine [19]. Furthermore, osmolality within the gastrointestinal tract is constantly changing as a result of water absorption [20].

Depending on bacterial species and strains, stress adaptation may lead to increased viability during drying [17,21–24], acid stress [25,26], and bile salts stress [27,28], thanks to cross protection. During osmotic stress, general stress proteins are overproduced [20,28,29]. Chaperones and proteases limit the impact of deleterious aggregation of denatured proteins [30]. During osmoadaptation, bacteria accumulate compatibles solutes [31], which can be imported inside the cell, or synthesized de novo. Compatibles solutes may preserve turgent pressure and enable cell growth and division [32]. Bacteria like *L. plantarum* can accumulate glutamate during osmoadaptation [33,34]. Some authors reported that *P. freudenreichii* is able to accumulate trehalose and glycine betaine (GB) under osmotic constraint [16,35]. During osmoadaptation, *P. freudenreichii* can accumulate energy storage molecules like glycogen and polyphosphate, in a very rich growth medium [29]. This specific growth conditions seemed promising in the aim to produce more resistant probiotic, as they enhanced *P. freudenreichii* resistance to acid, to bile salts stress and to spray-drying [16,36]. Osmoadaptation depending on both the strain and the culture medium, a screening is required to optimize probiotic products and to understand the effects of growth conditions. In this report, we investigated benefits and drawbacks of osmotic adjustment in two different strains of *P. freudenreichii*.

2. Materials and methods

2.1. Strains and pre-culture

Propionibacterium freudenreichii CIRM-BIA 129 (equivalent ITG P20) and CIRM-BIA 1025 (equivalent ITG P1) were provided, stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt

Table 1

Composition of the MMO (Medium Minus Osmoprotectants).

Constituent	Final concentration
Sodium lactate	12.8 g/L
KH ₂ PO ₄	0.6 g/L
Potassium acetate	0.4 g/L
MgSO ₄ ·7H ₂ O	50 mg /L
MnSO ₄ ·4H ₂ O	5 mg/L
FeSO ₄ ·7H ₂ O	2.5 mg/L
CuSO ₄	2.5 mg/L
Cobalt acetate	0.25 mg/L
ZnSO ₄	15 µg/L
H ₃ BO ₃	1 µg/L
Na ₂ MoO ₄	1 µg/L
Thiamine	50 µg/L
Pyridoxal	100 µg/L
Calcium pantothenate	50 µg/L
Riboflavine	50 µg/L
Nicotinamide	100 µg/L
p-Aminobenzoic acid	10 µg/L
Biotine	4 µg/L
Folic acid	20 µg/L
Cyanocobalamine	2 µg/L
L-Ala	50 mg/L
L-Arg	160 mg/L
L-Asn	150 mg/L
L-Asp	250 mg/L
L-Cys	140 mg/L
Gly	80 mg/L
L-His	100 mg/L
L-Ile	180 mg/L
L-Leu	300 mg/L
L-Lys	220 mg/L
DL-Met	60 mg/L
L-Phe	170 mg/L
L-Ser	180 mg/L
L-Thr	150 mg/L
L-Trp	50 mg/L
L-Tyr	60 mg/L
DL-Val	480 mg/L
Adenine	5 mg/L
Guanine	5 mg/L
Uracile	5 mg/L
Xanthine	5 mg/L

Alimentaire, INRA, Rennes, France). The strains are routinely cultivated in yeast extract lactate (YEL) medium [37] in this study, they are also cultivated in MMO (Medium Minus Osmoprotectants). MMO is a synthetic medium which was derived from CdM describe previously [12], MMO medium has the same composition as CdM but glutamate, glutamine and proline, which are considered as potent sources of osmoprotectants, were removed. *P. freudenreichii* was grown at 30 °C without agitation under microphilic condition. Composition of MMO is detailed in Table 1.

2.2. Bacterial growth under osmotic stress

P. freudenreichii CIRM-BIA 129 and CIRM-BIA 1025 were grown under different condition: in YEL medium (0.429 osmol), in YEL medium with 0.9 M NaCl (YEL + NaCl, 1.958 osmol) and in MMO, MMO with salt 0.4 M (MMO + NaCl, 0.958 osmol) and MMO with 0.9 M NaCl and 1 mM of GB (MMO + NaCl + GB 0.960 osmol). 0.4 M NaCl is the highest salt concentration allowing *P. freudenreichii* growth in MMO medium. 0.9 M NaCl is the highest salt concentration allowing *P. freudenreichii* growth in YEL medium. The GB concentration used in the chemically defined MMO medium was 1 mM, reported as the optimal concentration in previous reports including the actinobacteria *Brevibacterium ammoniagenes* [38] and *Propionibacterium freudenreichii* [39]. Bacterial populations were followed by Optical Density (OD) at 650 nm.

2.3. Stress challenges

Heat, oxidative, bile salts and acid challenge were applied to cultures at the beginning of stationary-phase (when maximal OD was reached). Heat challenge was performed by placing 2 mL (in a 15 mL Falcon tube) of *P. freudenreichii* culture in a water bath at 60 °C for 10 min [28]. Oxidative challenge was applied by adding 1.25 mM of hydrogen peroxide (Labogros, France) to 2 mL of *P. freudenreichii* culture during 1 h at 30 °C [40]. Acid challenge was applied by re-suspending *P. freudenreichii* in MMO medium adjusted to pH 2.0 by using HCl at 30 °C followed by a 1 h incubation [36]. Bile salts challenge was performed by adding 1 g·L⁻¹ of a bile salts mixture (an equimolar mixture of cholate and deoxycholate; Sigma Chemical, St. Louis, MO, USA) in the culture during 1 h at 37 °C [27]. CFU counting was performed after challenge. In order to calculate survival percentage, a CFU counting was made, with untreated culture left for the same time at 30 °C as a control.

2.4. Identification and quantification of osmoprotectants accumulated by *P. freudenreichii* CIRM-BIA 129

2.4.1. Extraction of accumulated osmoprotectants

P. freudenreichii CIRM-BIA 129 was grown in MMO, MMO + NaCl and MMO + NaCl + GB. During exponential phase (OD = 0.8), cells were harvested by centrifugation (8000g, 10 min). Cells were washed twice in a NaCl solution with the same osmolality than the culture medium. Cells were then re-suspended in 2 mL of distilled water, then 8 mL of absolute ethanol were added. The suspension was homogenized and centrifuged (8000g, 10 min) in order to remove cell fragments. The supernatant extract was evaporated during 7 h with a rotary evaporator. Dried extracts were then solubilized in deuterium oxide (Sigma-Aldrich, USA).

2.4.2. NMR (Nuclear Magnetic Resonance) analyses

All ¹H and ¹³C NMR spectra were recorded at 298 K on a Bruker Avance 500 spectrometer equipped with a 5 mm TCI triple-resonance cryoprobe (PRISM core facility, Rennes). ¹H spectra were acquired with a 6 kHz spectral width, 32 K data points and a total repetition time of 6.73 s. ¹³C spectra were acquired using a proton power-gated decoupling sequence with a 30° flip angle, a 30 kHz spectral width, 64 K data points and a total repetition time of 3.08 s. The data were processed with Topspin software (Bruker Biospin). Before applying the Fourier transform, free induction decays of ¹H spectra were treated with an exponential broadening of 0.3 Hz.

Samples were solubilized in D₂O. 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP-d₄) (Sigma-Aldrich, USA) served as an internal reference for ¹H and ¹³C chemical shifts.

Relative concentration of trehalose, glutamate and glycine betaine in the samples was determined by integration of their ¹H signals. Results are expressed as NMR relative units (RU).

2.5. Label free proteomics

2.5.1. Whole-cell protein extraction and protein tryptic digestion

The label free proteomics was have been conducted as Huang et al. 2018 [41]. At the beginning of stationary phase, *P. freudenreichii* cells were harvested by centrifugation and washed twice with PBS buffer (NaCl 8 g·L⁻¹, KCl 2 g·L⁻¹, KH₂PO₄ 2 g·L⁻¹, Na₂HPO₄ 12H₂O 35.8 g·L⁻¹). Cell pellets were then re-suspended in lysis solution (lysis solution: 0.5 mL pH 7.5, 157 g Tris-HCl, 0.03 g SDS, 0.3 g DTT and 9.5 mL H₂O), with 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, USA). The solution was frozen for 1 h, then sonicated (2 min 30 Hz), and cells were broken using zirconium beads in the homogenizer (Homogénéisateur Precellys Evolution – Bertin Instruments, France). The resulting SDS extracts were recovered by centrifugation (21,000 × g; 20 °C; 20 min) and then cleaned and quantified using the

two-dimensional (2-D) clean-up kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the 2-D quant kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), respectively. Tryptic digestion was performed on 100 µg of whole-cell proteins from each sample during 15 h at 37 °C using Sequencing Grade Modified Trypsin (Promega, Madison, USA) according to the manufacturer's instructions and as described previously [41]. Spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma-Aldrich, USA) was added in order to stop the digestion.

2.5.2. Nano-LC-MS/MS

Experiments were performed as previously described [41]. Experiments were performed using a nano RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nano-electrospray ion source. A preliminary sample concentration step was performed on a C18 pepMap100 reverse phase column (C18 column, 300-µm inner diameter (i.d.), 5 mm length, 5 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands). Peptides separation was performed on a reversed-phase column (PepMap 100 C18, 75 µm i.d., 250 mm length, 3 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) with a column temperature of 35 °C, using solvent A (2% (v/v) acetonitrile (Honeywell, USA), 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water). Peptides were separated using a gradient of 5 to 35% solvent B over 80 min followed by 35 to 85% solvent B over 5 min at a flow rate of 0.3 µL/min. Eluted peptides were directly electro sprayed into the mass spectrometer operating in positive ion mode with a voltage of 2 kV. Spectra were recorded in full MS mode and selected in a mass range 250–2000 m/z for MS spectra with a resolution of 70,000 at m/z 200. For each scan, the ten most intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at m/z 200 and the parent ion was subsequently excluded from MS/MS fragmentation for 20 s. The instrument was externally calibrated according to the supplier's instructions.

2.5.3. Protein identification

Proteins identification was performed as previously described [41]. Peptides were identified from the MS/MS spectra using X!Tandem pipeline software (Langella et al., 2017). The search was performed against a database composed of proteomes of strains *P. freudenreichii* CIRM-BIA 129 and CIRM-BIA 1025 (downloaded from NCBI.nlm.nih.gov on the 23rd of August 2018). Database search parameters were specified as follow: trypsin cleavage was used and the peptide mass tolerance was set to 10 ppm for MS and 0.05 Da for MS/MS. Oxidation of methionine and phosphorylation of threonine, serine and tryptophan were selected as a variable modification. For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation.

2.5.4. Protein quantification

Protein quantification was performed as previously described [41]. Every peptide identified by tandem mass spectrometry was quantified using the free MassChroQ software (Valot et al., 2011) before data treatment and statistical analysis within the R software (R 3.2.2, Project for statistical computing). A specific R package called 'MassChroQR' was used to automatically filter dubious peptides for which standard deviation of retention time was superior to 40 s and to regroup peptide quantification data into proteins. Two different and complementary methods of analysis were used, based on peak counting or XIC (extracted Ion Current). For peak counting analysis, variance analysis was performed on proteins with a minimum peak ratio of 1.5 between both culture conditions. Proteins with an adjusted p-value < .05 were considered significantly different. For XIC based quantification, normalization was performed to take into account possible global quantitative variations between LC-MS runs. Peptides shared between

different proteins were automatically excluded from the data set as well as peptides present in < 80% of samples. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein [42]. Analysis of variance was used to determine proteins with significantly different abundance between our two culture conditions.

2.6. Freeze-drying

P. freudenreichii strains were grown in the three different growth media. At the beginning of the stationary phase, cultures were harvested (8000g, 10 min, 30 °C). Pellets were then homogenized in a maltodextrin solution (100 g/L-1) (Roquette, France). The bacterial solutions were then freeze-dried (2253-04, Serail, France).

2.7. Statistical analysis

The data were from triplicate samples. All the results are presented as mean value with standard deviation. Statistical significance was set at $p < .05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows).

3. Results

3.1. *P. freudenreichii* growth in hypertonic media is strain-dependent

The growth of *P. freudenreichii* CIRM-BIA 129 and 1025 was analyzed in YEL medium containing increasing NaCl concentrations, or in MMO supplemented or not with 0.4 M NaCl and 1 mM GB (Fig. 1). The CIRM-BIA 129 strain was able to grow in the rich YEL medium supplemented by NaCl, up to 0.9 M, with a final OD close to the control without NaCl. High salt concentrations reduced significantly the growth rate (Fig. 1A). In the same conditions, the CIRM-BIA 1025 growth in YEL 0.4 M NaCl was similar to that of CIRM-BIA 129 but was completely inhibited in the presence of 0.9 M of NaCl, indicating that this strain is more susceptible to osmotic stress (Fig. 1B). In the chemically defined MMO medium, the two strains displayed a similar growth profile. With the addition of 0.4 M NaCl, both strains had a limited growth with a reduced finale OD and growth rate (Fig. 1C,D), while no growth occurred for both strains in the presence of 0.9 M NaCl (data not shown). Addition of GB partially restored growth of *P. freudenreichii* CIRM-BIA 129 in salted MMO. By contrast, growth of CIRM-BIA 1025 was similar despite the presence or not of GB (Fig. 1D).

3.2. Osmoprotectants accumulation in *P. freudenreichii* is strain-dependent

Intracellular accumulation of osmoprotectants was quantified by NMR analysis. *P. freudenreichii* CIRM-BIA 129 grown in MMO + 0.4 M NaCl accumulated trehalose (62.3 RU) and glutamate (37.7 RU) (Fig. 2). The strain CIRM-BIA 1025 accumulated the same compounds with nearly the same proportion, 60.9% and 39.1%, respectively. In the presence of glycine betaine, the CIRM-BIA 129 strain accumulated GB as the bulk majority of intracellular osmoprotectants (89.5 RU), with only limited amounts of glutamate (8 RU) and nearly no trehalose (2.5 RU). In the same conditions, the CIRM-BIA 1025 strain accumulated more diverse compatible solutes, with much less GB (29.4 RU) and a majority of trehalose (46 RU), while glutamate (24.5 RU) was also accumulated. These results suggest that both strains possess GB uptake system. However, the replacement of endogenous osmolytes by GB, classically observed in many bacteria, is only effective for CIRM-BIA 129.

3.3. Multiple stress tolerance of *P. freudenreichii* is strain- and medium-dependent

P. freudenreichii viability was monitored by numeration, before and

after stress challenges, in order to determine viability. We selected a series of stresses relevant to both technological and digestive processes. CIRM-BIA 129 tolerance towards heat, acid and oxidative lethal challenges was higher, when grown in salted MMO medium, compared to control MMO (Fig. 3A, B, C). This evidences osmotically induced multitolerance. It depends on the stress applied since the addition of NaCl, in the MMO medium, decreased CIRM-BIA 129 tolerance towards a bile salts lethal challenge, compared to control MMO (Fig. 3D). With the addition of GB, the viability of the CIRM-BIA 129 strain decreased for all lethal challenges, compared to MMO + NaCl medium. This indicates abrogation of osmotically induced multitolerance, as a result of GB accumulation. By contrast, neither the addition of NaCl, nor that of GB, had any impact on *P. freudenreichii* CIRM-BIA 1025 viability after heat challenge (Fig. 3E). These additions even decreased the viability after oxidative and acid challenges in this strain (Fig. 3F, G, H). Then, the response of 1025 and 129 are clearly different despite the accumulation of osmoprotectants, when submitted to osmotic stress.

3.4. Modulation of protein expression during osmoadaptation

To understand the difference observed between these two strains, we realized a proteomic differential analysis. We focused on proteins that were differentially express for at least one condition between MMO, MMO + NaCl and MMO + NaCl + GB, for *P. freudenreichii* CIRM-BIA 129 or for *P. freudenreichii* CIRM-BIA 1025, with a minimum ratio of 1.5 or < 0.66. A total of 387 proteins were significantly modulated (See Supplemental Table). Among these, 58 proteins were involved in energy production, conversion or carbohydrate and transport (Table 2), 53 were involved in amino acid metabolism and transport (Table 3) and 35 in post-translational modification, protein turnover, chaperone function (Table 4).

A PCA analysis was performed on the abundances of the 1362 propionibacterial proteins that were detected in this proteomic investigation (Fig. 4A). It revealed that *P. freudenreichii* CIRM-BIA 129 displayed three different profiles, according to the culture conditions. By contrast, CIRM-BIA 1025 only displayed two different profiles: one for the MMO medium, while the second profile was the same for the MMO + NaCl and for the MMO + NaCl + GB culture media (Fig. 4A). Salt addition strongly affected cellular proteome in both strains. Indeed, Fig. 4A clearly shows a shift of the global proteome towards increased values of the Axis 1, yet reduced values of the Axis 2, whatever the strain. However, glycine betaine addition further changed *P. freudenreichii* CIRM-BIA 129 global proteome by partially restoring the initial value of Axis 1. By contrast, glycine betaine addition add little or no effect on CIRM-BIA 1025 global proteome. This is consistent with the absence of effect of GB on CIRM-BIA 1025 growth. The corresponding heatmap (Fig. 4B) further evidences three very different proteomes in the CIRM-BIA 129 strain, while only two proteomic patterns (MMO + NaCl and MMO + NaCl + GB) are very close concerning the CIRM-BIA 1025 strain. We then selected 321 differential proteins exhibiting ratios below 0.66 or above 1.5. The corresponding Venn diagram, shown in Fig. 4C, further indicates strain-dependent proteomic readjustments. Among the 321 regulated proteins, only 60 were regulated in both strains. By contrast, 134 proteins were exclusively regulated in CIRM-BIA 129 and 127 exclusively in CIRM-BIA 1025. In CIRM-BIA 129, a high number of stress proteins was detected in the presence of salt without GB. By contrast, in CIRM-BIA 1025, a high number of stress proteomics were observed, whatever the presence or absence of GB.

When looking deeper into the expression of specific proteins, crucial differences, between strains, were evidenced, such as the induction of osmoprotectant uptake OpuA in CIRM-BIA 129, yet not in CIRM-BIA 1025, as a result of salt addition. This confirmed different behaviors of these strain. Furthermore, looking at proteins involved in energy production and conversion or carbohydrate and transport (Table 2), many adjustments were observed in *P. freudenreichii* CIRM-BIA 129, yet not in

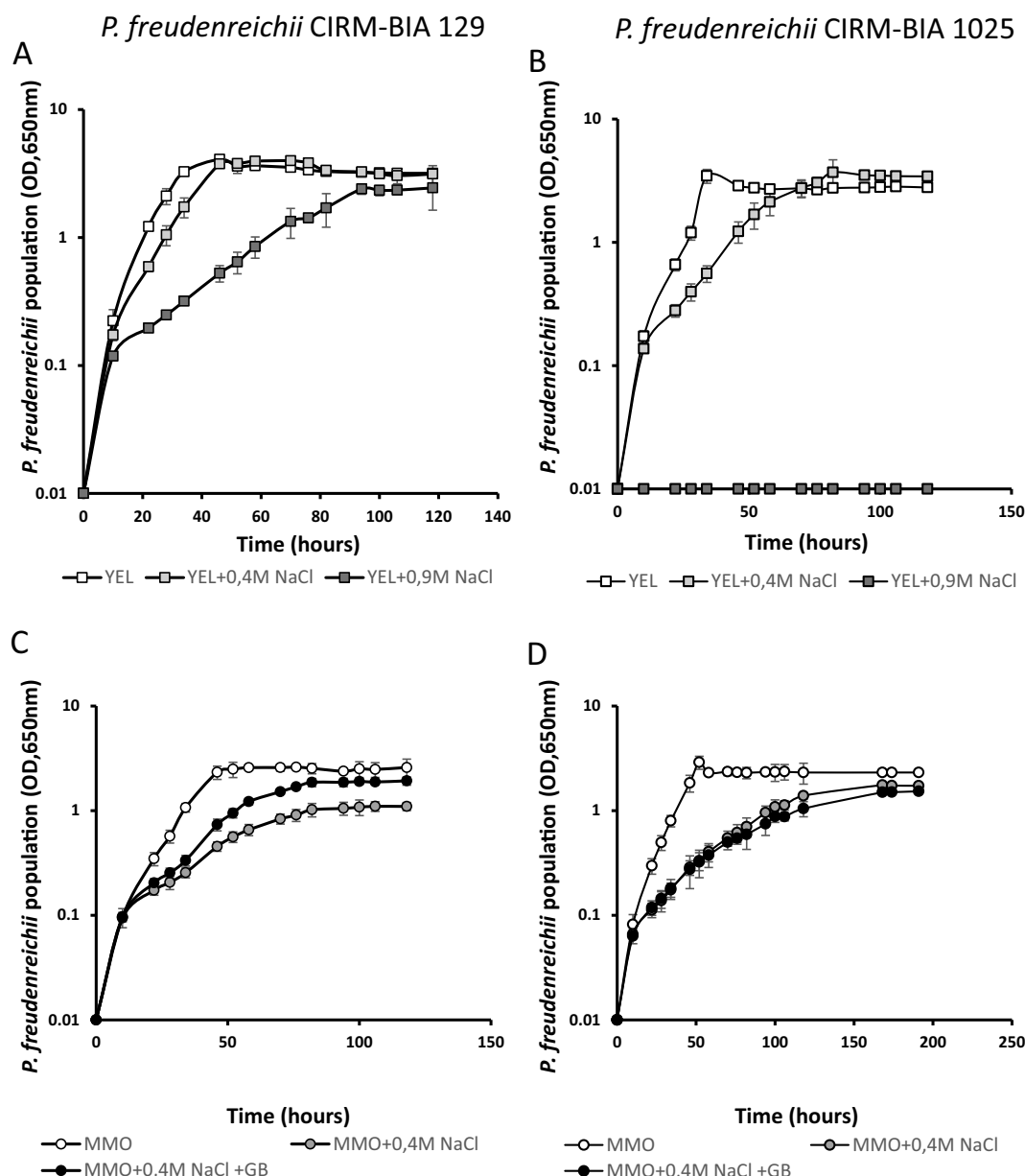


Fig. 1. *P. freudenreichii* growth in the presence of salt is strain-dependent.

P. freudenreichii CIRM-BIA 129 (A & C) and CIRM-BIA 1025 (B & D) were cultivated in YEL medium (A & B) with different salt concentrations (0, 0.4, and 0.9 M of NaCl) or in chemically-defined MMO medium, with or without salt (0.4 M NaCl), and with or without 1 mM glycine betaine (C & D).

the strain CIRM-BIA 1025. As an example, proteins involved in ATP synthesis (ATP synthase F1 sector, ATP synthase delta and B chain), in electron transfer chain (electron transfer flavoprotein, cytochrome *d* ubiquinol oxidase, oxidoreductase), and in carbohydrate utilization (enolase, galactokinase, beta-galactosidase), were modulated by NaCl addition, and restored by GB addition in the CIRM-BIA 129 strain, yet not in CIRM-BIA 1025 strain. By contrast, some modulations were specific of the strain CIRM-BIA 1025, such as the repression of propionic fermentation enzyme (methylmalonyl-CoA carboxytransferase 5S subunit) or induction of proteins involved in oxidative stress remediation (nitroreductase, aldo-keto reductase). About proteins involved in amino acid metabolism and transport (Table 3), transporters of osmoprotectants and/or of aminoacids were induced in the presence of NaCl, yet not in the presence of NaCl + GB, including betaine/carnitine/choline ABC transporter ATP-binding protein opuCA of glycine and betaine/carnitine/choline ABC transporter, while one involved in polar aminoacids (polar amino acid ABC transporter, binding protein

component) was repressed, in CIRM-BIA 129. These modulations did not occur in *P. freudenreichii* CIRM-BIA 1025. Proteins involved in osmoprotectants neosynthesis were also regulated in *P. freudenreichii* CIRM-BIA 129 (glutamine synthetase, glutamate deshydrogenase, glutamate synthase large subunit), yet not in 1025. In 1025, by contrast, proteins involved in signal transduction and regulation (nitrogen regulatory protein P II and bifunctional PLP-dependent enzyme with beta cystathionase and maltose regulon repressor activities) were specifically induced.

Concerning proteins involved in post translational modification, protein turnover, chaperone function (Table 4), general stress adaptation proteins were specifically modulated in CIRM-BIA 129, i.e. induced in the presence of NaCl, yet not in the presence of NaCl + GB, including groES protein 2 and thiol peroxidase. Addition of GB further repressed groEL protein 1, groEL protein 2, which also participate in general stress response. A different set of stress proteins were specifically induced in CIRM-BIA 1025, including heat shock protein, groES protein 1

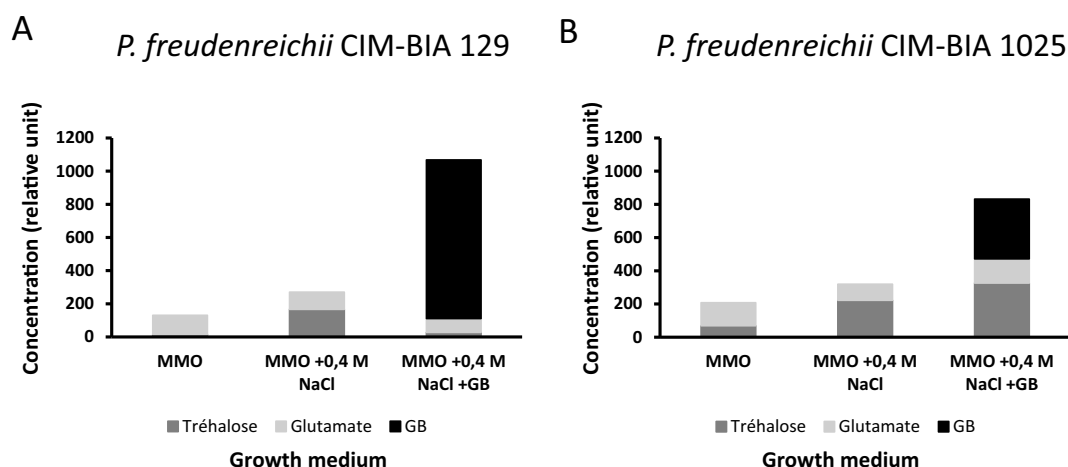


Fig. 2. *P. freudenreichii* compatibles solutes accumulation is strain and medium-dependent.

P. freudenreichii CIRM-BIA 129 (A) and CIRM-BIA 1025 (B) were cultivated in the different growth media described in Section 2.2. Cytoplasmic extract were made. Osmoprotectants were identified and quantified by NMR analysis in these extracts. Osmoprotectant accumulation are expressed as relative concentrations.

and chaperone protein Hsp20. Moreover, ATP-dependent protease Clp2, which is involved in misfolded proteins turnover, was induced by salt in both strains, but repressed by the addition of GB only in *P. freudenreichii* CIRM-BIA 129.

3.5. *P. freudenreichii* viability after freeze-drying

P. freudenreichii CIRM-BIA 129, when cultivated in MMO medium, displayed a low viability upon freeze-drying (3.3%) (Fig. 5). As previously shown for individual stress challenge (Fig. 3), viability was enhanced as a result of salt addition to this medium (9.4%). However, such improvement was reduced by the addition of GB, and the viability of *P. freudenreichii* CIRM-BIA 129 decreased to 6.8%. By contrast, the CIRM-BIA 1025 strain exhibited its best viability when cultivated in MMO medium (6.5%). With the addition of salt with or without GB, the *P. freudenreichii* CIRM-BIA 1025 viability decrease to 4%.

4. Discussion

4.1. Osmotic tolerance and adaptation is strain-dependent in *P. freudenreichii*

P. freudenreichii CIRM-BIA 129 and CIRM-BIA 1025 strains are both able to growth in the rich YEL medium with a low concentration of salt (0.4 M NaCl). The addition of a high concentration of salt (0.9 M NaCl) is more selective and allows growth of CIRM-BIA 129, yet not of CIRM-BIA 1025. This suggests that the rich YEL medium contains compatible solutes, which CIRM-BIA 129 would better use than CIRM-BIA 1025 does. The restoration of *P. freudenreichii* CIRM-BIA 129 growth in MMO + NaCl, as a result of GB addition, indicates a key role of GB in its osmoadaptation. By contrast, GB did not restore CIRM-BIA 1025 growth in MMO + NaCl. This suggested that GB is imported and accumulated by CIRM-BIA 129, yet not by CIRM-BIA 1025. This hypothesis was then shown to be false, as NMR evidenced accumulation of GB in both strains. However, GB levels were much lower in CIRM-BIA 1025 than in CIRM-BIA 129. This small accumulation had no positive impact on CIRM-BIA 1025 growth, in contrast with CIRM-BIA 129. Both strains accumulated glutamate, trehalose and glycine betaine. Glutamate accumulation in hyperosmotic conditions was already reported for *Lactobacillus plantarum* [32,33]. Trehalose is a key protectant molecule in *P. freudenreichii*, it can be accumulated during acid, cold, osmotic and oxidative treatments [29,35,43,44]. GB accumulation has already been observed during *P. freudenreichii* growth in hyper-concentrated rich

media [28], but its beneficial effect is shown here to be strain-dependent.

4.2. Benefits and drawbacks of osmoadaptation on cross-protection towards other stress are strain-dependent

NaCl adaptation enhanced *P. freudenreichii* CIRM-BIA 129 tolerance towards heat, oxidative and acid challenges, as well as towards freeze-drying. Restoration by GB in these hyperosmotic conditions however suppressed this cross-protection and even triggered hypersensitivity towards oxidative, bile salts, acid and freeze-drying challenges. Since GB replaces trehalose and glutamate accumulation inside the cells, we can suggest that these molecules could play a major role in cross protection [45]. By contrast, addition of salt failed to provide cross protection in *P. freudenreichii* CIRM-BIA 1025, and so did the addition of GB. Finally, osmoadaptation had a negative impact on tolerance of both strains towards freeze-drying (Fig. 5).

4.3. Proteomic patterns may explain differential adaptation and stress tolerance

Osmoadaptation triggered major modulations of cellular proteins' expression. Indeed, growth under hyperosmotic constraint and in the absence of osmoprotectant induced expression of many proteins involved in stress adaptation (ie osmoprotectant uptake systems) or reflecting metabolic readjustments. The heatmap in Fig. 4B and Venn diagram in Fig. 4C clearly evidence differences in terms of proteomics variations, among strains. Among the 321 stress proteins identified, only 23 were induced whatever the strain and medium. Such a discrepancy highlights the strain-dependence of osmoadaptation. Major proteome changes induced by stress in CIRM-BIA may explain the efficiency of osmoadaptation in this strain. This strain, which was shown to adapt hyperosmotic constraint and to transport GB, overexpressed proteins involved in carbohydrates transport and utilization, electron transfer chain and ATP synthesis (Table 2). Enolase, as an example, was previously shown to participate in multiple stress adaptation in *P. freudenreichii* [27] and in other food-grade bacteria [46]. This glycolytic enzyme is also a component of RNA degradosome, which is involved in RNA processing and gene regulation, in accordance with its role in stress tolerance acquisition in many bacteria [47]. Overexpression of ATPase subunits, allowing enhanced energy production, is a key parameter of adaptation, of intracellular homeostasis and of metabolic readjustments. It was already reported to be involved in acid adaptation

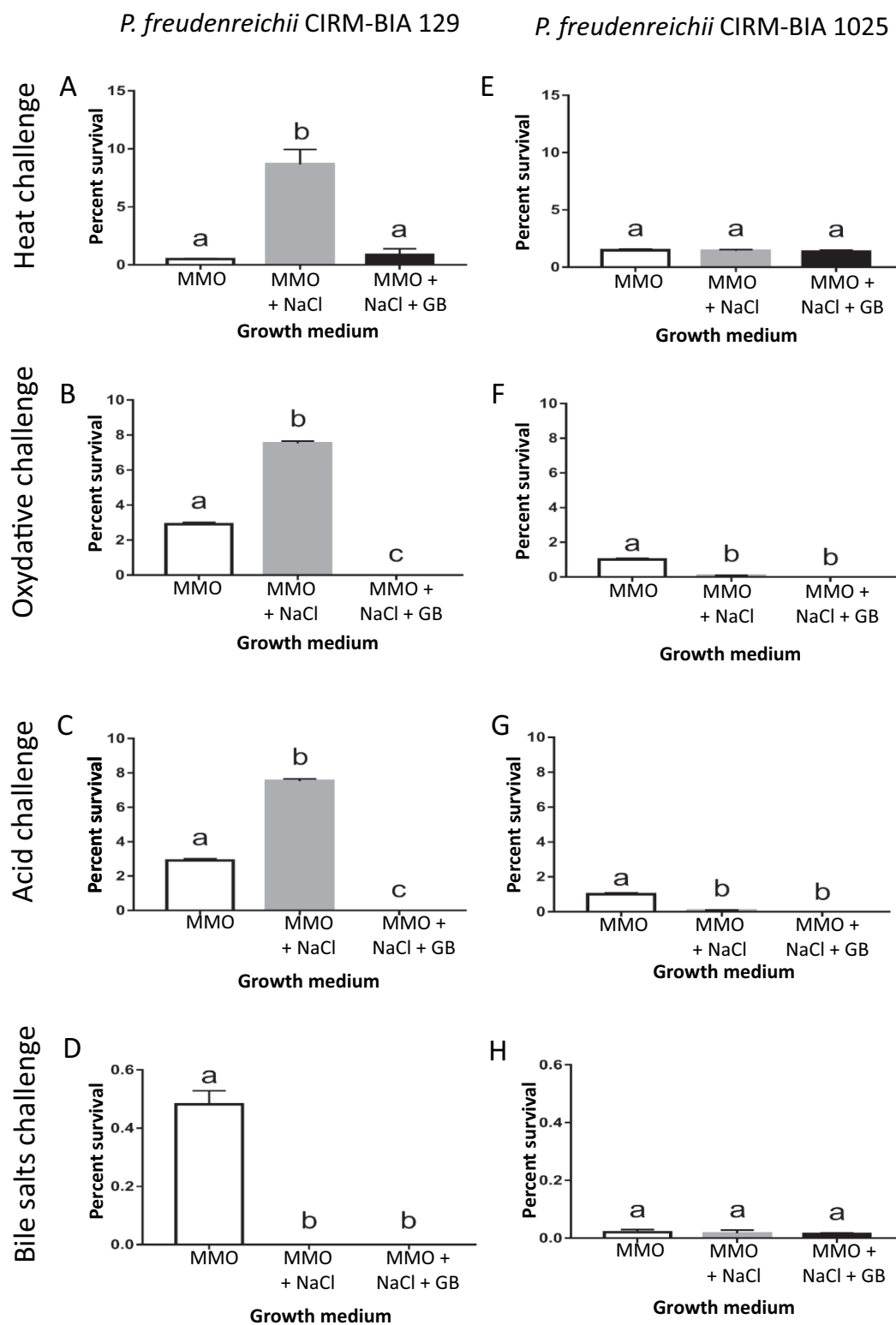


Fig. 3. Cross-protections conferred to *P. freudenreichii* are strain-dependent.

P. freudenreichii CIRM-BIA 129 (A, B, C, & D) and CIRM-BIA 1025 (E, F, G, & H) were cultivated until the beginning of stationary phase in three different growth media: MMO, MMO + NaCl and MMO + NaCl + GB. They were then subjected to heat (A & E, 60 °C for 10 min), oxidative (B & F, 1.25 M H₂O₂ for 1 h), bile salts (C & G, 1 g·L⁻¹ for 1 h) or acid challenges (D & H, pH 2.0 for 1 h) as described in materials and methods. Surviving propionibacteria were enumerated by CFU counting in treated and control samples. Results are expressed as percent of survival. Error bars represent the standard deviation for triplicate experiments. Significant difference are reported with different letters above the columns ($p > .05$).

Table 2
Salt and glycine betaine-mediated modulation of proteins belonging to the COG functional category C “energy production and conversion” or G “carbohydrate metabolism and transport”.

Accession	Description ^a	CIRM-BIA 129			CIRM-BIA 1025		
		MMO + NaCl/	MMO ^b	MMO + NaCl + GB/ GB/ MMO + NaCl ^d	MMO ^b	MMO + NaCl/ MMO ^c	MMO + NaCl + GB/ MMO + NaCl ^d
emb CDP49220.1	Phosphocarrier, HPr family	0.38	0.53	NS ^e	0.49	0.56	NS
emb CDP48824.1	Dihydroxyacetone kinase	0.38	NS	2.44	0.39	0.44	NS
emb CDP48902.1	Fructose-bisphosphate aldolase class I	0.41	NS	2.72	NS	NS	NS
emb CDP49440.1	Ferredoxin	0.43	0.45	NS	NS	NS	NS
emb CDP49591.1	Cytochrome d ubiquinol oxidase, subunit II	0.53	NS	2.10	NS	NS	NS
emb CDP49430.1	Glucose-1-phosphate adenylyltransferase	0.53	NS	2.12	0.51	0.57	NS
emb CDP49666.1	Coenzyme A transferase	0.54	NS	NS	NS	NS	NS
emb CDP48965.1	nucleoside-diphosphate-sugar epimerases	0.55	NS	NS	NS	NS	NS
emb CDP47837.1	Malate dehydrogenase	0.58	NS	NS	0.63	NS	NS
emb CDP49639.1	Gluconate kinase	0.59	NS	1.57*	0.63	NS	NS
emb CDP49605.1	3-carboxymuconate cyclase	0.60	0.63	NS	NS	NS	NS
emb CDP48837.1	Aldose 1-epimerase	0.60	NS	NS	NS	NS	NS
emb CDP48900.1	Citrate synthase	0.60	NS	1.50	0.53	0.55	NS
emb CDP47731.1	DhaK PTS-dependent dihydroxyacetone kinase, dihydroxyacetone-binding subunit	0.64	NS	1.65	NS	NS	NS
emb CDP48996.1	Galactokinase	0.64	NS	1.80	NS	NS	NS
emb CDP48782.1	Glycosyl hydrolase, family 13	0.65	NS	NS	NS	NS	NS
emb CDP49592.1	Cytochrome d ubiquinol oxidase subunit I	0.65	NS	NS	NS	NS	NS
emb CDP47594.1	Succinate dehydrogenase cytochrome B-558 subunit	0.66	NS	NS	NS	NS	NS
emb CDP48732.1	L-lactate dehydrogenase	0.66	NS	NS	NS	NS	NS
emb CDP47815.1	Pyruvate:ferredoxin oxidoreductase	0.66	NS	NS	NS	NS	NS
emb CDP47853.1	NADPH:quinone reductase related Zn-dependent oxidoreductase	NS	NS	NS	0.60*	NS	NS
emb CDP47989.1	Thiamine pyrophosphate (TPP family)	NS	NS	NS	1.56	1.54	NS
emb CDP48002.1	NADH:quinone oxidoreductase chain F	NS	NS	1.65	NS	NS	NS
emb CDP48006.1	NADH:quinone oxidoreductase chain B	NS	1.55	NS	NS	NS	NS
emb CDP48009.1	electron transfer oxidoreductase	NS	NS	1.61	NS	NS	NS
emb CDP48026.1	Zinc-containing alcohol dehydrogenase superfamily	NS	NS	NS	0.62	0.59	NS
emb CDP48084.1	FAD linked oxidase domain protein	NS	NS	1.67	NS	NS	NS
emb CDP48129.1	Xylulokinase protein, Carbohydrate kinase	NS	NS	1.64	0.61	0.66	NS
emb CDP48130.1	Glycerol-3-phosphate dehydrogenase	NS	1.93	2.07	NS	NS	NS
emb CDP48449.1	Succinate dehydrogenase, subunit B	NS	1.52	1.69	NS	NS	NS
emb CDP48511.1	Nitroreductase	NS	NS	NS	1.51	1.80	NS
emb CDP48893.1	Oxidoreductase	NS	1.58	NS	NS	NS	NS
emb CDP49268.1	Glycerol kinase	NS	NS	1.77	NS	NS	NS
emb CDP49349.1	Aldehyde dehydrogenase	NS	NS	1.73	NS	NS	NS
emb CDP49526.1	Beta-galactosidase	NS	NS	1.55	NS	NS	NS
emb CDP49788.1	Galactokinase	NS	NS	NS	1.74	1.82	NS
emb CDP49834.1	Ribose-5-phosphate isomerase 3	NS	NS	NS	2.14	1.99	NS
emb CEG97971.1	Inositol-1-monophosphatase	NS	NS	NS	0.58	0.62	NS
emb CEG98002.1	Putative aldo/keto reductase	NS	NS	NS	1.63	1.52	NS
emb CEG98016.1	Pyruvate phosphate dikinase	NS	NS	NS	0.52	0.60	NS
emb CEG99056.1	Phosphate acetyltransferase	NS	NS	NS	0.49	0.50	NS
emb CEG99057.1	Acetate kinase	NS	NS	NS	0.64	NS	NS
emb CEG98960.1	Methylmalonyl-CoA carboxytransferase 5S subunit.	NS	NS	NS	0.63	0.65	NS
emb CDP48804.1	ATP synthase B chain	1.61	1.75	NS	2.17	2.30	NS
emb CDP49578.1	Electron transfer flavoprotein-quinone oxidoreductase (FixC protein)	1.61	1.55*	NS	NS	NS	NS
emb CDP48974.1	Enolase 1	1.63	NS	0.60	NS	NS	NS
emb CDP49579.1	Electron transfer flavoprotein, carnitine metabolism (FixB protein)	1.63	NS	0.60	NS	NS	NS
emb CEG97844.1	ATP synthase F1 sector subunit beta	1.83	1.67	NS	NS	NS	NS
emb CDP48428.1	pyruvate dehydrogenase E1 component	1.85	NS	NS	NS	NS	NS
emb CDP48799.1	ATP synthase F1 sector epsilon subunit	1.85	1.70	NS	1.99	1.94	NS
emb CDP49189.1	Fructose-bisphosphate aldolase class II	1.88	NS	0.63	NS	NS	NS

(continued on next page)

Table 2 (continued)

Accession	Description ^a	CIRM-BIA 129		CIRM-BIA 1025			
		MMO + NaCl/ MMO ^b	MMO + NaCl + GB/ GB/ MMO + NaCl ^d	MMO + NaCl/ MMO ^b	MMO + NaCl + GB/ MMO ^c	MMO + NaCl + GB/ MMO + NaCl ^d	MMO + NaCl + GB/ MMO + NaCl ^d
emb CDP48899.1	Oxidoreductase	1,91	NS	NS	NS	NS	NS
emb CDP48803.1	ATP synthase delta chain	1,99	NS	NS	NS	NS	NS
emb CDP49838.1	Sulfite reductase	2,06	NS	NS	NS	NS	NS
emb CDP48122.1	Betaine-aldehyde dehydrogenase	0,59*	NS	NS	NS	NS	NS
emb CDP49431.1	glycosyltransferase	0,64*	NS	NS	NS	NS	NS
emb CDP49577.1	Ferredoxin-like protein fixX	2,06*	NS	NS	NS	NS	NS
emb CEG98306.1	sugar transporter, major facilitator superfamily	2,59*	NS	NS	NS	NS	NS

^aRatio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.
^b Determined by using UniProtKB database corresponding to ...
^c Ratio of the protein level in the MMO + NaCl medium compared to MMO medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl.
^d Ratio of the protein level in the MMO + NaCl + GB medium compared to MMO medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl + GB.
^e Ratio of the protein level in the MMO + NaCl + GB medium compared to MMO + NaCl medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl + GB.
^f Non significant.

in *P. acidipropionici* [48]. As another key difference between the two strains, CIRM-BIA 129 was able to import GB thanks to the ATP binding protein opuCA of the glycine betaine/carnitine /choline ABC transporter, which were induced here by salt (Table 3). This strain was already reported to overexpress opuCA in hyper concentrated sweet whey [28]. This induction explains the high GB accumulation in MMO + NaCl + GB (Fig. 2) and the restoration of growth (Fig. 1). Furthermore, CIRM-BIA 129 also overexpressed the binding protein of an ABC choline transporter, suggesting the ability to accumulate other osmoprotectants, when provided in the growth medium (Table 3). Salt further triggered over-expression of the GroES chaperone and of proteins involved in oxidative stress remediation (Table 4). By contrast, enzymes such as glutamine synthetase and glutamate dehydrogenase, which are susceptible to reduce intracellular compatible solute concentration, were repressed (Table 3). Finally, the overexpression of several proteins involved in stress adaptation is consistent with the enhanced tolerance towards stresses (Fig. 3) and towards freeze-drying (Fig. 5).

4.4. Glycine betaine modulates both stress proteome and resistance

P. freudenreichii CIRM-BIA 129 over-produced osmoprotectants transporters in MMO + NaCl. This over-production was however no more significant when GB was added, while GB restored growth (Table 3). Indeed, the absence of potent osmoprotectant in MMO + NaCl triggers the need for increased activity of such transporters. In many bacteria, such transporters are induced by the hyperosmotic constraint [49–51]. By contrast, once turgor pressure and growth are restored by GB, opuA expression is downregulated in response to the increase in the intracellular GB pool [49]. The “normalization” of the expression of many stress proteins, as a result of growth restoration by the availability of GB, is consistent with the loss of cross-protection towards stresses (Fig. 3) and towards freeze-drying (Fig. 5). Similarly, GB import limits Salmonella stress resistance, while salt adaptation without GB import leads to trehalose accumulation and to enhanced resistance in this bacterium [52]. Interestingly, the regulation of expression of the ATP-dependent stress protease Clp indicates that hyperosmolarity causes accumulation of misfolded proteins in both strains, and that GB-mediated restoration suppresses this intracellular stress in CIRM-BIA 129, yet not in CIRM-BIA 1025.

4.5. Salt stress proteome is strain-dependent

Our study clearly indicated that osmoadaptation is more efficiently induced by NaCl in CIRM-BIA 129 than in CIRM-BIA 1025. In hyperosmotic conditions, *P. freudenreichii* CIRM-BIA 1025, which failed to adapt to strong hyperosmotic constraint and to be protected by GB, overexpressed a different set of proteins. This included heat shock proteins Hsp20, which reflect intracellular macromolecules damages (Table 4). This also included proteins involved in intracellular detoxification such as aldo/keto reductase, as well as ribose-5-phosphate isomerase, which is involved in the pentose phosphate pathway, producing NADPH and remediating oxidative stress. Moreover, PLP-dependent cystathionase, with a cystathionine beta-lyase activity, which is involved in remediation of intracellular reactive metabolites spontaneously damaging macromolecules in *Salmonella* [53], was also induced by salt in CIRM-BIA 1025. Accordingly, PII nitrogen regulatory protein was strongly induced and plays a pivotal role in nitrogen stress remediation and stringent response [54,55]. Moreover, overexpression of chorismate mutase and of 3-phosphoshikimate 1-carboxyvinyl-transferase indicate induction of the shikimate pathway, which is used to protect against reactive oxygen species (ROS) generated by salt stress in plants [56]. Such over-expressions were not correlated with cross-protection of the strain CIRM-BIA 1025 towards challenge tests, in contrast with CIRM-BIA 129.

Table 3

Salt and glycine betaine-mediated modulation of proteins belonging to the COG functional category E “ amino acid metabolism and transport”.

Accession	Description ^a	CIRM-BIA 129			CIRM-BIA 1025		
		MMO + NaCl/ MMO ^b	MMO + NaCl + GB/ MMO ^c	MMO + NaCl + GB/ MMO + NaCl ^d	MMO + NaCl/ MMO ^b	MMO + NaCl + GB/ MMO ^c	MMO + NaCl + GB/ MMO + NaCl ^d
emb CDP48506.1	Alanine dehydrogenase	0,07	NS ^e	11,62	0,30*	0,32*	NS
emb CDP48687.1	Glycine cleavage H-protein (lipoate-binding)	0,38	0,46	NS	0,51	0,57	NS
emb CDP49124.1	Glutamine amidotransferase of anthranilate synthase or para-aminobenzoate synthase	0,45	NS	1,83	NS	NS	NS
emb CEH00437.1	Xaa-Pro aminopeptidase I	0,48	NS	1,78	NS	NS	NS
emb CDP48503.1	Amidohydrolase	0,53	NS	2,00	NS	NS	NS
emb CDP48646.1	anthranilate synthase component I	0,57	NS	NS	NS	NS	NS
emb CDP49409.1	Glutamine synthetase	0,59	1,51	2,56	NS	NS	NS
emb CDP47865.1	Cysteine synthase I	0,60	NS	NS	NS	NS	NS
emb CDP48633.1	Histidinol-phosphate aminotransferase	0,60	NS	NS	NS	NS	NS
emb CDP48419.1	4-aminobutyrate aminotransferase	0,61	NS	NS	NS	NS	NS
emb CDP49173.1	Dihydroxy-acid dehydratase	0,62	NS	NS	0,62	0,62	NS
emb CDP49134.1	L-serine dehydratase	0,63	NS	NS	0,62	0,62	NS
emb CDP48561.1	polar amino acid ABC transporter, binding protein component	0,65	NS	NS	NS	NS	NS
emb CDP49308.1	Cysteine desulphurases, SufS	1,66	NS	0,61	NS	NS	NS
emb CDP48772.1	3-isopropylmalate dehydratase small subunit	1,66	NS	0,62	NS	NS	NS
emb CDP49711.1	Nitrogen regulatory protein P-II	0,18*	2,16*	12,1*	4,41*	4,41*	NS
emb CEG99164.1	Alanine dehydrogenase	0,36*	NS	2,57*	0,25	0,30	NS
emb CEG98734.1	Glutamate synthase large subunit (Ferredoxin)	0,65*	NS	NS	NS	NS	NS
emb CDP49001.1	glutamate dehydrogenase	0,65*	NS	NS	NS	NS	NS
emb CEG99336.1	Aminopeptidase N, Lysyl aminopeptidase	NS	1,51	NS	NS	NS	NS
emb CDP49614.1	Argininosuccinate synthase	NS	1,70	1,65	NS	NS	NS
emb CEG97416.1	Protein of unknown function	NS	NS	NS	NS	0,50*	NS
emb CDP48480.1	Imidazole glycerol phosphate synthase subunit HisF	NS	NS	NS	1,50	NS	NS
emb CDP47871.1	Chorismate mutase	NS	1,60	NS	2,36	3,08	NS
emb CDP48779.1	3-isopropylmalate dehydrogenase	NS	0,64	NS	NS	NS	NS
emb CDP47561.1	N-acetyl-gamma-glutamyl-phosphate reductase	NS	NS	1,81	NS	NS	NS
emb CDP49606.1	Shikimate 5-dehydrogenase	NS	NS	NS	0,66	0,67	NS
emb CDP48345.1	Phosphoserine phosphatase/homoserine phosphotransferase bifunctional protein	NS	NS	NS	1,80	2,02	NS
emb CEG98743.1	Phosphoribosyl-AMP cyclohydrolase	NS	NS	NS	0,46	0,44	NS
emb CDP48360.1	3-phosphoshikimate 1-carboxyvinyltransferase	NS	NS	NS	1,52	1,60	NS
emb CDP48589.1	methionine synthase	NS	2,11	NS	NS	NS	NS
emb CDP47558.1	Acetylornithine and succinylornithine aminotransferase	NS	NS	1,75	NS	NS	NS
emb CEG99593.1	Aspartate ammonia-lyase (Aspartase)	NS	NS	0,63	NS	NS	NS
emb CDP47560.1	Arginine biosynthesis bifunctional protein ArgJ	NS	1,59	NS	NS	1,70*	NS
emb CDP48567.1	Bifunctional PLP-dependent enzyme with beta-cystathionase and maltose regulon repressor activities	NS	NS	NS	1,94	2,11	NS
emb CDP49050.1	Phosphoserine aminotransferase	NS	0,54	0,44	NS	NS	NS
emb CDP47562.1	Aspartate aminotransferase	NS	NS	NS	1,57	1,64	NS
emb CEG98065.1	Amino acid permease. Membrane protein	NS	NS	2,81*	NS	NS	NS
emb CEG99028.1	Amino acid permease-associated region. Membrane protein	NS	NS	NS	1,60	NS	NS
emb CDP49113.1	Propanediol utilization protein PduU	NS	NS	NS	NS	12,00*	NS
emb CEH00744.1	Thiamine pyrophosphate enzyme	NS	NS	NS	0,55	0,54	NS
emb CDP47931.1	solute binding protein of the ABC transport system	NS	0,63	NS	NS	NS	NS
emb CEG97415.1	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	1,65*	NS	NS	NS	NS	NS
emb CDP47878.1	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	1,68*	NS	NS	NS	NS	NS
emb CEG97413.1	binding protein of choline ABC transporter	2,15*	1,63*	NS	NS	NS	NS
emb CDP47876.1	binding protein of choline ABC transporter	2,34*	1,71*	NS	NS	NS	NS

*Ratio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.

^a Determined by using UniProtKB database corresponding to ...^b Ratio of the protein level in the MMO + NaCl medium compared to MMO medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl.^c Ratio of the protein level in the MMO + NaCl+GB medium compared to MMO medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl+GB.^d Ratio of the protein level in the MMO + NaCl+GB medium compared to MMO + NaCl medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl+GB.^e Non significant.

Table 4
Salt and glycine betaine-mediated modulation of proteins belonging to the COG functional category O “post translational modification, proteins turnover or chaperone functions”.

Accession	Description ^a	CIRM-BIA 129		CIRM-BIA 1025			
		MMO + NaCl/ MMO ^b	MMO + NaCl + GB/ MMO ^c	MMO + NaCl + GB/ MMO + NaCl ^d	MMO + NaCl/ MMO ^b	MMO + NaCl + GB/ MMO ^c	MMO + NaCl + GB/ MMO + NaCl ^d
emb CDP48273.1	Surface layer protein B	0.14	0.26	1.83	NS ^e	NS	NS
emb CDP48952.1	peptide-methionine (S)-S-oxide reductase	0.41	0.57	NS	0.47*	0.54*	NS
emb CDP48879.1	Thioredoxin	0.49	0.58	NS	NS	NS	NS
emb CDP48588.1	SppA, Periplasmic serine proteases	0.61	NS	NS	NS	NS	NS
emb CDP49020.1	Chaperone protein dnaJ 1	0.66	NS	NS	0.55	0.57	NS
emb CDP49048.1	Thioredoxin	0.67	NS	NS	NS	NS	NS
emb CDP47745.1	groES protein 2	1.51	NS	NS	NS	NS	NS
emb CDP49311.1	FeS assembly protein SufD	1.51	NS	NS	NS	NS	NS
emb CDP48411.1	SnpB SsrA-binding protein	1.71	NS	NS	NS	NS	NS
emb CDP47705.1	ATP-dependent Clp protease proteolytic subunit 2	1.78	NS	NS	1.54	1.61	NS
emb CDP49309.1	ABC-type transport system involved in Fe-S cluster assembly, ATPase component, SufC	1.85	NS	0.58	NS	NS	NS
emb CDP49617.1	thiol peroxidase	1.88	NS	0.59	NS	NS	NS
emb CDP49261.1	Secreted protein	0.57*	NS	NS	0.35*	0.41*	NS
emb CDP48858.1	Surface protein with SLH domain	0.61*	NS	NS	NS	NS	NS
emb CEH00247.1	Surface layer protein B	0.65*	NS	NS	NS	NS	NS
emb CDP47874.1	groEL protein 1	NS	NS	0.63	NS	NS	NS
emb CDP48789.1	Thioredoxin	NS	NS	0.65	NS	NS	NS
emb CEG99654.1	Heat shock protein 20 kDa 2	NS	NS	NS	1.56	NS	NS
emb CEG97258.1	Heat shock protein 20 kDa 1	NS	NS	NS	1.85	1.82	NS
emb CDP47875.1	groES protein 1	NS	NS	NS	1.96	1.94	NS
emb CDP47709.1	Trigger factor	NS	NS	0.66	NS	NS	NS
emb CDP47983.1	Alkyl hydroperoxide reductase subunit C	NS	NS	NS	1.78	1.79	NS
emb CDP49391.1	Probable peptidyl-prolyl cis-trans isomerase A	NS	0.65	NS	NS	NS	NS
emb CEH00703.1	Co-chaperone protein GrpE2	NS	NS	NS	1.91	2.08	NS
emb CDP49595.1	peptidyl-prolyl cis-trans isomerase	NS	0.51	0.64	NS	NS	NS
emb CEH01092.1	thiol peroxidase	NS	NS	NS	2.58	2.46	NS
emb CDP49795.1	Thioredoxin	NS	0.44	0.41	0.58	0.56	NS
emb CDP47990.1	Metalloprotease (Peptidase family M13)	NS	NS	NS	1.62	1.70	NS
emb CDP49400.1	HesB protein	NS	NS	NS	0.66	0.63	NS
emb CDP47657.1	Chaperone protein dnaJ 3	NS	NS	0.65	0.62	0.62	NS
emb CDP48201.1	peptidyl-prolyl cis-trans isomerase	NS	0.64	0.59	NS	NS	NS
emb CDP47586.1	Magnesium chelatase, subunit ChII	NS	NS	NS	3.59*	3.59*	NS
emb CDP49125.1	groEL protein 2	NS	NS	0.62	NS	NS	NS
emb CEG97257.1	Heat shock protein 20b 3 (20 kDa chaperone 3)	1.90*	1.90*	NS	NS	1.63	NS
emb CDP48339.1	Heat shock protein 20 kDa 3	1.97*	1.90*	NS	NS	NS	NS

*Ratio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.

^a determined by using a database composed of proteomes of strains *P. freudenreichii* CIRM-BIA 129 and CIRM-BIA 1025 (downloaded from NCBI.nlm.nih.gov on the 23rd of August 2018).

^b Ratio of the protein level in the MMO + NaCl medium compared to MMO medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl.

^c Ratio of the protein level in the MMO + NaCl + GB medium compared to MMO medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl + GB.

^d Ratio of the protein level in the MMO + NaCl + GB medium compared to MMO + NaCl medium. This ratio indicates induction (ratio > 1.5) or repression (ratio > 0.66) by NaCl + GB.

^e Non significant.

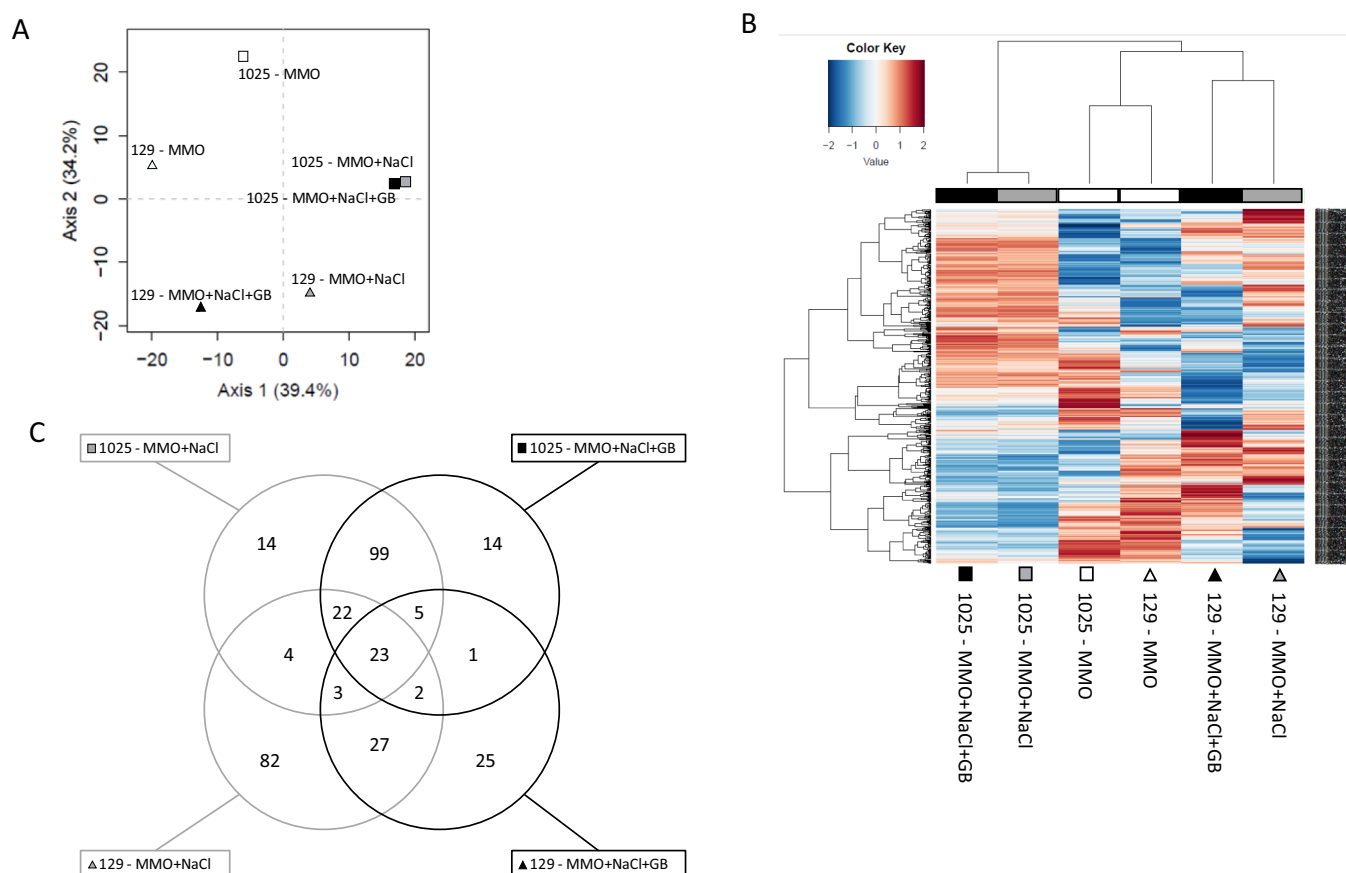


Figure 4

Fig. 4. Glycine betaine effect on the stress proteome is strain-dependent.

The PCA represents the distribution of the couple strain-growth medium, according to the protein abundances (A). Analyzed proteomes were extracted from *P. freudenreichii* CIRM-BIA 129 (square) or CIRM-BIA 1025 (triangle), cultivated in different growth medium: MMO (white), MMO + NaCl (grey), MMO + NaCl + GB (black). Proteins were extracted, identified and quantified as described in materials and methods. The heatmap (B) represents variations in relative abundance of all detected proteins, ranging from -2 (blue) to +2 (red).

The Venn diagram (C) represents the numbers of stress proteins exhibiting a modified expression in CIRM-BIA 129 or CIRM-BIA 1025, in presence of NaCl, with or without GB, compared to the control MMO medium (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

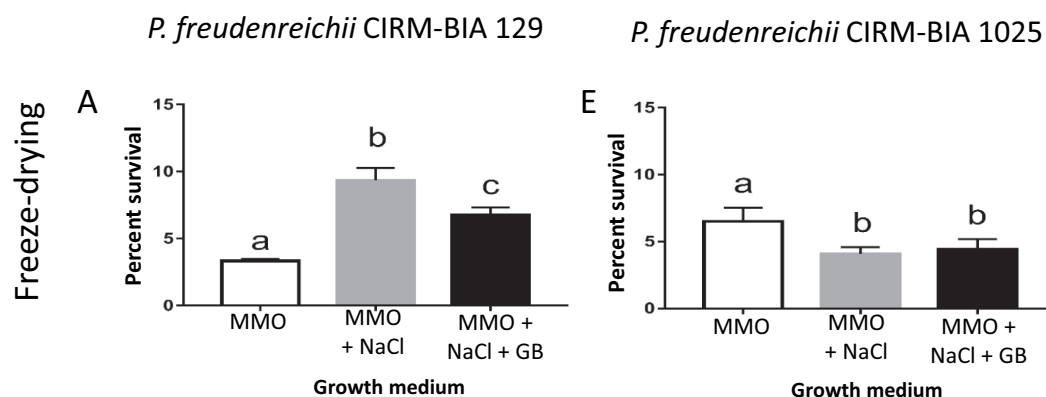


Fig. 5. Osmoadaptation modulates the viability of *P. freudenreichii* submitted to freeze-drying.

P. freudenreichii CIRM-BIA 129 and CIRM-BIA 1025 were cultivated until the beginning of stationary phase in three different growth medium: MMO, MMO + NaCl and MMO + NaCl + GB (A and B respectively). Propionibacteria were then freeze-dried as described in materials and methods. Results are expressed as percent survival. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p > .05$).

5. Conclusion

As a conclusion, this work highlights the strain-dependence of osmoadaptation, and of the resulting cross-protections, in *P. freudenreichii*. It confirms that strains selection is an important prerequisite to industrial production of bacteria. *P. freudenreichii* CIRM-BIA 129, which previously revealed a high probiotic potential, is able to adapt hyperosmotic conditions. Identification of relevant differential proteins provided new insights into strain-dependent osmotic adaptation. However, accumulation of GB, considered as universal osmoprotectant, has deleterious effects on stress tolerance in this strain. Finally, growth conditions constitute key parameters for freeze-drying optimization.

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Declaration of competing interests

The authors have no conflict of interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2019.103400>.

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