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Stress and osmoprotection in propionibacteria

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Abstract — *Propionibacterium* starters are subjected to many stresses during Swiss-type cheese manufacture. Among the phenomena which exert strong effects on *Propionibacterium* growth, osmotic stress influences the cell activity, the spatial cell distribution in the loaf and also drastically influences cheese ripening. In a chemically defined medium (M63), the generation time of *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 was doubled by 0.3 mol·L^{-1} sodium chloride and at 0.7 mol·L^{-1}, growth was almost totally inhibited. Growth in rich medium was more resistant to hyperosmotic conditions. ^{13}C Nuclear magnetic resonance (NMR) spectroscopy was used to investigate the intracellular solute pools in living cell suspensions and in hydroalcoholic extracts. Glutamate and trehalose were the main osmolytes found in the intracellular fraction of the cells in M63 containing 0.5 mol·L^{-1} NaCl. Addition of glycine–betaine, dimethylsulfoniopropionate or dimethylsulfonioacetate to this medium partially restored the growth. These efficient osmoprotectors were accumulated by the stressed cells. Moreover, glutamate and trehalose synthesis were also enhanced. In a rich medium, *P. shermanii* cells responded to changes in osmolarity by increasing the concentrations of specific solutes, and especially glycine–betaine, in order to maintain a constant turgor pressure. Implications of such behavior in cheese technology are discussed. © Inra/Elsevier, Paris.

***Propionibacterium* / trehalose / glycine–betaine / osmotic stress / NMR**

Résumé — Le stress osmotique et l'osmoprotection chez les propionibactéries. Au cours de la fabrication fromagère, les bactéries propioniques sont soumises à différents stress. Parmi ceux-ci figure le stress osmotique, résultat du saumurage, qui influence considérablement la croissance des cellules mais aussi leur distribution au sein de la meule et donc conditionne l'affinage du fromage. Dans un milieu chimiquement défini, le temps de génération de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 double en présence d'une concentration de chlorure de sodium de 0,36 mol·L^{-1}. À 0,7 mol·L^{-1} la croissance est totalement inhibée. Dans un milieu riche, les cellules sont plus résistantes à l'augmentation de la pression osmotique. Le tréhalose et le glutamate sont les prin-

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cipaux osmolytes accumulés lors d'une croissance en M63 + 0,5 mol·L^{-1} NaCl. Une addition de glycine–bétaïne, de diméthylsulfoniopropionate ou de diméthylsulfonioacétate au M63 restaure partiellement la croissance. Mais une addition de tréhalose n'apporte pas d'effet protecteur aux cellules. Une étude par résonance magnétique nucléaire du carbone 13 montre que ces osmolytes sont accumulés par les cellules. Lors de culture en milieu riche (YEL), les cellules de *Propionibacterium freudenreichii* subsp. *shermanii* accumulent de la glycine–bétaïne prélevée dans le milieu. Les implications potentielles du comportement de *Propionibacterium freudenreichii* subsp. *shermanii* en technologie fromagère sont discutées. © Inra/Elsevier, Paris.

***Propionibacterium* / tréhalose / glycine–bétaïne / stress osmotique / RMN**

1. INTRODUCTION

Propionic acid bacteria represent the main ripening flora of Swiss-type cheeses [15]. They are involved in lactate breakdown with propionate and acetate biosynthesis. The concomitant production of CO_2 is responsible for the formation of the expected eyes in the cheese body.

In cheese manufacture, *Propionibacterium* starters are subjected to high temperature shifts (up to 55 °C for 30 to 60 min) followed by a long cooling period (20 to 24 h), plus several variations during cold and warm room ripening. All these temperatures are far from the ideal growth temperature (30 °C). Moreover, H^+ ion evolution in the curd during lactic acid production and modifications in osmotic pressure caused by the immersion of the loaf in the brining solution for 48 to 72 h drastically influence cell viability and metabolism. In a study involving 36 French cheese factories, 80.5 % of the Emmental cheeses contained between 0.4 to 0.7 % NaCl with an average content of 0.58 % [4], but the salt distribution was far from homogeneous. The higher concentration was found in the rind (up to 1.8 %) and the diffusion was a slow process (incomplete after more than 60 d of ripening).

NMR spectroscopy is now widely used to study cellular physiology, energetics and carbon metabolism in microorganisms, in plant tissues and cell culture [22]. This technique is ideal for studying the accumulation of osmolytes in intact biological entities or in cell extracts.

Because of the economic importance of the Swiss-type cheese industry in France and in the world, we have initiated studies aimed at increasing our fundamental knowledge of the physiological and biochemical responses of *Propionibacterium freudenreichii* subsp. *shermanii* to osmotic, heat and pH stresses. Some responses to osmotic stress will be presented here.

2. MATERIALS AND METHODS

2.1. Organism and growth conditions

All the work was conducted with the type strain *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 grown either in Yeast Extract–Lactate medium (YEL; [21]) without Mn^{2+} to avoid paramagnetic effects (this omission had only a slight effect on cell growth) or in a chemically defined medium, M63 [23] or in reconstituted skim milk (100 g·L^{-1}). The pH was adjusted to pH 7.0 before sterilization (120 °C for 15 min for YEL and M63 and 110 °C for 15 min for the milk).

M63 was supplemented with a 6.7-g·L^{-1} solution of Bacto Yeast Nitrogen Base without amino acids (Difco, Osi, Paris, France) with 50 mmol·L^{-1} glucose (Sigma, Saint-Quentin Fallavier, France), previously sterilized by filtration using 0.2 µm cellulose acetate membranes (Sartorius, Palaiseau, France).

Incubation was performed, without shaking and pH regulation, at 30 °C in 1-L bottles. Stock cultures were maintained at –70 °C in a YEL medium containing 150 g·L^{-1} glycerol (Prolabo, Fontenay-sous-Bois, France).

2.2. Accumulation of labelled molecules

Transport and accumulation assays were accomplished with labelled molecules. [Me-^{14}C] dimethylsulfonioacetate (DMSA, 55 m Curie·mmol^{-1} was obtained from Isotopchim, France). [Me–^{14}C] glycine– betaine (55 mCurie·mmol^{-1}) was prepared from [1,2–^{14}C] choline (56 mCurie·mmol^{-1} obtained from CEA, France) using choline oxydase of *Alcaligenes* sp. (Sigma) according to the method of Ikuta et al. [16] and purified as described by Perroud and Le Rudulier [25]. [Me–^{14}C] dimethylsulfoniopropionate (DMSP) was synthesized from [carboxyl–^{14}C] acrylate. 20 µL of each labelled molecule was added to each culture. During growth, samples were removed and filtered and radioactivity was determined as already described [2].

For in vivo natural abundance ^{13}C-NMR experiments, cell suspensions, cell supernatant and extract supernatant analysis by ^{1}H and ^{13}C-NMR, and quantification of end products were conducted as previously described [8].

2.3. Cell extraction

Cells in the stationary phase were centrifuged (13 800 *g*, 10 min) and washed in an isotonic solution. The cell pellets were transferred into a glass tube with 80 % ethanol (grade reagent Merck, Nogent-sur-Marne, France) and stirred by vortex for 30 min at ambient temperature. They were evaporated to dryness by means of a rotary evaporator. The residues obtained were taken up in 0.5 mL D_2O (99.9 %D, Eurisotop, Saint Aubin, France) and stored at 4 °C until analysed.

2.4. Biomass measurements

Total cell biomass was determined as A_{650nm} measurements (Beckman, model DU 7400 spectrophotometer, Gagny, France).

3. RESULTS

3.1. Growth of *P. shermanii* in a defined medium with various NaCl concentrations

The development of *P. shermanii* in a chemically defined medium M63 with various amounts of sodium chloride is presented *figure 1*. The osmotic pressure developed by these culture media ranged from 0.06 to 1.44 osmol (kg H_2O)$^{-1}$. When the salt concentration increased in the medium, the growth rate and the total biomass produced decreased. A sodium chloride concentration of 0.3 mol·L^{-1} doubled the generation time of *P. shermanii* (14 to 30 h) and the final biomass level decreased by more than 83 %. At 0.7 mol·L^{-1} NaCl, growth was almost totally inhibited (*table I*).

In order to test if the observed effect was an osmotic effect or a salt effect, potassium chloride and sucrose were substituted for NaCl. The same growth inhibitions were obtained if KCl or the non-electrolyte sucrose was used in place of NaCl (data not shown).

3.2. Identification of solutes in osmotically stressed *P. shermanii* cells grown in M63

Hydroalcoholic extracts of stressed cells cultivated in M63 medium supplemented with 0.5 mol·L^{-1} NaCl and 100 mmol·L^{-1} lactate and non stressed cells (no NaCl in the growth medium) were analysed by ^{13}C {^{1}H}NMR spectroscopy. Trehalose was the most abundant compound for both stressed and unstressed conditions but its concentration increased by 28 % when the cells were stressed. Higher amounts of glutamate and alanine were also observed in the cells grown under hyperosmotic conditions.

3.3. Growth of *P. shermanii* in a rich medium with various NaCl concentrations

As observed with M63 medium, when the osmotic pressure was increased in the YEL medium (osmotic pressure in YEL alone was 0.35 osmol (kg H_2O)$^{-1}$), the growth rate and the total biomass produced decreased. But, to double the generation time of *P. shermanii*, a NaCl concentration of 0.79 mol·L^{-1} was needed and the final

biomass level decreased by 53 %. The addition of NaCl over 1.5 mol·L^{-1} in the classical medium of *Propionibacterium* totally inhibited the growth.

3.4. Accumulation of compatible solutes in osmotically stressed *P. shermanii* cells grown in YEL with 0.36 M NaCl

The intracellular concentration of glycine–betaine increased substantially as observed by in vivo ^{13}C-NMR spectra presented in *figure 2*. The areas of the proline and glutamate resonances increased only a little between 0 and 3.6 mol·L^{-1} NaCl. The characteristic resonances of trehalose C1 to C6 were not evident in vivo.

3.5. Growth of *P. shermanii* in milk with various NaCl concentrations

Addition of 0.25 mol·L^{-1} NaCl doubled the generation time of *P. shermanii* in reconstituted milk. Growth was almost completely inhibited at 1.0 mol·L^{-1} (data not shown).

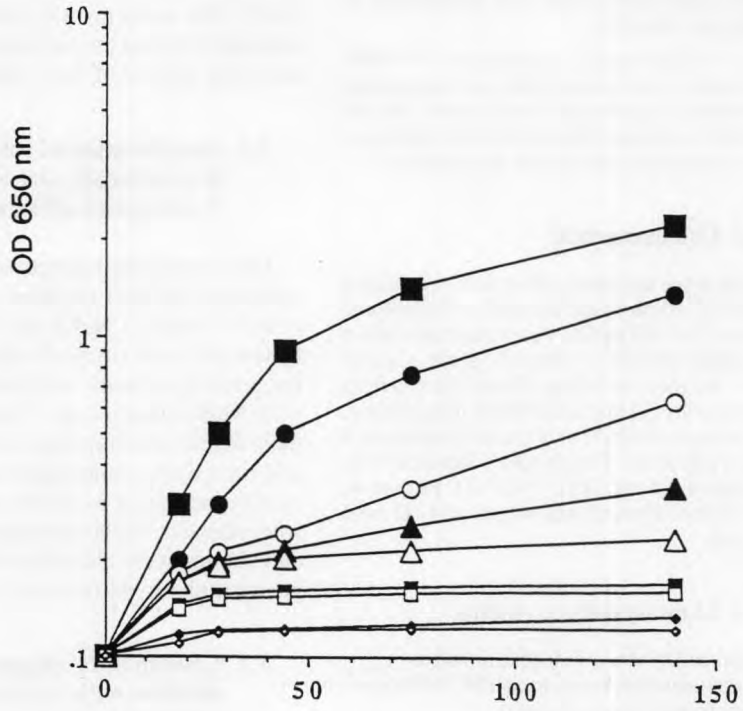

Figure 1. Effect of NaCl on growth (OD_{650}) of *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. Cells were grown at 30 °C in M63 medium without or with NaCl. ■ Without added NaCl; ● 0.1 mol·L^{-1} NaCl; ○ 0.2 mol·L^{-1} NaCl; ▲ 0.3 mol·L^{-1} NaCl; △ 0.4 mol·L^{-1} NaCl; ■ 0.5 mol·L^{-1} NaCl; □ 0.6 mol·L^{-1} NaCl; ◆ 0.7 mol·L^{-1} NaCl; ○ 0.8 mol·L^{-1} NaCl.

Figure 1. Effet de la concentration en chlorure de sodium sur la croissance de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. Les cellules étaient cultivées à 30 °C sur milieu M63. ■ sans addition de NaCl ; ● 0,1 mol·L^{-1} NaCl ; ○ 0,2 mol·L^{-1} NaCl ; ▲ 0,3 mol·L^{-1} NaCl ; △ 0,4 mol·L^{-1} NaCl ; ■ 0,5 mol·L^{-1} NaCl ; □ 0,6 mol·L^{-1} NaCl ; ◆ 0,7 mol·L^{-1} NaCl ; ○ 0,8 mol·L^{-1} NaCl.

Table I. Growth parameters for *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 grown in M63 with different NaCl concentrations.

Tableau I. Paramètres de la croissance de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 en milieu M63 en présence de différentes concentrations de NaCl.

NaCl molarity ($mol \cdot L^{-1}$)	Osmolarity (osmol ($kg\ H_2O$)$^{-1}$)	Generation time (h)	Final biomass ($OD_{650\ nm}$)
0	0.06	14 ± 0.7	2.10 ± 0.1
0.1	0.18	20 ± 0.9	1.40 ± 0.05
0.2	0.36	26 ± 1.1	0.65 ± 0.02
0.3	0.54	30 ± 1.1	0.35 ± 0.02
0.4	0.72	37 ± 1.5	0.25 ± 0.02
0.5	0.9	40 ± 1.7	0.17 ± 0.02
0.6	1.1	45 ± 1.7	0.16 ± 0.02
0.7	1.26	NC	0.12 ± 0.02
0.8	1.44	NC	0.12 ± 0.02

NC: Not calculable / NC : non calculable.

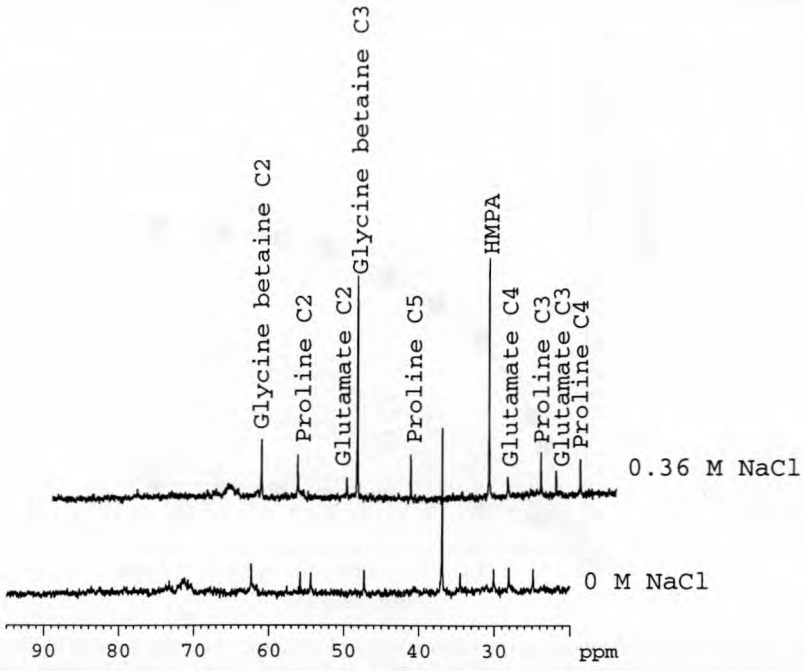

Figure 2. In vivo natural abundance ^{13}C {^{1}H}–NMR spectra of *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. The cells were grown in YEL and YEL supplemented with 0.36 $mol \cdot L^{-1}$ NaCl at 24 °C. The spectra were collected with 512 scans. HMPA, Hexamethyl–phosphoramide.

Figure 2. Spectres RMN ^{13}C {^{1}H} in vivo, en abondance naturelle de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. Les cellules étaient cultivées à 24 °C sur milieu YEL et YEL supplementé avec 0,36 $mol \cdot L^{-1}$ NaCl. Les spectres étaient réalisés par accumulation de 512 scans. HMPA, Hexaméthyl–phosphoramide.

3.6. Effect of exogenous compounds on growth of *P. shermanii* cells subjected to osmotic stress in M63

When the cells were cultivated in M63 in the presence of 0.5 mol·L^{-1} NaCl, the generation times increased from 14 to 40 h. But some compatible solutes added to the growth medium exerted a protective effect as observed in *figure 3*. The mostly efficient class of compounds was composed of dimethylsulfonioacetate (DMSA), glycine–betaine and dimethylsulfoniopropionate (DMSP) which allowed a decrease in the generation time to 26 ± 2 h. Carnitine and pipecolate exerted a slight protective effect on the growth rate (generation time of 35 ± 1 h) but allowed a substantial increase in the final biomass (*table II*). Even if carnitine and pipecolate concentrations in the medium were increased up to 10 mmol·L^{-1}, their efficiency remained low. The most inefficient group was composed of trehalose, proline, mannitol, ectoine, choline, taurine and sorbitol which caused little or no relief of the inhibition caused by NaCl.

3.7. Accumulation of compatible solutes in osmotically stressed *P. shermanii*

Hydroalcoholic extracts of cells cultivated in M63 medium with 0.5 mol·L^{-1} NaCl and glycine–betaine, DMSA or DMSP were analysed by ^{13}C-NMR spectroscopy (*figure 4*). In each case, the added osmoprotective agent was accumulated by the

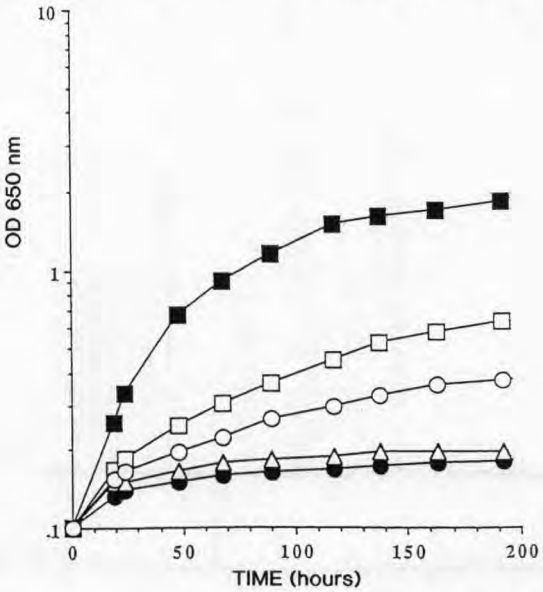

Figure 3. Effects of exogenous compatible solutes (1 mmol·L^{-1}) on the growth of *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. Cells were grown in M63 medium without ■ or with ● 0.5 mol·L–1 NaCl; addition to M63 medium + 0.5 mol·L^{-1} NaCl of: trehalose, △; dimethylsulfonioacetate, □; carnitine, ○.

Figure 3. Effets de l'addition dans le milieu de culture de composés osmoprotecteurs (1 mmol·L^{-1}) sur la croissance de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. ■ Les cellules étaient cultivées sur milieu M63 sans addition de NaCl. L'osmolarité du milieu M63 était augmentée par addition de 0,5 mol·L^{-1} NaCl. ● cellules cultivées sans addition d'osmolyte ; △ cellules cultivées en présence de trehalose ; □ cellules cultivées en présence de diméthylsulfonioacétate ; ○ cellules cultivées en présence de carnitine.

Table II. Growth parameters for *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 grown in M63 + 0.5 mol·L^{-1} NaCl with different osmoprotective agents.

Tableau II. Paramètres de la croissance de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027 en milieu M63 additionné de 0,5 mol·L^{-1} NaCl, en présence de différents osmoprotecteurs.

Growth conditions NaCl (mol·L^{-1})–osmolytes (1 mmol·L^{-1})			Generation time (h)	Final biomass (OD_{650})
0		0	14 ± 0.7	2.1 ± 0.1
0.5		0	40 ± 1.7	0.17 ± 0.02
0.5	+	Glycine–betaine	25 ± 1.1	0.65 ± 0.02
0.5	+	DMSP	24 ± 1.1	0.75 ± 0.02
0.5	+	DMSA	29 ± 1.1	0.67 ± 0.02
0.5	+	Pipecolate	35 ± 1.5	0.40 ± 0.02
0.5	+	Carnitine	36 ± 1.5	0.40 ± 0.02
0.5	+	Proline	40 ± 1.7	0.24 ± 0.02
0.5	+	Trehalose	39 ± 1.7	0.22 ± 0.02
0.5	+	Mannitol	39 ± 1.7	0.23 ± 0.02
0.5	+	Choline	42 ± 1.7	0.23 ± 0.02
0.5	+	Taurine	40 ± 1.7	0.24 ± 0.02
0.5	+	Ectoine	44 ± 1.7	0.24 ± 0.02
0.5	+	Sorbitol	39 ± 1.7	0.20 ± 0.02

DMSA, dimethylsulfonioacetate.
DMSP, dimethylsulfoniopropionate.

cells. Moreover, trehalose was also detected in these cells.

The rate of accumulation of osmoprotective agents was assessed using agents labelled with carbon 14. The maximum rates were detected after 24 h of growth. The highest rate was observed for glycine–betaine at 290 nmol·mg^{-1} cell dry weight. DMSP and DMSA were accumulated at 190 and 80 nmol·mg^{-1} cell dry weight, respectively.

3.8. Metabolism of choline by *P. shermanii*

When cells of *P. shermanii* were grown with ^{14}C–choline in M63 medium, the compound was incorporated into the intracellular soluble fraction but at a low level (18 % of the total initial radioactivity used). Moreover, this incorporation decreased when the osmotic pressure increased in the medium (only 7 % of the total initial radioactivity used at 0.3 and 0.5 mol·L^{-1} NaCl). When the soluble intracellular fraction of unstressed cells was studied by electrophoresis, more than 84 % of the radioactivity was found in the glycine–betaine spot; the remainder was in the choline spot. This percentage decreased when the salt concentration increased (down to 67 ± 2 %).

4. DISCUSSION

In the defined medium M63 used in the first part of this study, the halotolerance of *P. shermanii* was low, even if direct comparisons with other bacteria grown in different media are difficult to make. Moreover, we did not observe any stimulation of growth even at low salt concentration which contrasts with observations made for several other microorganisms including lactic acid bacteria [3]. The initial presence of other salts in M63 is probably the main reason for this absence of stimulation.

Though chemically defined media are necessary when complete knowledge of the

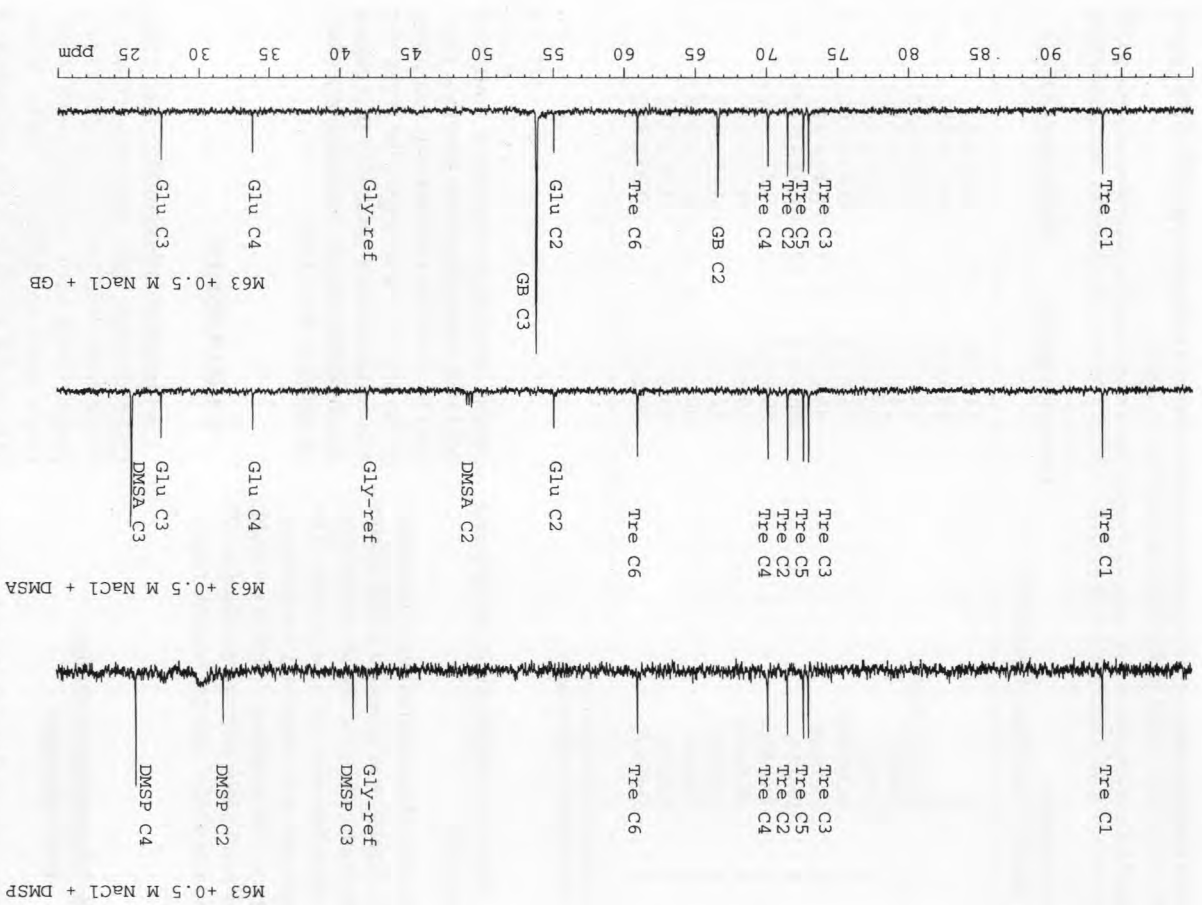

Figure 4. Accumulation of compatible solutes in osmotically stressed *P. shermanii*: ^{13}C {^{1}H}–NMR spectra of intracellular extract of *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. Cells were grown in M63 plus 0.5 mol·L^{-1} NaCl up to the stationary phase. Glycine (Gly) was used as internal reference. Abbreviations: DMSP, dimethylsulfoniopropionate; DMSA, dimethylsulfonioacetate; Tre, trehalose; Glu, glutamate; GB, glycine–betaine.

Figure 4. Accumulation d'osmolytes dans des cellules stressées de *Propionibacterium freudenreichii* subsp. *shermanii* CIP 103027. Les cellules ont été récoltées à la phase stationnaire. La Glycine (Gly) était utilisée comme standard interne à l'analyse. Abréviations : DMSP, diméthylsulfoniopropionate ; DMSA, diméthylsulfonioacétate ; Tre, Tréhalose ; Glu, Glutamate ; GB, Glycine–bétaïne.

composition is required, especially to be able to follow a tracer, care must be taken when correlations have to be done with real 'natural' growth conditions, like those prevailing in cheese. Salt tolerance in the rich YEL medium was clearly different than that in M63. Higher salt concentrations were needed to affect growth to the same extent. Moreover, the osmotic susceptibility of the cell growth in milk was intermediate between those in M63 and YEL (data not shown). The sensitivity of *Propionibacterium* to NaCl was previously demonstrated to be strain and pH–dependent [29]. Concentrations of NaCl as low as 0.5 % were sufficient to reduce the growth of some strains [24], while Antila [1] reported inhibition at 3 % NaCl.

The characterization of an organism's salt tolerance largely depends on the availability of protecting compounds and the presence of suitable uptake and/or biosynthesis systems. For example, the upper limits of salt tolerance for *Lactobacillus bulgaricus* in MRS [9] is 0.5 mol·L^{-1} while it is 1.5 mol·L^{-1} and 2.5 mol·L^{-1} for *L. plantarum* and *L. halotolerans*, respectively [18]. Then, *P. shermanii* is in the average susceptibility. Growth of *P. shermanii* in YEL under hyperosmotic conditions is allowed, at least, by the choline (37 mg·L^{-1}) and glycine–betaine (152 mg·L^{-1}) present in yeast extract. The glycine–betaine concentration in cow milk is unknown (but of < 2.3 mg·L^{-1} in human milk [5]) but choline average concentration in cow milk is 79 ± 7 mg·L^{-1} (0.66 mmol·L^{-1}) [10] and many choline derivatives such as phosphocholine, glycerophosphocholine, and phosphatidylcholine are probably available in milk [26]. Moreover, cow milk contains other potential osmoprotective molecules such as carnitine (10 to 17 mg·L^{-1}, 62 to 105 µmol·L^{-1}) [12]. To what extent propionibacteria can use them (each one or a mix) to enhance their osmotic tolerance is unknown. The question is also open for other bacteria including lactic acid bacteria.

The osmolarity of the aqueous phase of Emmental cheese was experimentally determined as 1.2 osmol (a_w of 0.98), which was equivalent to a NaCl concentration of 0.7 mol·L^{-1} [28]. At this concentration the growth of *P. shermanii* was totally inhibited in the defined M63 medium but not in YEL or milk. Probably, this aqueous phase of Emmental cheese, such as milk, contains several compounds able to 'protect' the cells against this osmotic pressure, and allows propionibacteria to grow. Accumulation of choline, glycine–betaine and trehalose by *P. shermanii* cells grown on the aqueous phase of Emmental has already been published [7].

Many microorganisms accumulate compatible solutes during periods of decreased water activity. These small organic compounds contribute to the osmotic balance of the extracellular environment, enhance the stability of enzymes and maintain the integrity of cellular membranes. These functions are related to the molecular structure of the compatible solutes, which all have specific characteristics in common. They have an overall neutral charge at physiological pH values and may combine polar and relatively hydrophobic moieties. Glycine–betaine was the major solute found in *Lactobacillus plantarum, L. bulgaricus, L. halotolerans* and *Enterococcus faecium* subjected to salt stress [18]. In addition, *L. plantarum* cell survival after air drying was also enhanced by such accumulation [17].

Even though *P. shermanii* exhibited a moderate resistance to osmotic pressure, it accumulated one or more intracellular solutes to fight against increased osmotic strength. ^{1}H-NMR analyses demonstrated the presence of increased concentrations of trehalose, glutamate and alanine in stressed cells grown in M63 without glycine–betaine. Nevertheless, these concentrations were low and the observed protective effect was weak. The biosynthesis of trehalose by propionibacteria has already been observed after cold shock [6] and growth in non-optimal

growth conditions [27]. Many organisms accumulate or synthesize trehalose when exposed to adverse growth conditions. Moreover, glutamate together with trehalose are the main osmolytes in *E. coli* growing in a minimal medium without the preferred osmoprotectant glycine–betaine [20]. The pathway for stress-induced trehalose synthesis in *Propionibacterium* has been published [6, 7].

Alanine concentration increased in *P. shermanii* cells under hyperosmotic conditions. Alanine was also accumulated by *L. plantarum* when the osmolarity of the medium was raised [14]. In both species, glycine–betaine is preferentially accumulated under osmotic stress conditions.

Several osmolytes partly alleviate the growth inhibiting effect of NaCl. Among them, DMSA, glycine–betaine and DMSP are accumulated by *P. shermanii* cells from the medium. DMSA and DMSP are two methylated sulfur compounds and are apparently interchangeable with glycine–betaine as osmolytes [11]. The improvement of growth rate in hyperosmotic environment by addition of external osmolytes to the medium was first observed in *E. coli* with glycine–betaine [20]. Only a few microorganisms are able to biosynthesize glycine–betaine from simple carbon and nitrogen sources. Most of them accumulate it from the medium or/and synthesize it from choline or by methylation of glycine [13]. *P. shermanii* demonstrated a low capacity (or an absence) of glycine–betaine synthesis from choline in M63 medium. Nevertheless, even if the cells seem to incorporate choline at a low uptake rate, they are able to accumulate [7] and convert it to glycine–betaine later. The fact that choline uptake and metabolism of glycine–betaine decreased when the osmotic pressure increased was surprising and must be confirmed. Choline uptake and oxidation are enhanced in *E. coli* under similar osmotic stress conditions [19].

Further experiments are needed to determine how choline and glycine–betaine transport (or biosynthesis) activities are regulated under stress conditions and how the different osmolyte intracellular concentrations increase during *P. shermanii* growth. Moreover, the cross reactions between osmotic and other stresses such as heat stress need investigation.

ACKNOWLEDGEMENTS

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