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NOTE

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Effects of pressure on cell morphology and cell division of lactic acid bacteria

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Abstract The effect of pressure and temperature on the growth of the mesophilic lactic acid bacteria *Lactococcus lactis* and *Lactobacillus sanfranciscensis* was studied. Both strains were piezosensitive. *Lb. sanfranciscensis* failed to grow at 50 MPa and the growth rate of *Lc. lactis* at 50 MPa was less than 30% of that at atmospheric pressure. An increase of growth temperature did not improve the piezotolerance of either organism. During growth under high-pressure conditions, the cell morphology was changed, and the cells were elongated as cell division was inhibited. At atmospheric pressure, temperatures above the optimal temperature for growth caused a similar effect on cell morphology and cell division in both bacteria as that observed under high-pressure conditions. The segregation and condensation of chromosomal DNA were observed by DAPI staining and occurred normally at high-pressure conditions independent of changes in cell morphology. Immunofluorescence microscopy of *Lc. lactis* cells demonstrated an inhibitory effect of high pressure on the formation of the FtsZ ring and this inhibition of the FtsZ ring formation is suggested to contribute to the altered cell morphology and growth inhibition induced by high pressure.

Keywords Cell morphology · FtsZ · High pressure · *Lactobacillus sanfranciscensis* · *Lactococcus lactis*

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Introduction

Pressure is an important thermodynamic variable which ranges from 0.1 MPa at the surface to 110 MPa in the deepest ocean trench. This parameter acts to decrease the total volume of a system at equilibrium in the case of liquids and solutions. The physical basis of pressure effects on biological systems is well documented as arising from the repressive effects of increased pressure on biochemical processes, which are accompanied by positive volume changes. However the pressure-induced phenomena that occur in living microorganisms have not yet been fully elucidated (Abe and Horikoshi 1998; Abe et al. 1999; Kato and Bartlett 1997). Deep-sea microorganisms have developed the ability to survive under extreme pressure conditions. Piezophiles are defined as microorganisms that possess optimal growth rates at pressures above atmospheric pressure (Abe and Horikoshi 2001; Yayanos 1995). Piezotolerant microorganisms are capable of growth at high pressure as well as at atmospheric pressure and grow at 50 MPa at a rate that is above 30% of their growth rate at atmospheric pressure, as long as they have otherwise optimal growth conditions. Piezosensitive microorganisms cease to grow at 40–50 MPa.

Various physiochemical conditions can have a great influence on the effect of high pressure on microbial growth rate (Abe et al. 1999; Abe and Horikoshi 2001). Piezophiles are generally most piezotolerant at temperatures a few degrees above the optimum growth temperature, and moderate pressure increases both the optimal and maximum growth temperature by a few degrees (Zobell 1970; Yayanos et al. 1979; Marquis 1984; Yayanos 1986; Kato et al. 1996).

Piezophilic and piezotolerant microorganisms have considerable potential for use in biotechnology because of the improvement in industrial processes through application of high pressure and the potential for novel natural products (Abe et al. 1999; Kato and Bartlett 1997; Kato et al. 2000). Furthermore, pressure

in the range of 50–150 MPa may be applied in food biotechnology to control the metabolic activity of bacteria in order to enhance the formation of metabolites with positive impact on food quality. Casal and Gomez (1999) proposed the use of lactic acid bacteria cells attenuated by high pressure to accelerate cheese ripening. Sublethal pressure lowered the lactic-acid-producing activity without causing damage to the peptidolytic acitivity. Korakli et al. (2002) proposed the use of sublethal pressure to alter the composition of metabolites formed from maltose by *Lactobacillus sanfranciscensis*. However, whereas it is well documented that short-term treatment at elevated pressure above 200 MPa inactivates food-related bacteria, enabling pressure applications for food preservation, few data are available on the response of food-fermenting microorganisms to sublethal pressure (Abe et al. 1999).

Lactococcus lactis is one of the economically most important lactic acid bacteria because of its worldwide use in cheese processing. *Lc. lactis* strains are used as starter cultures because of their acidification activity and their contribution to cheese flavor development. *Lb. sanfranciscensis* belongs to the predominantly microbial flora of traditionally prepared wheat and rye sourdoughs. In sourdoughs with a tradition of continuous propagation, lactic acid bacteria as well as yeast contribute to the formation of aroma volatiles or precursors available for thermal transformation to aroma compounds (Hammes and Gänzle 1997).

This study aimed to investigate the effect of pressure and temperature on mesophilic bacteria used in food biotechnology, *Lc. lactis* and *Lb. sanfranciscensis*, at the level of growth rate, cell morphology, and cellular organization. The process of cell division was monitored using fluorescence and immunofluorescence microscopy (IFM) to detect DNA segregation and formation of the FtsZ ring, respectively. The FtsZ protein is a tubulin-homolog with GTPase activity and is widely conserved in bacteria. Macromolecular assembly of FtsZ is a prerequisite for membrane constriction during cell division (Erickson 1997; Sun and Margolin 1998; Errington et al. 2003).

Effect of pressure and temperature on growth

Lc. lactis MG 1363 and *Lb. sanfranciscensis* TMW1.53 were grown at 30°C under anaerobic conditions in M17 broth (Merck, Darmstadt, Germany) supplemented with 1% glucose (GM17) and MRS-4 broth (Gänzle et al. 1998), respectively. To determine the maximal pressure of growth for these organisms, fresh broth inoculated with 0.1% of a stationary culture was subjected to hydrostatic pressure ranging from 0.1 to 100 MPa at 30° ±0.5°C, as described (Abe 1998). Samples were taken after 20 and 48 h (*Lc. lactis*) or 24 and 48 h (*Lb. sanfranciscensis*) and the viable cell counts were determined. The cell counts of *Lc. lactis* after 20 and 48 h of incubation at different pressure levels are shown in Table 1 (left column). The cell counts after 20 h of incubation were reduced concomitant with the increase in pressure. At 50 MPa, cells grew to only 2.3% of cell counts at atmospheric pressure. After 48 h the cells incubated between 0.1 and 40 MPa had already entered the lytic phase, and an increase of the cell counts at 50 MPa compared with the cell counts after 20 h was observed. Viable cell counts were reduced by 3–5 log cycles after incubation at 60–100 MPa for 48 h.

Lb. sanfranciscensis was more piezosensitive than *Lc. lactis,* and did not grow at 50 MPa (Table 1, right column). Pressure conditions between 0.1 and 30 MPa had no significant effect on growth. At 40 MPa, the generation time was about 24 h and the final cell density after 48 h was 1% of the cell counts after cultivation at ambient pressure. Growth was fully inhibited at pressure of 50 MPa but the cells remained viable. Incubation at a pressure of 60 MPa or higher pressure caused a reduction of cell viable counts by up to 90%.

Deep-sea bacteria exhibit optimal high-pressure growth near their upper temperature limit for growth (Kato et al. 1995, 2000; Kato and Bartlett 1997). To determine the effect of increasing temperatures for growth under high-pressure conditions in *Lc. lactis* and *Lb. sanfranciscensis*, cultivation experiments were performed at temperature and pressure conditions above the optimal conditions of growth. The optimal and

Table 1 Cell counts of *Lactococcus lactis* MG 1363 and *Lactobacillus sanfranciscensis*after incubation at 30°C and various pressure levels

Pressure level (MPa)	*Lc. lactis* MG 1363		*Lb. sanfranciscensis*	
	CFU after 20 h[a]	CFU after 48 h[a]	CFU after 24 h[a]	CFU after 48 h[a]
0, 1	2.49×10^{9}	1.1×10^{8}	9.10×10^{8}	7.80×10^{8}
10	1.78×10^{9}	1.6×10^{8}	8.90×10^{8}	2.20×10^{9}
20	1.86×10^{9}	3.2×10^{8}	7.80×10^{8}	8.90×10^{8}
30	1.56×10^{9}	2.0×10^{8}	2.40×10^{8}	3.10×10^{8}
40	7.2×10^{8}	4.7×10^{8}	6.10×10^{6}	1.14×10^{7}
50	5.68×10^{7}	1.8×10^{8}	1.81×10^{5}	2.03×10^{5}
60	5.3×10^{4}	2.48×10^{3}	1.23×10^{5}	5.80×10^{4}
70	4.62×10^{3}	1.14×10^{3}	1.58×10^{5}	9.6×10^{4}
80	3.56×10^{3}	2.3×10^{2}	2.02×10^{5}	1.05×10^{4}
90	5.76×10^{3}	8.0×10^{2}	6.0×10^{5}	5.5×10^{4}
100	3.1×10^{4}	2.0×10^{1}	3.0×10^{5}	5.8×10^{4}

[a] Corresponding results were obtained by optical density measurements of the cultures

maximum temperatures of growth for *Lc. lactis* under aerobic conditions were determined as 32° and 40°C, respectively, and the optimum growth rate was 1.7 h^{-1} (data not shown). The optimum and maximum temperatures for growth of *Lb. sanfranciscensis* are 32° and 41°C, respectively (Gänzle et al. 1998).

The effects of pressure and temperature on the anaerobic growth rate in *Lc. lactis* and *Lb. sanfranciscensis* were determined in the large-scale cultivation system "DEEP-BATH" (deep-sea baro/thermophiles collecting and cultivating system; Canganella et al. 1997; Moriya et al. 1995; Yanagibayashi et al. 1999). Fresh broth was inoculated with 0.1% of cells in stationary phase and cultivated for up to 48 h. The maximum specific growth rates μ_{max} were calculated based on optical density at 660 nm readings as described (Gänzle et al. 1998).

The combined effects of pressure and temperature on the growth rate of *Lc. lactis* is shown in Fig. 1. The growth rate at 0.1 MPa and 30°C was 1.7 h^{-1} and it was reduced with an increase in pressure. The upper limit of pressure for growth was at 50 MPa, and the growth rate was 14% of that in optimal conditions. At an incubation temperature of 40°C a rapid reduction of the growth rate was observed with the rise in pressure, and the growth was fully inhibited at 50 MPa. Further experiments were performed at 35°C to determine the inhibitory effect under high-pressure conditions caused by a temperature between the optimal range and the upper limit. The growth rate at 0.1 MPa was 1.39 h^{-1} and at 50 MPa, the growth rate was lower than the growth rate at 50 MPa and 30°C. Thus, an increased temperature did not increase the piezotolerance of *Lc. lactis*.

The combined effects of pressure and temperature on the growth rate were also determined for *Lb. sanfranciscensis*. At ambient pressure and 30°C, the growth rate of *Lb. sanfranciscensis* was 0.66 h^{-1} and the growth rate was reduced proportional to the increase in pressure. No growth was observed at 50 MPa (data not shown). As in the case of *Lc. lactis*, the piezotolerance of *Lb. sanfranciscensis* was not enhanced by raising the incubation temperature to levels above the optimum temperature for growth at ambient pressure (data not shown).

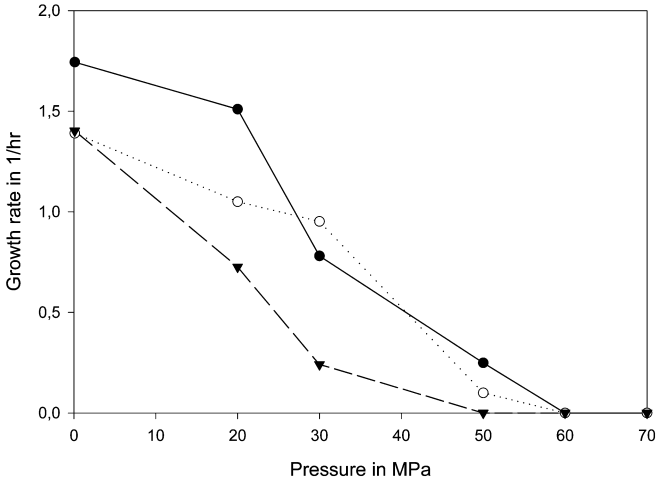

Fig. 1 Effect of pressure and temperature on the growth rate of *Lactococcus lactis* MG 1363. Growth rate at 30°C (*filled circles*), at 35°C (*empty circles*), and at 40°C (*inverted triangles*)

Morphological changes and immunofluorescence microscopy

Cells of *Lc. lactis* grown between 0.1 and 50 MPa were observed with a three-phase-contrast microscope immediately after decompression. Additionally, *Lc. lactis* cells were stained with FtsZ antibodies and DAPI, and observed by fluorescence microscopy. Figure 2 A-C depicts cells of *Lc. lactis* grown at 0.1, 30, and 50 MPa. At ambient pressure, *Lc. lactis* cells are present in form of diplococci. An increase in growth pressure induced chain formation. The size and frequency of the chains depended on the pressure level. At 10 and 20 MPa, chains consisting of 3–4 cells were observed, but the diplococcal appearance was predominant. At 30 MPa, approximately 50% of the cells were in chains, these chains were formed with 4–6 cells. In addition a deformation of cell morphology was observed; the cells adopted a slight rod-shape form (Fig. 2B). Cells grown at 40–50 MPa were mainly in chains composed of 3–14 cells. A change in the cell morphology was noticed, they lost their spherical shape, adopting a rod shape in the direction of the chain length. The presence of oversized cells was apparent (Fig. 2C). Chain formation and changes in the cell morphology were also observed at temperatures of growth above the optimal range. The combination of higher pressure and temperature enhanced the changes of cell morphology (data not shown).

To gain insight into the mechanisms governing the abnormal morphology of *Lc. lactis* grown under high-pressure conditions, chromosomal DNA segregation and condensation during cell division was visualized by fluorescence microscopy of exponentially growing cells stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) and the formation of the FtsZ ring was determined by immunofluorescence microscopy (IFM) employing anti-FtsZ antibodies. The method used for IFM was developed for the Gram-negative *Escherichia coli* (Sato et al. 2002; Ishii et al. 2002). To allow IFM staining of the Gram-positive *Lc. lactis*, cells were additionally treated for 7.5 min with lysozyme solution (2 mg/ml in 25 mM Tris-HCL, 50 mM glucose, and 10 mM EDTA) after fixation to poly-L-lysine-treated slides. The FtsZ of *Lc. lactis* has not been characterized on protein level; therefore, the *Lc. lactis* FtsZ was targeted with *E. coli* FtsZ rabbit IgG (Sato et al. 2002) or *Shewanella violacea* FtsZ rabbit IgG (Ishii et al. 2002) and visualized with the secondary antibodies Alexa Fluor488 goat anti-rabbit IgG (Molecular Probes, Eugene, USA).

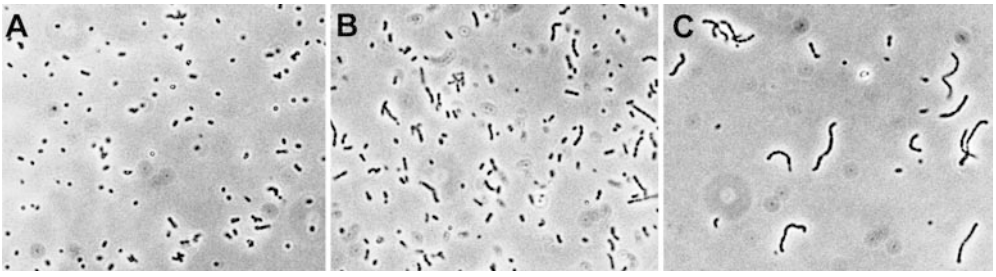

Fig. 2 Three-phase-contrast microscopic images of *Lactococcus lactis* MG 1363 grown at 0.1 MPa (**A**), 30 MPa (**B**), and 50 MPa (**C**)

According to the National Center for Biotechnology Information (NCBI) and the Japanese Genetic Center (DDBJ), *Lc. lactis* FtsZ (Y15422) shares approximately 40% DNA identity and 30% protein identity with *E. coli* FtsZ (X55034), and 57.5% DNA identity and 48% protein identity with *S. violacea* FtsZ (AB052554; Ishii et al. 2002). Western blotting analyses of *Lc. lactis* cell lysates were performed to verify the immunoreaction with antibodies targeting both the *E. coli* and the *S. violacea* FtsZ proteins. *Lc. lactis* FtsZ reacted positively to FtsZ antibodies of *E. coli* and more strongly to the antibodies of the piezophilic bacterium *S. violacea* DSS12 (data not shown). Therefore, the antibodies of *S. violacea* DSS12 were used in further IFM experiments.

Figure 3 shows phase-contract, DAPI fluorescence and immunofluorescence (IMF) images of selected cells of *Lc. lactis* cells grown at 0.1, 20, and 50 MPa to the exponential phase of growth. Diplococci were the prevalent form in cells grown at 0.1 MPa. During cell division, the segregation and condensation of DNA is indicated by separated nucleoids in DAPI-stained cells and the presence of FtsZ ring between the daughter cells is noticeable in IFM images (Fig. 3B, C. The presence of FtsZ rings was noticeable in 12% of cells growing at 0.1 MPa (total of 200 observations, Fig. 3C and data not shown). Prior to cell division, the intensity of DAPI fluorescence increases because of DNA replication, the nucleoids are not clearly divided and no FtsZ ring was observed. At 20 MPa, the diplococcal appearance remained predominant and chains of four cells as well as oversized cells were also observed. DAPI staining did not show differences between 20 MPa-grown cells and those cells grown at ambient pressure (Fig. 3D, E). However, the formation of FtsZ rings was not observed (total of 100 observations, Fig. 3F, data not shown). After growth at 50 MPa, *Lc. lactis* grew in long chains and oversized cells were observed. The division between cells was difficult to determine with light microscopy, but DAPI staining indicated the presence of several nucleoids in one chain corresponding to duplication, segregation and condensation of DNA (Fig. 3G, H). However, FtsZ rings were observed in fewer than 0.5% of the cells (total of 800 observations, Fig. 3I and data not shown).

Non-pressure-adapted (mesophilic) microorganisms commonly show growth inhibition at about 40–50 MPa (Fujii et al. 1996; Gross and Jaenicke 1994). In this study, we have shown that at optimal temperature for

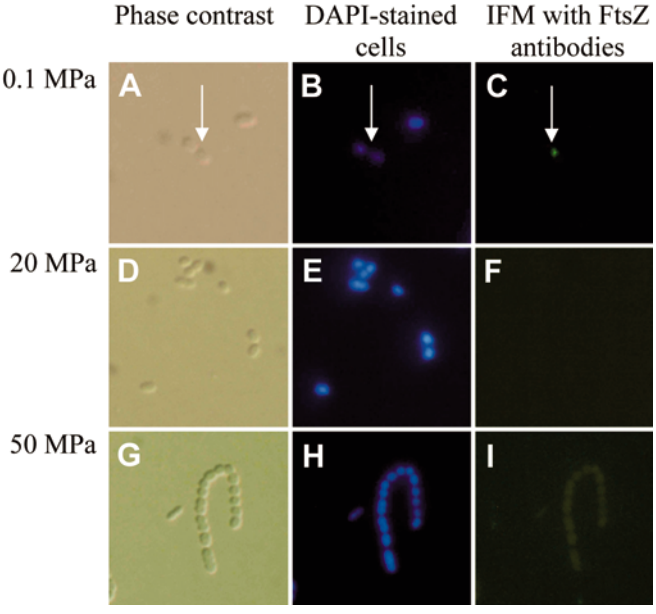

Fig. 3 Microscopic image of *Lactococcus lactis* MG 1363 grown at 0.1 MPa (**A**–**C**), 20 MPa, (**D**–**F**), and 50 MPa (**G**–**I**). **A**, **D**, **G**: three-phase microscopic images, **B**, **E**, **H**: fluorescence microscopy of cells stained with DAPI, **C**, **F**, **I**: immunofluorescence microscopy with Sv FtsZ antibodies. The *arrows* in **A**, **B**, and **C** highlight dividing cells where FtsZ ring formation is apparent

growth of the mesophilic strains *Lc. lactis* MG 1363 and *Lb. sanfranciscensis* were able to grow at pressures up to 50 and 40 MPa, respectively. According to the definition of Abe and Horikoshi (2001), both strains are piezosensitive. The cell morphology was altered after growth under pressure conditions, the cells were elongated and the cell division was inhibited. This is in accordance with findings for the Gram-negative bacterium *E. coli*, for which it was shown that its growth under pressure conditions is accompanied by formation of filaments (Sato et al. 2002). Data presented here and previous experiments with *Lc. lactis* and *Lb. sanfranciscensis* (Korakli et al. 2002; Molina-Gutierrez et al. 2002) demonstrate that growth of the mesophilic lactic acid bacteria is inhibited at much lower pressures (50 MPa) than overall metabolic activity (150 MPa) and cell viability (200 MPa).

The morphologic changes observed under pressure conditions suggest that some of the cytoskeleton and/or autolytic proteins were affected by high pressure. In *Lc. lactis*, autolytic activity is required for cell separation

and decreased autolysin activity is associated with filament formation (McDonald 1971; Langsrud et al. 1987). The muramidase AcmA is the only autolysin in *Lc. lactis* and an *acm*A deletion mutant strain grew in long chains (Buist et al. 1995). Therefore, our microscopic observation of *Lc. lactis* may indicate that the biosynthesis or the activity of AcmA enzyme was impaired by high pressure. Chromosomal DNA stained by DAPI was observed in all cells, indicating that DNA was replicated and segregated irrespective of the morphological changes induced by high-pressure conditions.

High hydrostatic pressure has been shown to enhance the growth rate and produce a shift of the optimal temperature of growth from piezophilic bacteria (Canganella et al. 1997; Kato et al. 2000). High pressure and heat are parameters with opposite effects on membrane fluidity, and the physiological adaptation of piezophiles involves the regulation of the membrane fluidity by incorporation of increased proportions of poly- and monounsaturated fatty acids (Bartlett 2002; Allen and Bartlett 2000; Delong and Yayanos 1985). The extension of growth pressure with increased temperature in piezophiles may be attributed to this adaptive response with respect to the composition of the cytoplasmic membrane. Mesophilic bacteria adapt their membrane composition in response to shifts in growth temperature; however, they are unable to mount a specific pressure response in order to maintain membrane fluidity in high-pressure conditions (Allen and Bartlett 2000). Therefore, the piezotolerance of *Lc. lactis* MG 1363 and *Lb. sanfranciscensis* was not improved at temperatures above their optimal range (see this study).

Protein–protein interactions in multimeric enzymes, ribosomes, cytoskeleton proteins, and proteins that act in signal transduction pathways; are thought to be sensitive to increasing pressure. Hydrostatic pressure causes the dissociation of numerous multimeric proteins because the processes are typically accompanied by negative volume changes (Abe and Horikoshi 2000; Gross et al. 1993; Gross and Jaenicke 1994). Recently, Sato et al. (2002) studied cell division of *Escherichia coli* under high-pressure conditions. By the use of IFM it was shown that the macromolecular assembly of FtsZ to a cytokinetic ring was inhibited by high-pressure conditions. Furthermore, no chromosome segregation occurred under high-pressure conditions. Because the FtsZ ring assembly is an early event in cell division (Erickson 1997; Sun and Margolin 1998; Errington et al. 2003), Sato et al. (2002) suggested that the cells might be "frozen" at early stage in the cell cycle by pressure application. In contrast, the FtsZ protein of the deep-sea isolate *S. violacea* is functional at 50 MPa (Ishii et al. 2002). The presence of a gene encoding a putative FtsZ homolog in *Lc. lactis* MG1363 has previously been reported (Arnau and Sorensen 1997), and this is the first study to demonstrate that the FtsZ protein is functional in lactic acid bacteria at ambient pressure. In *Lc. lactis* cells grown under high-pressure conditions, cell segmentation and segregation of chromosomal DNA was apparent, indicating that the cytoskeletal assembly apparatus involved in cell division retained some of its functionality. Rapid re-assembly of the FtsZ ring after decompression, followed by fragmentation of filamentous cells within minutes, as observed in *E. coli* (Sato et al. 2002) was not observed in *Lc. lactis*. However, a strong reduction of the number of FtsZ rings in exponentially growing cells of *Lc. lactis* MG1363 under high-pressure conditions indicated that aggregation of the FtsZ protein was inhibited by high pressure. Thus, a pressure-mediated inhibition of the cytoskeleton assembly during cell division contributes to the growth arrest of *Lc. lactis* at high pressure. Generally, levels of FtsZ expression in *E. coli* and *Bacillus subtilis* are constant irrespective of the growth rate (Weart and Levin 2003) and sublethal heat stress induced overexpression of the *ftsZ*-gene in *Lc. lactis* MG1363 (Arnau and Sorensen 1997). The cell morphology of *Lc. lactis* MG1363 grown at elevated pressure resembles that of cells grown at supraoptimal temperatures, i.e., under growth conditions of *ftsZ* overexpression. Therefore, inhibition of FtsZ assembly rather than a reduced expression of the *ftsZ* gene are likely to explain the altered cell morphology.

High pressure induces unique stress responses in mesophilic bacteria, such as synthesis of specific proteins, accumulation of osmolytes, and changes in metabolism (Abe and Horikoshi 2000; Fujii et al. 1996; Iwahashi et al. 1997, 2000; Korakli et al. 2002; Molina-Gutierrez et al. 2002; Welch et al. 1993). Our work has shown that pressure alters the morphology and functionality of cytoskeleton proteins of lactic acid bacteria. This study provides new insights into pressure-induced response of lactic acid bacteria and lays the foundation for future processes in food biotechnology for altered properties of these organisms under high-pressure conditions.

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