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Full Length Research Paper

Physiological responses of salt stress and osmoprotection with proline in two strains of lactococci isolated from camel's milk in Southern Algeria

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The response of *Lactococcus* sp. strains isolated from camel milk to salt stress was characterized in M17 medium. We studied the growth of 20 strains in saline M17 medium, M17 medium containing various concentrations of NaCl (1.1, 1.2, 1.4, 1.6 and 1.7 M), and determined the minimal inhibitory concentration (MIC) of NaCl. Among the 20 studied strains, 2 strains presented a considerable growth in the presence of high concentrations of NaCl: The MIC reached 1.7 and 1.6 M for the CHT1 and CHT4 respectively. We studied the growth of 2 strains when proline was added in the medium, the bacteria actively accumulates proline from saline M17 medium. The results confirm the effectiveness of proline as an osmoprotectant. The optimal osmoprotection of both strains was obtained when concentration of proline is 70 mM. Analysis of the cellular content by thin layer chromatography (TLC) showed that internal concentration of proline was higher in cells grown in presence of salt. Among the responses to stress is the production of protein that ensured correct folding of new proteins and prevents aggregation of proteins altered, the SDS PAGE analysis of the proteins contents of two strains CHT1, CHT4, indicated the appearance of high and low molecular mass new proteins.

Keywords: Salt stress, bacterial growth, osmoregulation, proline, stress protein synthesis.

INTRODUCTION

Bacterial cells had developed powerful strategies to proliferate and survive under stressful conditions. The growth of bacteria depends on nutritional and environmental conditions that they meet. Microorganisms can develop in optimal way only in a range limited by physico-chemical factors (temperature, pH, salinity, etc). Stress responses of bacteria have been studied (Welch, 1993) and seemed to be implicated in important phenomena such as cellular survival, species perpetuation, and evolution of genera (Tamara, 1996). The optimal conditions for growth are rare when the bacteria are used in industry and these processes can then act as stress conditions as underlined by Kleerebezem et al. (2002). From an industrial point of view, it is important to select strains that perform well in fermentation and are resistant to the adverse conditions that occur during the fermentation process (Sanders et al., 1998).

One of the most powerful adaptative strategies that bacterial cells have evolved to counteract low activities of their growth media is the accumulation to high intracellular levels of a set of organic solutes (osmoprotectants) that are synthesized *de novo* or actively taken up from the growth medium (Csonka and Epstein, 1996). The exogenous osmoprotectants belong to a few classes of organic compounds that are neutral at physiological pH and compatible with cellular functions. In bacteria, the regulation of osmoprotectants transport was described and hence the transport systems were found to be induced or activated by high osmolarity of the medium (Perroud and Le Rudelier, 1985; Hutkins et al., 1987; Abee et al., 1990; Fougere and Le Rudelier, 1990). In lactic acid bacteria, the principal osmoprotectants are glycine betaine, carnitine and proline (Van Der Heide and Poolman, 2000). The osmoprotection with exogenous proline were

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described in many bacteria. Proline is also an osmoprotectant in bacteria which possess an osmotically induced or activated proline transport system (Bae and Miller, 1992; Jewell and Kashket, 1991; Milner et al., 1987; Townsend and Wdikinson, 1992). Some bacteria under osmotic stress increase the proline pool of endogenous origin by regulation of proline metabolism (Kawahara et al., 1989).

In addition, bacteria maintain protein homeostasis under normal conditions and during injury or stress using various mechanisms including the action of group of regulatory proteins called molecular chaperones (Ellis and Van der Vies, 1991). This adaptation phenomenon appears to involve multiple genes encoding stress proteins, which can be specifically induced by a particular stress factor (specific stress proteins) or induced by several conditions (general stress proteins). One of the major stress responses in lactic acid bacteria is the synthesis of proteins involved in the protection and repair of macromolecules. As with other bacteria, one of the major stress response systems in acid lactic bacteria is a protein quality control system including molecular chaperones and proteases (Sugimoto et al., 2008).

Few studies have been devoted to the camel milk and its microflora. The majority of studies conducted on camels concentrate mainly on its anatomical features and physiological adaptations to desert conditions. We are interested in lactic acid bacteria strains isolated from camel milk of southern Algeria. This milk is a little bit salted in comparison with others milks (example from cow, goat or sheep), likely due to preferential and large consumption by camel of common salt-tolerant plant *Limoniastrum guyonianum* in the region of Timimoun, Southern Algeria. Several strains of *Lactococcus* sp. isolated from camel's milk of Timimoun are able to resist to salt stress (Zadi-Karam and Karam, 2006). The ability to grow in media containing up to 1.2 M NaCl is a known major trait that characterizes enterococci but not others lactic acid bacteria (Mundt, 1986). Enterococci are ubiquitous microorganism that may be responsible for diseases, although, some strains also participate in the ripening of cheeses and in the stimulation of the growth of *Lactobacillus* and of other naturally lactic bacteria present in this environment.

In this study, we reported the behavior of *Lactococcus* sp. CHT1 and CHT4 under hypersaline conditions. We also characterized the uptake of several osmoprotectants and their efficiency in growth recovery. In addition, the influence of the most effective osmoprotectant, proline, was studied on both salt-induced cross protection and stress protein synthesis.

MATERIALS AND METHODS

Bacterial strains and conditions of culture

We used 20 strains of *Lactococcus* sp. isolated from milk of female camel of Timimoun, Southern Algeria (Karam and Karam, 2006). The identification of the strains growing in the presence 1.1 M NaCl and at 45°C was determined using the API 20 Strep identification microsystem. Identification of Lactococci resistant to 1.1 M encountered among the NaCl-resistant shell of salt concentration was confirmed by electrophoresis analysis of soluble proteins in denaturing conditions, and in comparison with reference strains (Karam and Karam, 2006).

Bacteria were cultured in M17 medium (Terzaghi and Sandine, 1975). M17 contained (per liter distilled water) 2.5 g tryptic casein peptone, 2.5 g pepsin meat peptone, 5 g soy papain peptone, 2.5 g yeast extract, 5 g meat extract, 19 g sodium glycerophosphate, 0.25 g magnesium sulfate, 0.5 g ascorbic acid and 5 g lactose. The pH of medium was adjusted to 7.2 with 0.1 M NaOH. The osmolarity of the medium was increased by addition of NaCl (1.1, 1.2, 1.4, 1.6 or 1.7 M) and bacteria were grown at 30°C. Growth was monitored by measuring OD_{600nm} (UV-Vis Jasco V530). Minimal inhibitory concentration (MIC) of NaCl was determined as the lowest concentration that prevents bacterial growth.

Osmoprotection by proline

Lactococcus sp. was grown at 30°C without shaking in 5 ml of M17 medium containing the MIC of NaCl (MIC of NaCl for CHT1 and CHT4 were 1.7 and 1.6 M, respectively) and the proline as osmoprotectant at 40, 50, 60, 70 or 100 mM. After 72 h of incubation at 30°C, the growth was estimated by measuring absorbance at 600 nm and the optimal concentration of proline was determined.

Protein extraction and analysis

Strains were grown as aforementioned to an OD_{600nm} of 1 in M17 medium and were harvested by centrifugation (7000 g, 15 min). The pellet obtained was resuspended in 300 µL of sterile distilled water. The cellular lyses were obtained after 30 cycles of freezing (1 h at -20°C) – defrosting (30 min at 30°C) – stirring (Assistant Reamix 2789, 2 min, 2500 rpm). Lysis extent was followed by measuring absorbance at 600 nm. After centrifugation (12000 g, 15 min), the supernatants were recuperated and quantified for proteins by the method described by Bradford (1976). The protein profiles of strains cultured with or without stress conditions were compared by SDS-PAGE on a 10% polyacrylamide gel according to Laemmli (1970). An amount of 20 µg of protein of each sample was deposited in a well of the gel with a micro-syringe. After electrophoresis, gels were stained with Coomassie blue and then discolored with acetic acid-methanol solution.

Analysis of organic solutes

Thin layer chromatography was employed to look for the accumulation of proline in bacteria exposed to salt stress. Samples of 3 µL of supernatants were spotted on a silica gel (Kieselgal 60F 254 Merck) and migration was done using 80:20:20 solvent mixture of *n* butanol/acetic acid/water. After migration, the gel was air dried. Amino acids spots were revealed with a ninhydrin solution (0.2% in a 4:1 mixture acetic acid-ethanol).

RESULTS AND DISCUSSION

Bacterial growth in saline conditions

Sensitivity of strains to NaCl is shown in Figure 1. Growth in M17 medium without NaCl (control) depended on the strain; most of them showed a good growth (OD_{600nm} >1),

Figure 1. Growth of bacteria according to saline concentrations.

while a few (CHT8, CHT11, CHT14 and CHT18) showed a limited growth (OD_{600nm} up to 0.8). Additions of 1.1 or 1.2 M NaCl to the M17 medium affected growth of most strains, but slightly modified growth of only CHT1 and CHT4. For concentration of 1.4 M NaCl few strains grew, some strains do not grow at 1.6 M NaCl, while for others growth inhibition was at 1.7 M NaCl (Figure 1).

As shown in Figure 2, the most important growth in the salt stress conditions was obtained with strains CHT1 and CHT4. After 72 h incubation, we observed the growth of

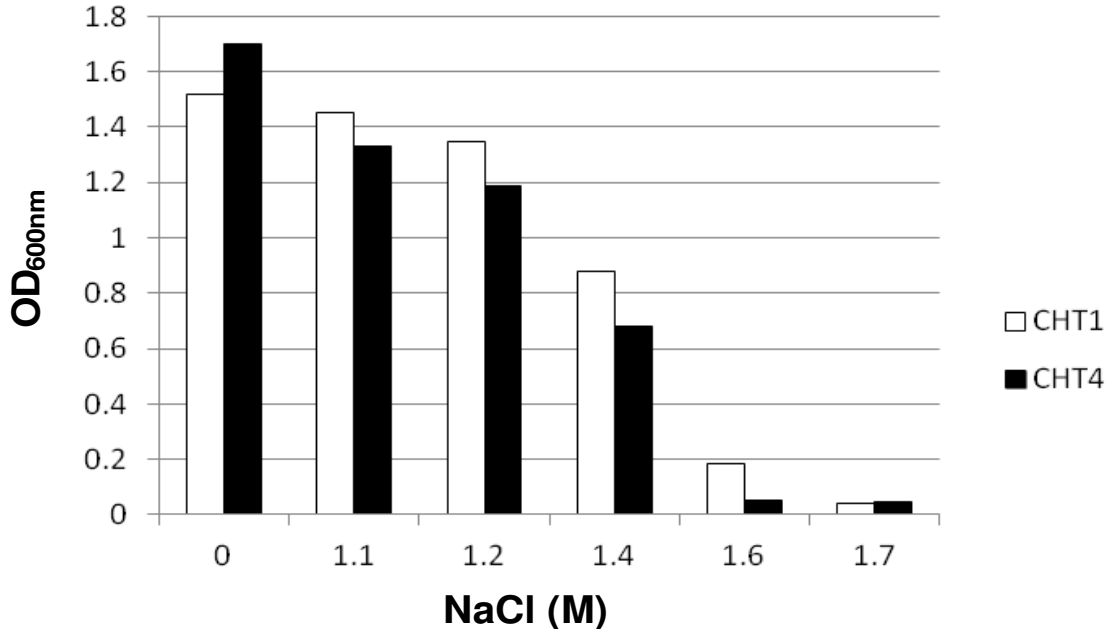

Figure 2. Growth of CHT1 and CHT4 strains in various concentrations of NaCl.

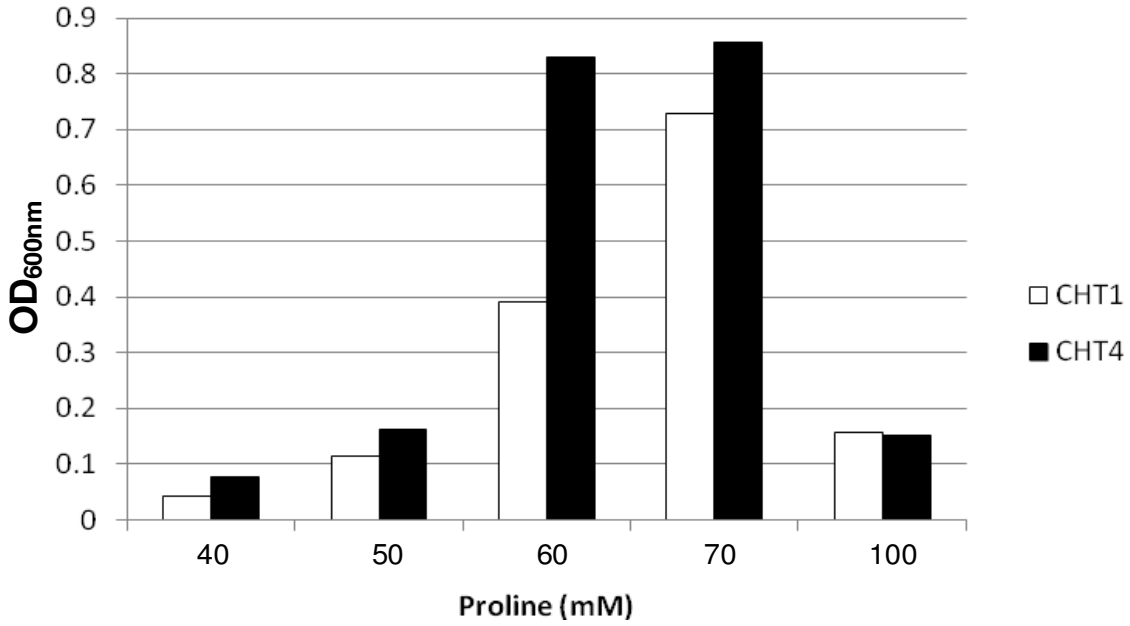

Figure 3. Effects of proline on stress and cultures.

these two strains CHT1 and CHT4 at 1.1, 1.2 and 1.4 M NaCl and found that MIC of NaCl for CHT1 and CHT4 were 1.7 and 1.6 M, respectively.

Effect of osmoprotectant on bacterial growth

Lactic acid bacteria are not capable of synthesizing osmoprotectant in situations of osmotic stress (Romeo et al., 2003). Addition of proline to the various salt concentrations used in the medium made it possible to raise growth inhibition; the concentration of NaCl for CHT1 and CHT4 were 1.7 and 1.6 M, respectively (Figure 3). The higher level of bacterial growth was observed for the strains with 70 mM proline. These results confirm the osmoprotectant effect of proline and corroborate with

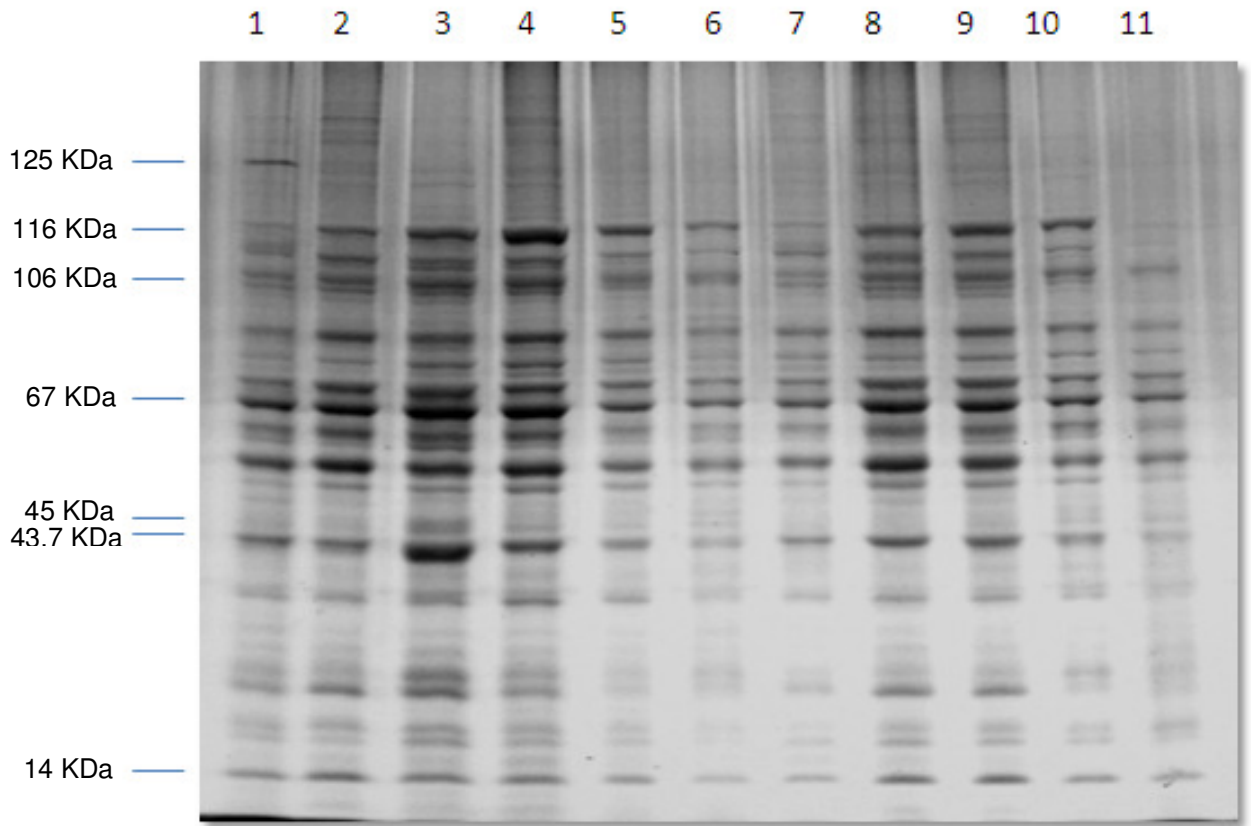

Figure 4. Protein profiles from CHT1 and CHT4 cells under normal and stress conditions. Lanes 1 to 6: Proteins from CHT1; lanes 7 to 11: Proteins from CHT4. Lane **1:** Free salt strain**;** lane **2:** 1.1 M NaCl**;** lane **3:** 1.2 M NaCl; lane **4:** 1.4 M NaCl; lane **5:** 1.6 M NaCl; lane **6:** 1.7 M NaCl and 70 mM proline; lane **7:** free salt strain; lane **8:** 1.1 M NaCl; lane **9:** 1.2 M NaCl; lane **10:** 1.4 M NaCl**;** lane **11:** 1.6 NaCl and 70 mM proline.

those of Romeo et al. (2001) who noted that proline and glycine betaine ensured a good osmoprotection in lactic acid bacteria.

Variation of cell protein content

Proteins contents of CHT1 and CHT4 cells grown in free salt or saline M17 medium, with or without osmoprotectant, were compared by electrophoretical analysis (Figure 4). We observed the disappearance of a 125 kDa protein when the bacteria CHT1 was under salt stress and the appearance of a 45 kDa protein when bacteria were grown in 1.2 M NaCl. We also observed also the appearance of a 106 kDa new protein when the strain CHT4 was under 1.1 and 1.2 M NaCl. For both strains we noticed that the intensity of protein concentrations increases with salt (116, 67 and 43.7 kDa) and this intensity decreases in the presence of proline.

Our results indicate differences in terms of protein content of *Lactococcus* sp. CHT1 and CHT4 under different salt stress conditions: There was production of some new proteins not present in the free salt medium (for instance proteins 106 and 45 KDa) and inhibition of production of some others proteins that were produced in the free salt medium (for instance proteins 125 KDa). There was also an increase, as well as a decrease in the level of expression of some proteins (for instance proteins 116, 67, 43.7 KDa). All these differences may be directly associated with the bacterial response of to salt stress. The production of novel proteins or the increased production of already existing proteins, which are only produced under stress conditions, is responsible for stress responses. The decrease in production or the inhibition of production of certain proteins is most probably the result of high levels of proteins modification or gene regulation, caused by a decrease in metabolic activity.

Variation of cell amino acid content

We compared the cellular amino acid content of CHT1 and CHT4 cells grown at 30°C in salt free or saline M17 (different concentrations and MIC of NaCl) medium supplemented with 70 mM proline (Figure 5). Comparison of cellular content led to the following observations: Both strains exhibited almost the same cellular content when grown in different conditions. Four spots (called A, B, C

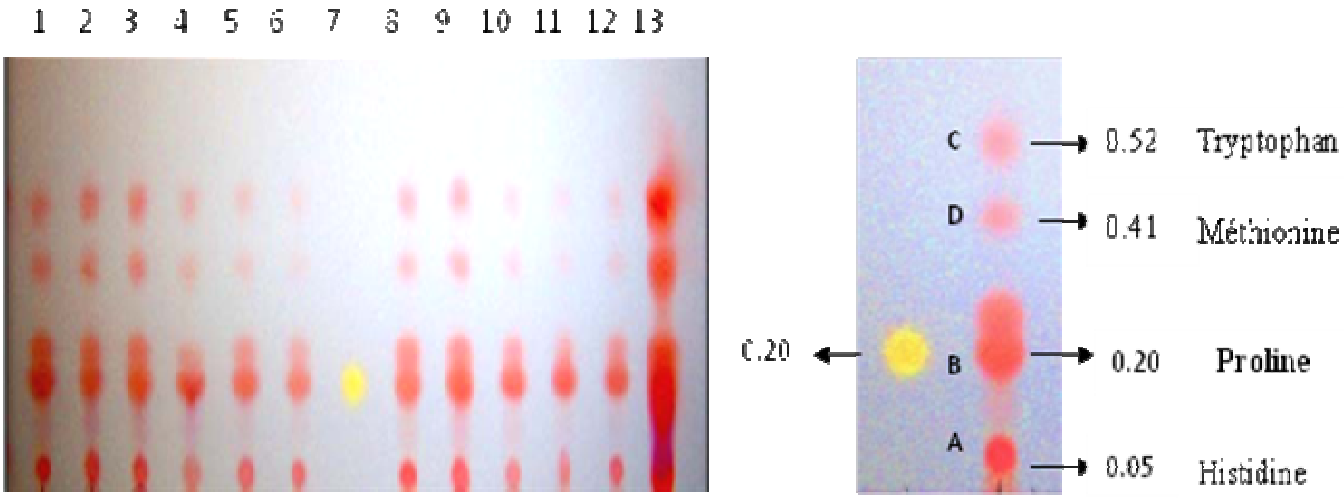

Figure 5. Analysis of amino acids content by TLC. Lanes 1 to 6: CHT1 extracts; lanes 8 to 12: CHT4 extracts. Lane **1**: Free Salt; lane **2**: 1.1 M NaCl; lane **3**: 1.2 M NaCl; lane **4**: 1.4 M of NaCl; lane **5**: 1.6 M NaCl; lane **6**: 1.7 M NaCl and 70 mM proline; lane **7**: PURE proline; lane **8**: Free salt; lane **9**: 1.1 M NaCl; lane **10**: 1.2 M NaCl; lane **11**: 1.4 M NaCl; lane **12**: 1.6 M NaCl and 70 mM proline; lane **13**: Sterile M17 medium. A, B, C and D indicate position of histidine, proline, tryptophan and methionine, respectively and refer to their Rf. Yellow spot (Rf = O.20) indicates proline spot.

and D) are found within the two strains, corresponding respectively to: A histidine (Rf = 0.05), B proline (Rf = 0.20), C methionine (Rf = 0.41), and D tryptophan (Rf = 0.52).

Studies on lactic acid bacteria showed that there is no pathway for biosynthesis of compatible solutes in these bacteria (Romeo et al., 2001). Hence, it seems that proline, which was detected in our samples, is exogenous and that its accumulation is done from the culture medium (M17). The presence of proline inside the cells in the absence and presence of salt confirms that bacteria use proline for growth and also for osmoprotection. More also, the presence of proline in free salt strains is related to the metabolism of lactic acid bacteria through their specific peptidases for proline peptide release (Monnet, 1993).

Conclusion

Thus far, two strains were isolated from camel's milk in Timimoun (southern Algeria), a little salty environment, and identified as Lactococci. Our study shows that these strains are able to grow in saline medium up to 1.4 M NaCl. This unexpected result is in part due to their ability to accumulate exogenous proline that acts as an osmo-protectant, and also to synthesize a variety of "salt stress'' proteins. The relationship between synthesis of these proteins and salt resistance, however, need to be further analysed.

REFERENCES

Abee T, Palmen R, Hellingwerf KJ, Konings WN (1990). Osmoregulation in *Rhodobacter sphaeroides*. J. Bacteriol. 172: 149-154.

Bae JH, Miller KJ (1992). Identification of two proline transport systems in *Staphylococcus aureus* and their possible roles in osmoregulation. Appl. Environ. Microbiol. 58: 471-475.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantifies of protein utilizing the principle of protein-dye binding. Anal. Biochem.72: 248-254.

Csonka LN, Epstein W (1996). Osmoregulation. In *Escherichia coli and Salmonella. Cell. Molec. Biol.* Washington: American Society for Microbiology. pp. 1210-1223.

Ellis RJ, Van Der Vies SM (1991). Molecular chaperones. Annu. Rev. Biochim. 60: 321-347.

Fougere F, Le Rudelier D (1990). Uptake of glycine betaine and its analogs by bacteroids of *Rhizobium meliloti*. J. Gen. Microbiol. 136: 157-163.

Hutkins RW, Ellefson WL, Kashket ER (1987). Betaine transport imparts osmotolerance on a strain of *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 53: 2275-2281.

Jewell JB, Kashket ER (1991). Osmotically regulated transport of proline by *Lactobacillus acidophilus* IFO-3532. Appl. Environ. Microbiol. 57: 2829-2833.

Zadi-Karam H, Karam ND (2006). Bactéries lactiques du lait de chamelle d'Algérie : mise en évidence de souches de *Lactococcus* résistantes au sel. *Tropicultura*, 24(3): 153-157.

Kawahara Y, Ohsumi T, Yoshihara Y, Ikeda S (1989). Proline in the osmoregulation of *Brevibacterium lactofermentum*. Agric. Biol. Chem. 53: 2475-2479.

Kleerebezem M, Boels IC, Groot MN, Mierau I, Sybesma W, Hugenholtz J (2002). Metabolic engineering of *Lactococcus lactis:* the impact of genomics and metabolic modeling. J. Biotechnol. 98: 199-213.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680-685.

Milner JL, McClellan DJ, Wood JM (1987). Factors reducing and promoting the effectiveness of proline as an osmoprotectant in *Escherichia coli* K12. J. Gen. Microbiol. 133: 1851-1860.

Monnet V (1993). Les péptides des lactocoques. Le lait. 73: 97-108.

Mundt JO (1986). Enterococci, In: Bergey's manual of systematic bacteriology, Baltimore, USA:Williams and Wilkins, 2: 1063-1065

Perroud B, Le Rudelier D (1985). Glycine betaine transport in *Escherichia coli:* osmotic modulation. J. Bacteriol. 161: 393-401.

Romeo Y, Bouvier J, Gutierrez C (2001). La réponse au stress osmotique des bactéries lactiques *Lactococcus lactis* et *Lactobacillus plantarum*. INRA. EDP Sciences. Lait. 81: 49-55.
Romeo Y, Bouvier J, Gutierrez C ,Bouvier I (2003). Osmotic stress response of lactic acid bacteria. In minerals and dairy products. F. Gaucheron ed; Technical and Doc, pp. 645-661.
Sanders JW, Venema G, Kok J, Leenhouts K (1998). Identification of sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene. Mol. Gen. Genet. 257: 681-685.
Sugimoto S, Kenji S, Al-Mahin A (2008). Molecular chaperones in lactic acid bacteria: physiological consequences and biochemical properties. J. Biosci. Bioen. 106(4): 324-336.
Tamara (1996). Du nouveaux sur l'origine des espèces. *La Recherche* (Paris). 291:52–59.
Terzaghi BE, Sandine WE (1975). Improved medium for *lactic streptococci* and their bacteriophage. Appl. Microb. 29: 807-813.
Townsend DE, Wdikinson BJ (1992). Proline transport in *Staphylococcus aureus*-a high-affinity system and a low-affinity system involved in osmoregulation. J. Bacteriol. 174: 2702-2710.
Van der Heide T, Poolman B (2000).Osmoregulated ABC-transportsystem of *Lactococcus lactis* senses water *via* changes in the physical state of the membrane. Proc. Nalt. Acad. Sci., USA.97:7102-7106.
Welch W (1993). How cells respond to stress. Sci. Am. 268: 34-41.