Acid stress susceptibility and acid adaptation of Propionibacterium freudenreichii subsp. shermanii

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Acid stress susceptibility and acid adaptation of *Propionibacterium freudenreichii* subsp. *shermanii*

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Abstract — The probiotic application of dairy propionibacteria as well as their use in cheese technology implies exposure to various environmental stresses, including acidic pH. The acid tolerance response (ATR) of *Propionibacterium freudenreichii* was investigated. One strain present in Swiss-type cheese proved to be acid-tolerant, since no lethal effect was observed during exposure at pH 3. Moreover, survival at pH 2 (acid challenge) was conferred by pre-exposure to a moderate acid stress (acid adaptation). This adaptative response was triggered quickly, and showed a maximal efficiency upon exposure to pHs between 4 and 5. Stationary phase ATR and acid habituation were also demonstrated, and conferred increased survival at pH 2 without pre-exposure. Exponentially-growing bacteria were partially protected towards acidity by pre-exposure to other stresses (heat, starvation, but not hyperosmolarity). A comparative study of different strains revealed that acid stress susceptibility is strain-dependent within this species. Adaptation and survival at low pH is likely to determine the efficacy of a *P. freudenreichii* strain both as a cheese starter and as a probiotic.

Swiss-type cheese / *Propionibacterium* / acid tolerance response / stress / probiotic

Résumé — Sensibilité et adaptation vis-à-vis du stress acide chez *Propionibacterium freudenreichii* subsp. *shermanii*. L'application probiotique des bactéries propioniques laitières, de même que leur utilisation en technologie fromagère, entraîne leur exposition à divers stress environnementaux, dont le stress acide. Nous avons étudié l'acquisition de tolérance en réponse au stress acide (ATR) chez *Propionibacterium freudenreichii*. Une souche présente dans un fromage à pâte pressée cuite s'est révélée tolérante, puisque aucun effet létal n'a été observé pendant une exposition à un pH de 3. De plus, la pré-exposition à un pH modérément acide (adaptation) a permis la survie à pH 2 (épreuve). Cette réponse adaptative est déclenchée rapidement et présente un optimum d'efficacité entre pH 4 et 5. L'ATR de phase stationnaire ainsi que l'habituation à l'acidité ont également été démontrées et améliorent la survie à pH 2 sans pré-traitement. Les bactéries récoltées en phase exponentielle de croissance sont partiellement protégées de l'épreuve acide par pré-exposition à d'autres stress (chaleur,

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carence nutritive, mais pas le stress hyperosmotique). Une étude comparative de différentes souches a révélé que la susceptibilité au stress acide dépendait de la souche au sein de cette espèce. L'adaptation et la survie à bas pH déterminent probablement l'efficacité de *P. freudenreichii* à la fois en tant que levain et en tant que probiotique.

Emmental / *Propionibacterium* / adaptation / stress acide / probiotique

1. INTRODUCTION

In their natural environments or during industrial processes, bacterial cells are often subjected to a variety of abiotic stresses. In order to survive, bacteria have developed a set of mechanisms leading to protection against severe injury after an unfavorable environmental factor has been sensed. Stress adaptation implies the complex regulation of gene expression [34], and stress-activated genes seem to be well conserved within the prokaryotes. However, striking differences are observed between bacterial species, and even between strains of the same species in terms of stress susceptibility [16].

Acidification is widely used in the food industry as a means of preservation, and prevents spoilage by contaminating microorganisms. Fermentation of lactose by lactic acid bacteria in dairy products, in particular, leads to the accumulation of the end-product lactic acid. Furthermore, bacteria provided in fermented food are exposed in the human stomach to hydrochloric acid, lowering the pH to values around 1–2. Adaptation to acidic conditions thus seems necessary for efficient dairy starters. It is also of prime necessity for bacteria, either detrimental (pathogenic) or beneficial (probiotic), in order to reach the intestine [11].

The ability, for bacteria exposed beforehand to a moderate acid stress, to survive a subsequent exposure to an otherwise lethal acid shock is referred to as the acid tolerance response (ATR). ATR has been well documented for a substantial number of gastrointestinal or food-borne pathogenic bacteria such as *Escherichia coli* [35], *Salmonella typhimurium* [8], *Aeromonas hydrophila* [14], *Vibrio parahaemolyticus* [39], *Helicobacter pylori* [21], *Listeria monocytogenes* [4, 23] and *Enterococcus faecalis* [6], as well as the oral cariogenic *Streptococcus mutans* [12].

Less extensive research has been performed on the adaptative response to acid stress in beneficial lactic acid bacteria. ATR has been reported in *Lactobacillus acidophilus* [17] and *Lactococcus lactis* [13, 28]. In the latter microorganism, acid tolerance closely depends on the ability to regulate intracellular pH [24], which has been shown to be mainly achieved by an inducible proton-translocating ATPase [22]. The acid stress susceptibility of probiotics such as bifidobacteria, however, remains a limitation to their use in fermented dairy products, and their viability decreases during cold storage of acidified foods [2].

The Gram-positive, anaerobic aerotolerant bacterium *Propionibacterium freudenreichii* has to cope with injurious stresses linked to the manufacture of Swiss-type cheeses. During this process, it has to cope with thermal treatment (52 °C, 30 to 60 min), slightly acidic environments (down to pH 5.2, caused by the starter lactic acid bacteria) and saline stress caused by immersion (48 to 72 h) in saturated brine [20]. Only after these steps do the propionibacteria grow, convert the lactic acid to propionic and acetic acids as well as CO_2, leading to the characteristic flavor and the opening of Swiss-type cheeses. More recently, propionibacteria have been reported as probiotics which are able to modulate both enzymatic activities and microbial flora within

the gut [19, 26]. The acknowledged beneficial effect of propionibacteria partly relies on their ability to stimulate bifidobacteria both in vitro and in the human intestine [31]. This raises the question of the susceptibility of propionibacteria to stresses imposed within the human digestive tract, which includes acid stress during transit in the stomach.

Much has to be learnt about the ability of propionibacteria to withstand low pH environments. Some species can be used for the production of propionic and acetic acid by fermentation [3, 25]. However, their significant susceptibility to the accumulation of end-product organic acids is a limitation to this application. As shown by Rehberger and Glatz [29], *P. jensenii* and *P. thoenii* survive at lower pH values than *P. acidipropionici*. Surprisingly, the capacity to produce large amounts of acids does not coincide with the ability to survive low pH. So far, the classical cheese starter *P. freudenreichii* species has not been characterized with regard to its acid stress response.

In this report, we present an investigation of acid stress susceptibility and adaptation in a strain of *P. freudenreichii* subsp. *shermanii* used in Swiss-type cheese technology.

2. MATERIALS AND METHODS

2.1. Cheese experiments

An Emmental Swiss-type cheese produced in the west of France was purchased from a local supermarket and analyzed immediately. 10-g portions of cheese were sliced and homogenized for 30 s using a Waring blender (New Hartford, CO, USA) in 90 mL of either 2% trisodium citrate for enumeration of propionibacteria, or HCl solution for acid stress studies. In the latter case, HCl concentrations of 100, 88, 73 and 48 mmol·L^{-1} led to a final homogenate pH of 2, 2.5, 3 and 4, respectively. The viability of dairy propionibacteria was monitored 0, 15, 30, and 60 min after homogenization. To this aim, samples were removed, diluted in peptone–water, pH 7, containing 0.9% NaCl, and poured into LGA medium containing 1.5% agar. This selective medium, allowing enumeration of propionibacteria from complex mixtures [37], contained per L: 10 g lithium lactate, 10 g peptone, 10 g yeast extract, 6 g glycerol, 1 g milk powder, 50 mg bromo cresol purple, 328 mg K_2HPO_4 and 56 mg $MnSO_4$ and was adjusted to pH 7. Colony-forming units (CFU) were determined after 6 d of anaerobic incubation at 30 °C.

2.2. Pulse-field gel electrophoresis

Propionibacteria colonies isolated on LGA medium from the homogenized cheese were cultured in yeast extract lactate (YEL) medium. DNA samples were prepared according to Gautier et al. [9], and digested for 4 h at 37 °C using the restriction enzyme *Xba* I (Boehringer Mannheim, Meylan, France). Electrophoresis was run for 20 h at 14 °C on 1% agarose gels submitted to 200 V, with pulses at 2 and 20 s, using a Chef DR II system (Bio-rad, Richmond, UK). The TL size standard used was developed in our laboratory and consisted of *P. freudenreichii* subsp. *shermanii* ITGP18 chromosomal DNA digested with *Xba* I [9].

2.3. In vitro growth conditions

The *P. freudenreichii* TL162 strain was used in the in vitro studies. The culture medium used was YEL broth which contained, per L: 12.5 g sodium lactate, 10 g tryptone (Biokar Diagnostics, France), 10 g yeast extract (Biokar Diagnostics, France), 328 mg K_2HPO_4 and 56 mg $MnSO_4$. Unless otherwise indicated, the pH was adjusted to 7 using HCl prior to sterilization by filtration (Millipore, 0.45 μm). Growth was carried out at 30 °C without shaking and monitored spectrophotometrically at 650 nm.

2.4. Adaptation conditions

Log-phase cells were obtained as follows. A starter culture (10 mL in YEL medium) was diluted 1 000-fold in fresh YEL medium. During exponential growth, this preculture was again diluted 1 000-fold in 100 mL fresh medium. When the culture reached a cell density of 5×10^8 cells per mL (OD_{650} = 0.5), bacteria were harvested by centrifugation (6 000 × *g*, 30 °C, 5 min). For acid adaptation, cells were resuspended in an equal volume of lactate broth (YEL devoid of yeast extract) adjusted using HCl at pH values between 3 and 6. Unless otherwise indicated, adaptation took place during 30 min at 30 °C before cells were harvested for extreme acid challenge. Heat shock was carried out by incubating log-phase bacteria for 30 min at 55 °C in their culture medium. Starvation was caused by a 3-h incubation in phosphate-buffered saline solution (PBS) (137 mmol·L^{-1} NaCl, 27 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} KH_2PO_4, 8.1 mmol·L^{-1} Na_2HPO_4, pH 7.4). Moderate osmotic stress was applied for 30 min at 30 °C in the presence of 0.3 mol·L^{-1} NaCl or 0.6 mol·L^{-1} sucrose in YEL medium, or in a defined sodium lactate solution (per L: 12.5 g sodium lactate, 328 mg K_2HPO_4 and 56 mg $MnSO_4$, pH 7). None of these treatments was responsible for a detectable decrease in cell viability.

2.5. Extreme acid challenge

Adapted and non-adapted cells were harvested by centrifugation and resuspended in an equal volume of lactate broth adjusted at pH 2 using HCl unless otherwise specified. Viable-cell counts were determined after 0, 15, 30 and 60 min of acid challenge. Samples were diluted in peptone-water, pH 7, containing 0.9% NaCl and poured into YEL medium containing 1.5% agar. CFU were determined after 6 d of anaerobic incubation at 30 °C. The data shown represent the means of at least 3 independent experiments.

3. RESULTS

3.1. Acid stress susceptibility of dairy propionibacteria present in cheese

The ability of propionibacteria present in commercially available Emmental Swiss-type cheese to survive severe acidic conditions (such as those encountered in gastric juice) was investigated. The number of viable propionibacteria cells counted on the selective LGA medium after homogenization under neutral conditions was 3×10^8 to 5×10^8 CFU per g of cheese. Then we determined the percentage of survival of propionibacteria after homogenization of the cheese in 10 vol. HCl solution of various concentrations (Fig. 1). No significant loss

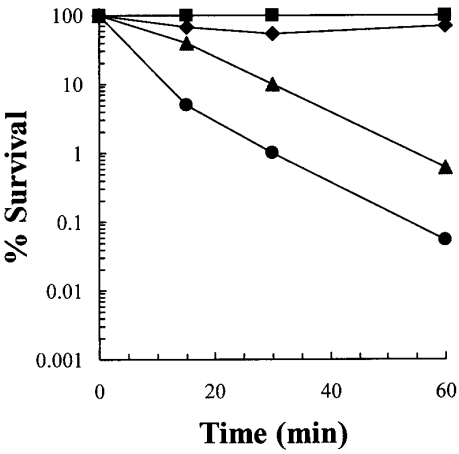

Figure 1. Effect of acidic pH on cell viability of dairy propionibacteria in cheese. Emmental Swiss-type cheese was homogenized in different solutions of HCl, and viability was monitored during 60 min. The HCl concentrations were 48, 73, 88 and 100 mmol·L^{-1}, leading to a final pH of 4 (■), 3 (◆), 2.5 (▲), and 2 (●), respectively.

Figure 1. Effet du pH acide sur la viabilité des bactéries propioniques laitières présentes dans l'Emmental. L'Emmental était broyé dans différentes solutions de HCl et la viabilité suivie pendant 60 min. Les concentrations de HCl étaient 48, 73, 88 et 100 mmol·L^{-1}, conduisant respectivement à des pH finaux de 4 (■), 3 (◆), 2.5 (▲), et 2 (●).

of viability was observed at pH 3 or 4, and a similar propionibacteria number was recovered per g of cheese whatever the incubation time. In contrast, rapid bacterial death was detected in cheeses acidified to a final pH of 2.5. Propionibacteria viability fell exponentially, resulting in a 2-log reduction in CFU counts. This was faster at pH 2, and a 3-log decrease was observed in the latter case (Fig.1).

3.2. Identification of the predominant propionibacteria strain in cheese

To further investigate acid stress tolerance in dairy propionibacteria, we conducted an identification of the *P. freudenreichii* strains present in the studied cheese. Clones were isolated from the homogenate and the corresponding DNA restriction patterns were analyzed using PFGE, along with those of propionibacteria strains known to be widely commercialized as cheese starters in the west of France. Figure 2 illustrates a representative result of this analysis. Eight out of 9 isolates analyzed on this gel (lanes 2 to 10) showed a restriction pattern similar, if not identical, to the industrial strain TL162 of *P. freudenreichii* subsp. *shermanii* (lane 1). This restriction pattern has previously been shown to be highly strain-specific within the *P. freudenreichii* species [9]. The TL162 strain could hence be considered as the predominant propionibacterial flora in this cheese. The following experiments on acid tolerance and acid adaptation were thus conducted in vitro using pure cultures of the TL162 strain.

3.3. Extreme acid stress susceptibility and acid tolerance response of log-phase strain TL162

Acid stress is considered as the exposure to pH values below the growth range. We thus investigated the ability of strain TL162 to grow at various pHs in YEL medium. Optimal growth was observed in the pH

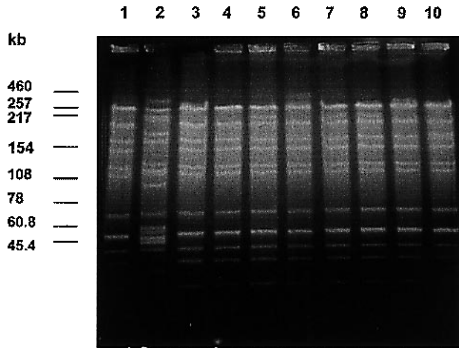

Figure 2. Pulsed-field gel electrophoresis separation of *Xba* I restriction fragments of genomic DNA. Lane 1, industrial strain TL162 of *P. freudenreichii* subsp. *shermanii*; lanes 2 to 10, clones isolated from the cheese homogenate. On the left are shown the relative positions of fragments contained in the TL size standard.

Figure 2. Analyse par électrophorèse en champs pulsés des fragments de restriction *Xba* I d'ADN génomiques. Piste 1, souche industrielle TL l62 de *P. freudenreichii* ; pistes 2 à 10, clones isolés de l'homogénat d'Emmental. Les positions relatives des fragments d'ADN contenus dans le standard de taille TL sont indiquées sur la gauche.

range 6 to 8 (generation time 4 h 30 min). However, strain TL162 was able to grow at pHs down to 5 with a prolonged generation time (8 h 40 min). The pH conditions generating extreme acid stress for strain TL162 were then determined. Log-phase harvested bacteria were exposed during 60 min to different pHs below 4 in acidified lactate broth, and cell viability was monitored during this challenge. While no significant loss of viability was observed at pH 3 or 4, exponential cell death occurred in more acidic environments (Fig. 3). Indeed, a 1-log decrease in cell viability was observed at pH 2.5 in 1 h and a 4-log decrease at pH 2 for unadapted cells (i.e., cells directly transferred from the pH 7 growth medium to the acidic challenge medium). The acid stress undergone at pH values above 2.5 was considered moderate, as no significant effect on cell viability was observed, even during longer periods (data not shown). The

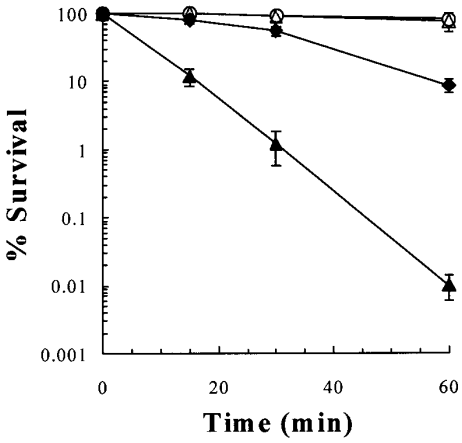

Figure 3. Demonstration of ATR in *P. freudenreichii* strain TL162. Log-phase cells were, harvested and viability was monitored during 60 min acid challenge at pH 3 (○), 2,5 (◆), or 2 (▲). Alternatively, log-phase cells were pre-adapted during 30 min at pH 4.5 (△) before acid challenge at pH 2.

Figure 3. Démonstration de l'ATR chez *P. freudenreichii* souche TL162. Des cellules en phase exponentielle de croissance étaient récoltées et la viabilité suivie pendant 60 min d'épreuve acide à pH 3 (○), 2,5 (◆) ou 2 (▲). En parallèle, ces cellules étaient pré-adaptées pendant 30 min à pH 4,5 (△) avant l'épreuve acide à pH 2.

conditions for lethal acid challenge in the following experiments were then pH 2, 60 min at 30 °C in acidified sodium lactate broth. The ability of a sub-lethal acid stress (adaptation) to induce tolerance to potentially lethal acid concentrations in *P. freudenreichii* was also explored. After a 30-min exposure to a moderate acid stress at a sub-lethal pH (4.5), 78% of the cells survived a challenge at pH 2 for 60 min (Fig. 3). This reveals the existence of a log-phase ATR in *P. freudenreichii* subsp. *shermanii* strain TL162.

3.4. Characterization of log-phase ATR

Adaptation by a sub-lethal acid pre-stress was carried out at different times and pH values and the adaptation factor, which is defined as the ratio of survival percentages between adapted and non-adapted cells, was determined. As shown in Figure 4A, protection was obtained in the pH range 3 to 6 with tolerance factors above 200. Compared to non-adapted cells (tolerance factor = 1), the most efficient ATR was observed between pH 4 and 5 with tolerance factors above 6 000. Figure 4B shows the kinetics of acquisition of acid tolerance during pre-exposure to pH 4.5 prior to extreme acid challenge at pH 2. ATR was triggered as early as 3 min following pre-exposure, and resistance increased exponentially during 10 min to reach a maximum at 30 min. No improvement of acid tolerance was observed after longer periods of adaptation, and the level of tolerance was maintained (data not shown). The conditions for optimal ATR were thus pre-exposure of bacteria to pH 4.5 during 30 min at 30 °C.

3.5. Acid tolerance induced by other environmental factors

The ability, for bacteria grown at a pH below the optimum conditions, to better tolerate acid stress (acid habituation) has been described elsewhere. Thus, *P. freudenreichii* was cultivated at the minimal pH allowing exponential growth (pH 5) prior to extreme acid challenge. As shown in Figure 5, these acid-habituated bacteria (grown at pH 5) were more resistant to extreme acid conditions than control bacteria (grown at pH 7). Indeed, 32% of acid-habituated cells versus 0.01% of control cells survived challenge at pH 2.

By comparison, ATR at pH 5 resulted in 69% survival and ATR at pH 4.5 in 78% survival. Thus a brutal pH downshift triggered a better acid tolerance than growth at acidic pH. Moreover, better tolerance (90% survival at pH 2) was achieved after pre-exposure to pH 4.5 of acid-habituated cells.

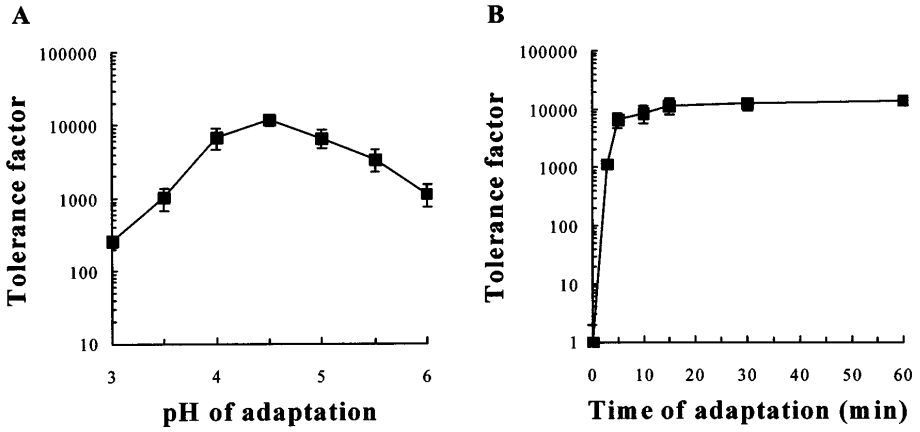

Figure 4. pH-dependence and kinetics of ATR in *P. freudenreichii* strain TL162. Acid adaptation was performed on log-phase cells during 30 min at various pH values between 3 and 6 (**A**), or at pH 4.5 for various times (**B**) prior to acid challenge during 60 min at pH 2. The tolerance factor was defined as the ratio of survival percentages between adapted and non-adapted cells.

Figure 4. Dépendance vis-à-vis du pH et cinétique de l'ATR chez *P. freudenreichii* souche TL162. Des cellules en phase exponentielle de croissance étaient pré-adaptées pendant 30 min à différents pHs compris entre 3 et 6 (**A**) ou à pH 4,5 pendant des temps différents (**B**) avant l'épreuve acide d'une heure à pH 2. Le facteur de tolérance était défini comme le rapport entre les pourcentages de survie des cellules adaptées et des cellules non-adaptées.

These results indicate that acid-habituated *P. freudenreichii* cells also display ATR.

Because overlaps between multiple stresses often occur in bacteria, we intended to determine whether the other constraints imposed on *P. freudenreichii* during cheese-making could confer tolerance towards acid shock. To that aim, non-adapted cells were heat-shocked prior to acid challenge. Heat shock (30 min at 55 °C) partially protected the bacteria from acid-induced mortality (Fig. 5). Cross-protection was obtained for temperatures between 40 and 55 °C, with an optimum of efficiency for this last value (2.1% survival at pH 2). Lower temperatures were ineffective at inducing acid tolerance, while conditions above 56 °C caused a dramatic decrease in cell viability (data not shown). Starvation-induced multiresistance was also observed in other bacteria. We therefore investigated the ability of starved cells to survive extreme acid challenge. Stationary-phase cells were significantly more resistant (8% survival) than log-phase cells. Similarly, cells starved for 3 h in PBS were partially protected (7% survival) against acid challenge (Fig. 5).

Osmotic stress has also been shown to trigger cross-protection in other bacteria. We therefore investigated the effect of moderate hyperosmotic stress on extreme acid stress survival. *P. freudenreichii* was more sensitive to acid challenge after exposure to 0.3 $mol \cdot L^{-1}$ NaCl (Fig. 5). Indeed, only 0.001% of the salt-stressed cells survived acid challenge, while 0.01% of the control cells were able to do so. Because this weakening effect could be due either to osmotic or to ionic stress, 0.6 $mol \cdot L^{-1}$ sucrose was also used as a non-ionic osmoticum and led to a similar increased acid susceptibility. This was observed either in the complex YEL medium or in the defined lactate solution (data not shown). No loss of viability was caused by these hyperosmotic treatments alone.

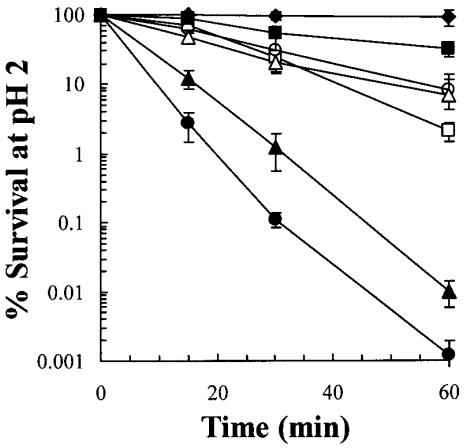

Figure 5. Acid tolerance induced by other factors. Log-phase non-adapted cells were heat-shocked at 55 °C (□), starved in PBS (△), exposed to 0.3 mol·L^{-1} NaCl (●), or non-treated (▲), and cell viability was monitored during subsequent acid challénge at pH 2. Alternatively, cells were grown at pH 5 (■), or harvested during stationary phase (○) prior to acid challenge. Lastly, cells grown at pH 5 were acid-adapted during 30 min at pH 4.5 before acid challenge at pH 2 (◆).

Figure 5. Tolérance vis-à-vis du stress acide induite par d'autres facteurs. Des cellules en phase exponentielle de croissance étaient stressées thermiquement à 55 °C (□), carencées dans du tampon PBS (△), exposées à 0,3 mol·L^{-1} de NaCl (●) ou non-traitées (▲) avant l'épreuve à pH 2 pendant laquelle la viabilité était suivie. En parallèle, des cellules étaient cultivées à pH 5 (■) ou récoltées en phase stationnaire de croissance (○) avant l'épreuve acide. Enfin, des cellules cultivées à pH 5 étaient pré-adaptées à pH 4,5 avant l'épreuve acide (◆).

3.6. Comparative analysis of different dairy propionibacteria strains

Acid stress sensitivity has been shown to vary between strains of the same bacterial species. We thus conducted a comparative study of different propionibacteria. Growth rate was monitored in YEL medium where pH was adjusted to different values (Tab. I). The results obtained were strain-dependent. Some *P. freudenreichii* strains, such as TL162, could grow at pH 5 while others, such as CNRZ725, needed a pH above 5.75 for exponential growth. All the tested strains displayed optimal growth in the pH range 6 to 8 (data not shown). In addition, the survival percentage of log-phase non-adapted cells was determined for all strains after a lethal acid challenge (1 h at pH 2 in lactate broth). Again, the survival percentage varied between 3.26×10^{-1} (strain ITGP6) for the most tolerant and 3.22×10^{-5} for the least acid tolerant strain (TL166) of *P. freudenreichii*. For the two *P. acidipropionici* strains studied, no viable cells could be detected after the lethal acid challenge.

4. DISCUSSION

In this report, we evaluated the acid stress susceptibility of a dairy propionibacterium used as a starter in Swiss-type cheese technology. This strain (TL162) was shown to survive the various technological stresses well, and to reach a final density of 3×10^8 to 5×10^8 CFU per g ripened cheese. The constitutive susceptibility of this strain was shown to be remarkably low, since pH 3 exerted no significant lethal effect on it, neither as provided in the cheese, nor as log-phase cells cultivated in YEL medium. In our experiments, pH 2 could be considered as lethal for non-adapted mid-log phase cells. It should be noted that the acid challenge imposed in this study was particularly harsh, since weak organic acids, either present in the medium (lactic acid), or produced by the bacterium (propionic and acetic acids) were present throughout these experiments. It is generally admitted that weak acids, under their protonated form, diffuse across the cell membrane and worsen the biological effect of low pHs [1].

Exponential-phase ATR was also demonstrated in this strain, showing that *P. freudenreichii* TL162 can adapt to severe acidic environments. ATR was achieved very rapidly, and substantial protection was

Table I. Growth rate (generation·h^{-1}) at acidic pH, and percentage of survival at pH 2, of different dairy propionibacteria[1].

Tableau I. Taux de croissance à pH acide (génération·h^{-1}), et pourcentage de survie à pH 2, de différentes bactéries propioniques[1].

Strain[2]	Growth rate (pH 5)	Growth rate (pH 5.5)	% Survival (after 1 h at pH 2)
P. acidipropionici NCDO1072	0	0.12	ND[3]
P. acidipropionici CNRZ80	0	0.13	ND
P.f. subsp. *freudenreichii* CNRZ81	0	0.10	3.83×10^{-2}
P.f. subsp. *freudenreichii* ITGP18	0.04	0.16	2.35×10^{-3}
P.f. subsp. *shermanii* CNRZ725	0	0	3.03×10^{-2}
P.f. subsp. *shermanii* ITGP20	0	0	1.84×10^{-3}
P.f. subsp. *shermanii* TL166	0	0.09	3.22×10^{-5}
P.f. subsp. *shermanii* ITGP10	0.05	0.14	3.85×10^{-3}
P.f. subsp. *shermanii* ITGP23	0	0.15	4.17×10^{-3}
P.f. subsp. *shermanii* ITGP1	0.09	0.15	9.41×10^{-2}
P.f. subsp. *shermanii* ITGP6	0.08	0.15	3.26×10^{-1}
P.f. subsp. *shermanii* CIP103027	0.03	0.16	1.85×10^{-2}
P.f. subsp. *shermanii* TL162	0.11	0.17	1.01×10^{-2}

[1] Culture collections: CIP, Collection Institut Pasteur, Paris, France; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; ITG, Institut Technique du Gruyère, Rennes, France; TL, Technologie Laitière, INRA, Rennes, France. All these strains were grown on YEL medium at various initial pHs. Each result is the mean of at least three different experiments, and no significant variation of the pH was observed during exponential growth.
[2] *P.*: *Propionibacterium;* and *P. f.*: *Propionibacterium freudenreichii*.
[3] ND: Not detectable.

[1] Collections de souches : CIP, Collection Institut Pasteur, Paris, France ; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France ; ITG, Institut Technique du Gruyère, Rennes, France ; TL, Technologie Laitière, INRA, Rennes, France. Toutes ces souches étaient cultivées sur milieu YEL à différents pH initiaux. Chaque résultat constitue la moyenne d'au moins trois expérimentations différentes et aucune variation significative du pH n'était observée pendant la croissance exponentielle.
[2] *P.* : *Propionibacterium* et *P. f.* : *Propionibacterium freudenreichii*.
[3] ND : non détectable.

observed after 3 min of acid adaptation. Optimal efficiency was obtained with adaptation pHs between 4 and 5, as described for the other bacterial species cited above. By contrast, the pH value (pH 2) at which adapted TL162 cells could survive without significant loss of viability was remarkably low.

The efficiency of this adaptative response might be correlated to the surprising properties of a recently discovered acid-tolerant propionibacterium, *P. cyclohexanicum*, which is closely related to *P. freudenreichii*. The growth of *P. cyclohexanicum* occurs at pH 3.2 to 7.5, with an optimum between 5.5 and 6.5 [15]. It can withstand extreme heat stress (90 °C, 10 min) and shows a membrane fatty acid composition distinct from that of dairy propionibacteria. Thus, it seems that remarkably efficient adaptative mechanisms occur in propionibacteria and afford survival in various environments.

In *S. typhimurium*, acid adaptation confers resistance to lethal heterologous challenges, but heterologous adaptations do not induce acid tolerance [16]. Consistently, acid stress has been proposed to be the most

general stress in this bacterium. Similarly, ATR provides protection towards acid and other stresses in *L. monocytogenes* [4]. In *E. faecalis*, on the other hand, no cross-protection between acid and other stresses (heat and bile salts) has been detected [6]. In *P. freudenreichii*, we demonstrated that it was possible to induce tolerance to acid by heterologous stresses. However, none of these stresses was able to promote survival at pH 2, as does ATR, even after longer periods of heterologous adaptation. Partial protection was observed after a moderate heat shock, suggesting that general stress factors (such as those under the dependence of an alternative σ factor) confer acid tolerance in exponentially-growing cells. Stationary phase ATR was also demonstrated in *P. freudenreichii* and was independent of exposure to acidic pH. Starving mid-log phase cells in PBS had a very similar effect. This suggests that the mechanism(s) involved in stationary phase acid tolerance is (are) mainly due to starvation, a stress known to trigger multiresistance in *E. faecalis* [10].

In contrast to the other stimuli, osmotic stress, either in a complex or in a defined medium, rendered *P. freudenreichii* more sensitive to acid challenge. Moderate hyperosmotic stress has been shown to trigger tolerance against various stresses other than acidity in a variety of bacteria, either Gram-positive or Gram-negative [5, 36, 38]. However, the presence of osmoprotectants was shown to inhibit these cross-protections, at least in *S. typhimurium* [7] and *E. faecalis* [27]. Moreover, salt stress failed to provide acid tolerance in enteric bacteria [16, 33] and increased acid sensitivity in *L. monocytogenes* [18]. The actual benefit of osmotic stress and adaptation to bacterial survival in adverse environments thus remains uncertain [27].

We have also shown that *P. freudenreichii*, when cultivated at the lowest pH allowing growth (pH 5), was less sensitive to acid stress. This phenomenon, referred to as acid habituation (AH), has been described in *E. coli*. AH in this bacterium involves both protein synthesis-dependent and -independent mechanisms [32, 35] and is clearly separate from ATR. In our study, AH at pH 5 was shown to be less effective than ATR at the same pH. In addition, ATR after AH provided a higher tolerance than each of these treatments alone. More than one response to low pH thus might co-exist in *P. freudenreichii*.

The minimal pH allowing exponential growth as well as the ability to survive under extreme acid stress has been shown to be strain-dependent within the species *P. freudenreichii*. This variability might well be related to the great differences observed between strains regarding their ability to produce volatile fatty acids and CO_2 in the acidified curd, and hence their suitability for Swiss-type cheese technology [30]. The strain TL162 appears to be one of the most tolerant, which is in accordance with its technological performance. Indeed, this *P. freudenreichii* subsp. *shermanii* strain has been selected for its suitability for cheesemaking, and is widely commercialized as a starter (PAL cheese starter; Standa Industrie).

In conclusion, effective tolerance towards acid stress is a promising result for the use of dairy propionibacteria as a probiotic food complement. The level of protection afforded by ATR is higher than that described for other bacteria, either those that are favorable or detrimental to human health. Swiss-type cheese has been shown to contain high amounts of propionibacteria for which the level of acid tolerance is higher than that of in vitro cultivated log-phase propionibacteria, but lower than that allowed by ATR. Interestingly, some of the stresses encountered in Swiss-type cheese technology have been shown to be cross-protective. Salt stress, in contrast, has a negative effect, and this has been mimicked by using sucrose instead of salt, suggesting that mainly hyperosmotic stress is responsible for this effect. Thus, the cellular response to technological stresses,

as well as its consequence on acid tolerance should be considered for the development of effective probiotic preparations aimed at protecting *P. freudenreichii* from acid injury within the human digestive tract.

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