Acta Alimentaria, Vol. 32 (4), pp. 373–381 (2003)

EFFECTS OF PASTEURIZING LEVELS OF HIGH HYDROSTATIC PRESSURE ON *BACILLUS SUBTILIS luxAB* SPORES

J. FARKAS*, É. ANDRÁSSY, A. SIMON and L. MÉSZÁROS

Department of Refrigeration and Livestock Products' Technology, Szent István University,
H-1118 Budapest, Ménesi út 45. Hungary

(Received: 12 February 2003; accepted: 1 July 2003)

A recombinant *Bacillus subtilis* strain containing a plasmid encoding a *luxAB* fusion, which gave bioluminescence upon addition of an exogenous long-chain aldehyde as substrate for the endogenous luciferase enzyme, was used as test organism. Its populations were treated with 300 MPa for 20 min, or 600 MPa for 20 min at around room temperature, and this treatment is foreseen as a quality-friendly, non-thermal pasteurisation of foods. Besides the estimation of viable cell counts, the extent of pressure-induced germination and post-process development were investigated by phase-contrast microscopy, turbidimetry and luminometry. Increased heat sensitivity of pressurized spore populations was observed both by viable cell counting during a linearly programmed elevation of temperature and a simultaneous differential scanning calorimetry. This was related to pressure-induced germination of spores, although a small fraction remained ungerminated. The luciferase pool built into the spores during their formation seemed to have withstood pressurization. Spore germination was accompanied by the emergence of bioluminescence which also indicated sensitively the characteristic changes of metabolic activity running parallel with the development of untreated cell populations and that of the survivors of the hydrostatic pressure treatments when the cells were incubated in a nutrient broth.

Keywords: *Bacillus subtilis, luxAB* recombinant, high hydrostatic pressure, spore germination, bioluminescence, differential scanning calorimetry

Bacterial spores present the greatest challenge for inactivation by high hydrostatic pressure (HHP), one of the new non-thermal food preservation techniques. It was observed already by the scientists who pioneered food preservation by HHP treatment that bacterial spores survive the direct application of even extremely high (more than 1000 MPa) hydrostatic pressures (JOHNSON & ZOBELL, 1949; TIMSON & SHORT, 1965). However, at certain intermediate pressure levels (several hundred MPa), the treatment may initiate germination of spores, thereby making them heat sensitive (CLOUSTON & WILLS, 1969; GOULD & SALE, 1970). It is assumed that this effect is the result of increased solvation of spore components (HOOVER, 2002).

As the light emission phenomenon is a sensitive expression of bioluminescent microorganisms, we attempted to use spores of a *luxAB* transformant of *Bacillus subtilis* to investigate pressure-induced spore germination. *LuxAB* bacteria do not possess a long-chain aldehyde as inner substrate which is needed for the expression of their

* To whom correspondence should be addressed.
Fax: +36-1-372-6321; e-mail: jfarkas@alarmix.net

0139-3006/2003/$ 20.00

luciferase activity, however, their bioluminescence can be induced proportionally with their viable cell counts if such aldehydes (e.g. nonanal) are added to their cultures (HILL et al., 1993; 1994). Because of lack of metabolism in the spore state, *luxAB* spores are dark even in presence of their luciferase content until rehydration of the spore protoplast, but this substrate induced bioluminescence appears in case of rehydration of the spore core connected with the germination process. The aim of our study was to determine the extent of how much the spore germination of *Bacillus subtilis luxAB* spores can be induced by medium levels of HHP treatments, foreseen as a quality friendly, non-thermal pasteurization of food (HASSAN et al., 2002; MOHÁCSI-FARKAS et al., 2002). The decrease of heat resistance as an effect of pressure-induced germination and post-germinative development of pressurized populations were investigated in comparison with non-pressurized controls.

1. Materials and methods

1.1. Test organism

A bioluminescent derivative of *Bacillus subtilis,* which contains a plasmid pSB340 encoding a *lux*AB fusion (WANG & DOI, 1984) was used as test organism. The plasmid confers also erythromycin resistance on the bacterium. The strain was obtained from the Department of Applied Biochemistry and Food Science of the University of Nottingham, Loughborough, U.K. Our previous studies with it (FARKAS et al., 2002) showed a good linear correlation between the bioluminescence intensity of its vegetative cultures in the range of 10^2–10^9 CFU ml^{-1} cell-concentrations.

1.2. Culture conditions and preparation of spore suspension

Spore populations were produced on agar-solidified plates of modified Schaeffer's medium with a mineral supplement (PRIEST, 1989). The inoculated agar plates were incubated at 30 °C until the cultures contained at least 99% phase-bright spores as examined by phase-contrast light microscopy. The spores were washed from the agar plates with cold, sterile distilled water and centrifuged in a Beckman J2-21 centrifuge at 4000×*g* for 20 min at 5 °C. The spore pellet was re-suspended and debris of sporangia was digested by filter-sterilised lysozyme of 0.1 mg ml^{-1} (Reanal, Budapest, Hungary) at 5 °C for 24 h. After the lysozyme treatment, the washing procedure was repeated 4-times and the spores were finally re-suspended in 1/15 Sörensen's phosphate buffer at pH 7.0.

1.3. Pressure treatment

The spore suspension was aseptically transferred in 8 ml portions into plastic pouches and heat sealed. These sealed pouches were pressurized using a "Food-Lab 9000" type pressure rig (Stansted Fluid Power Ltd., Stansted, U.K.). The pouches were submerged in a hydrostatic fluid medium of ethanol with 15% castrol oil. The temperature of the

pressurizing medium was kept between 10 and 25 °C by controlling the temperature of the pressure rig. 300 MPa for 20 min and 600 MPa for 20 min treatments were applied, respectively.

1.4. Estimation of survivors

Survivors were estimated in serial dilutions (diluent: 0.1% peptone and 0.9% NaCl in distilled water) by plating them into Difco nutrient agar and incubating them at 30 °C for 96 h.

1.5. DSC-studies

The buffered dense suspensions (approx. 10^{10} cells ml^{-1}) of untreated or pressurized spores, and for comparison those of vegetative cells, were investigated with a SETARAM "MicroDSC-III" microcalorimeter. Portions of 0.9 ml of the suspensions were dispensed into hermetically sealed stainless-steel sample holders of the micro-calorimeter interfaced to a computer for data acquisition and data processing, then heated from approx. 25 °C to about 110 °C at a scanning rate of 1.0 °C min^{-1}, using the same volume of cell-free buffer as a reference sample. After the first DSC run, the sample holders were cooled by the instrument to their initial temperature, and then re-heated at the same scanning rate as in the first run to investigate the reversibility of heat transitions observed at the first run.

Thermal death of untreated and pressurized cells parallel with the DSC experiments was estimated by heating buffered suspensions of approx. 10^8 CFU ml^{-1} under a programmed heat treatment in a NESLAB EX-110 type "thermostat" with a similar heating rate as in the micro-calorimeter. During this heat treatment sample aliquots were removed at pre-determined temperatures, cooled on ice and survivors were counted as described above.

1.6. Study of germination and post-germinative development of untreated and pressurized spore populations by microscopy, turbidimetry and luminometry

Post-processing germination and post-germinative development of untreated and pressurized spore populations were followed after inoculation into pH 7.5 Difco nutritent broth supplemented for plasmid maintenance with erythromycin (1 μl ml^{-1}, Merck 1.8890215) and incubation at 30 °C using a water bath shaker (New Brunswick Co., type 676). Changes in the cell-state distribution of the cultures, as an effect of the pressure treatment and subsequently during the post-processing incubation in the nutrient medium, were estimated by phase-contrast microscopy, determining the proportion of the phase-bright (refractile) spores, the phase dark (germinated) spores, the outgrowing cells and the vegetative cells. Changes of absorbance of aliquots of the cultures were measured in a Spectronic Unicam Helios-β spectrophotometer at 520 nm, and the bioluminescence was monitored by measuring the light emission (luciferase activities) in triplicate samples by a Turner Mod. TD-20e luminometer, integrating the

light output values (mVs) for the first 10 sec as an effect of addition of 50 µl of 1% nonanal (Merck) solution (in EtOH) to 0.3 ml of aliquots taken from the culture of the test organism.

2. Results and discussion

2.1. Effects of pressurization on the viability and cell state of Bacillus subtilis luxAB *spores*

Viable cell counting showed that the HHP treatments of 300 MPa for 20 min and 600 MPa for 20 min reduced the viable cell counts of the spore suspension by approx. 17 and 23%, respectively. However, approx. 94 and 96% of the pressurized cells were unable to survive a heat treatment at 80 °C for 10 min after 300 MPa for 20 min and 600 MPa for 20 min, while untreated spores were heat resistant: surviving completely such a heat treatment (data not shown).

2.2. Development of untreated and pressurized spore populations in nutrient medium

Germination of *luxAB* spores runs parallel with their bioluminescence activity of the population because electron transport starts in a very early stage of germination (HILL et al., 1994). Results of our comparative phase-contrast microscopic, turbidimetric, and luminometric investigations during incubation of untreated or pressurized *Bac. subtilis luxAB* cell suspensions in nutrient broth at 30 °C are shown in Figs 1, 2 and 3. WUYTACK and co-workers (1998) found also comparable germination efficiencies of *Bacillus subtilis* spores under the pressure range of 100–600 MPa for 30 min at 40 °C.

The microscopic and the turbidimetric investigations showed in harmony that, unlike the untreated inoculum which contained refractile spores, the majority of the pressurized spores were phase-dark. Their suspension showed thereby a very much reduced turbidity. While the very low initial luminometric signal obtained with untreated spores was equal with the background level of the medium, the pressurized population gave initial bioluminescence levels which were already close to those of the non-pressurized samples after 20 min incubation in the nutrient broth. All three types of measurements reflected well the various stages of transition via outgrowth to vegetative growth. The development of the pressurized population lagged behind that of the untreated one, in accordance with the initial inactivation occurred during pressurization.

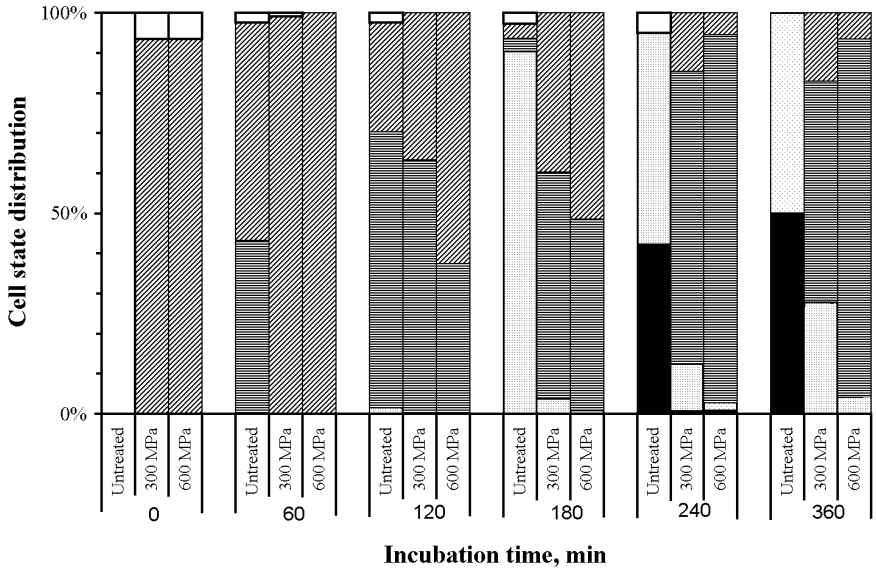

Fig. 1. Changes in cell-state distribution of untreated, at 300 MPa for 20 min and at 600 MPa for 20 min treated *Bacillus subtilis luxAB* spores during incubation in nutrient broth at 30 °C. □: Refractive spores; ▨: dark (germinated) spores; ▤: outgrowing cells; ▩: vegetative cells; ■: vegetative growing cells

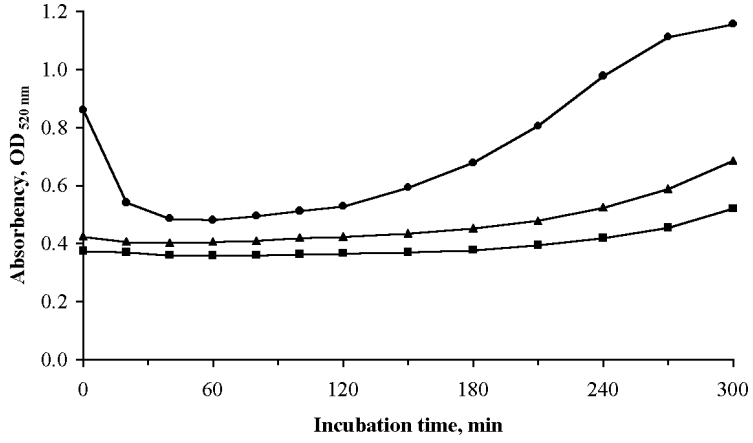

Fig. 2. Turbidimetric signal of untreated, at 300 MPa for 20 min and at 600 MPa for 20 min treated *Bacillus subtilis luxAB* spores during incubation in nutrient broth at 30 °C. ●: Untreated; ▲: at 300 MPa for 20 min treated; ■: at 600 MPa for 20 min treated

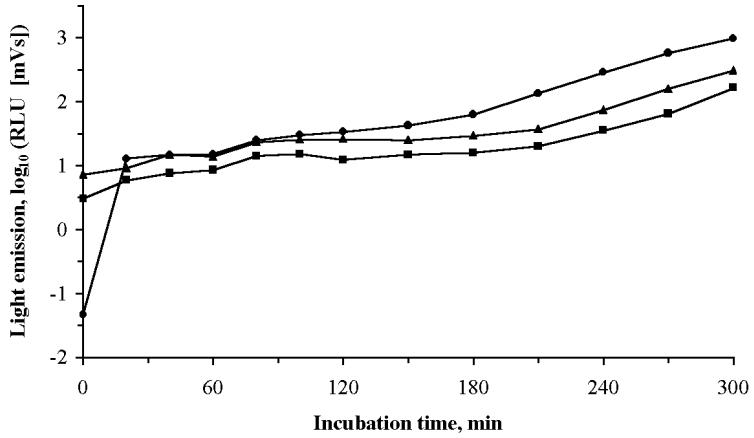

Fig. 3. Luminometric signal of untreated, at 300 MPa for 20 min and at 600 MPa for 20 min treated *Bacillus subtilis luxAB* spores during incubation in nutrient broth at 30 °C. ●: Untreated; ▲: at 300 MPa for 20 min treated; ■: at 600 MPa for 20 min treated

2.3. DSC thermograms and heat destruction measurements

The difference thermograms obtained by subtracting the re-scans from the initial scans reflected distinct differences between the untreated and the pressurized suspensions (Fig. 4). The DSC sample of the untreated spore suspension showed only two endothermic peaks. The first endothermic transition commenced at about 95 °C. The pressurized spore suspensions had a slowly increasing exothermic heat flux with a maximum at 52–53 °C, probably representing a temperature range of respiration activity of the germinating spores, and an endothermic peak at around 73–75 °C, resembling somewhat the major endothermic transition found by DSC of the vegetative cells.

The losses of colony forming abilities of the non-treated and the pressurized suspensions as a function of the temperature elevation during the course of the DSC scanning are shown in Fig. 5. These studies revealed that about 20% increase of the colony counts occurred in the non-pressurized samples in the 50–80 °C range, probably as a heat activation effect, whilst a drastic reduction of the viable cell counts started only after the temperature reached 95 °C (i.e. simultaneously with their first DSC-endotherm, see Fig. 4). In the pressurized suspensions a considerable portion of the population became non-viable already from 50–60 °C onward (interestingly, more in the 300 MPa-samples than in the 600 MPa-samples) and the majority (more than 90%) of the population became non-viable when the temperature reached 85 °C.

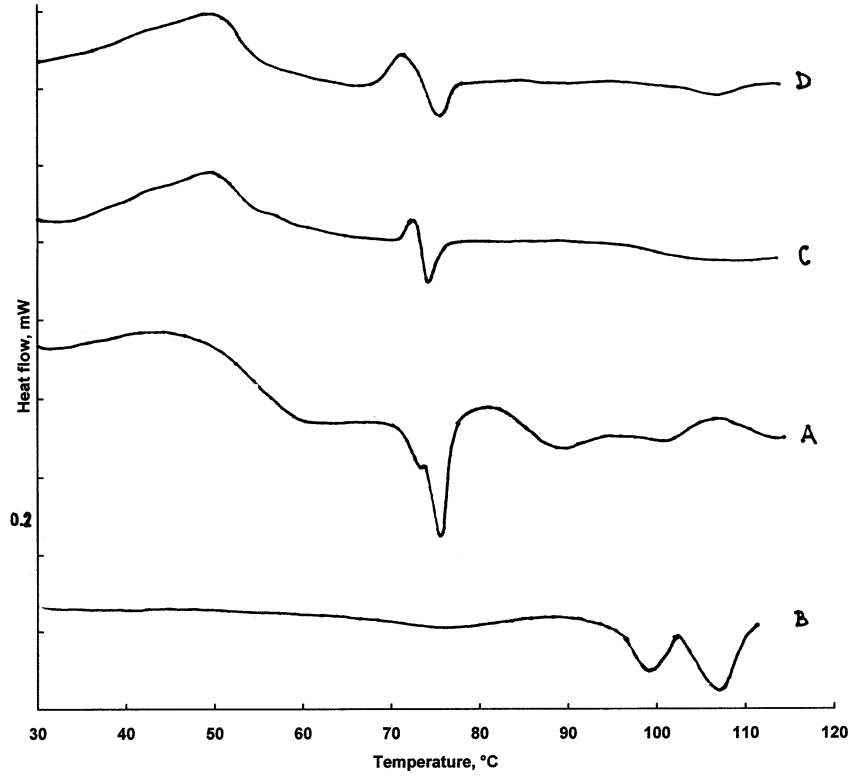

Fig. 4. DSC thermograms of HHP treated spore suspensions as compared to those of untreated suspensions of spores and vegetative cells. A: vegetatives; B: spores, untreated; C: spores, 300 MPa for 20 min treated; D: spores, 600 MPA for 20 min treated

The step-wise character of the loss of viability in the pressurized populations can be explained by their heterogeneous cell-state distribution. These observations are similar to the findings of WUYTACK and co-workers (1998) who observed also that pressure-germinated *Bacillus subtilis* spores were rapidly inactivated at 55 °C, and the low pressure (100 MPa)-germinated spores were qualitatively different from the high pressure (600 MPa)-germinated spores.

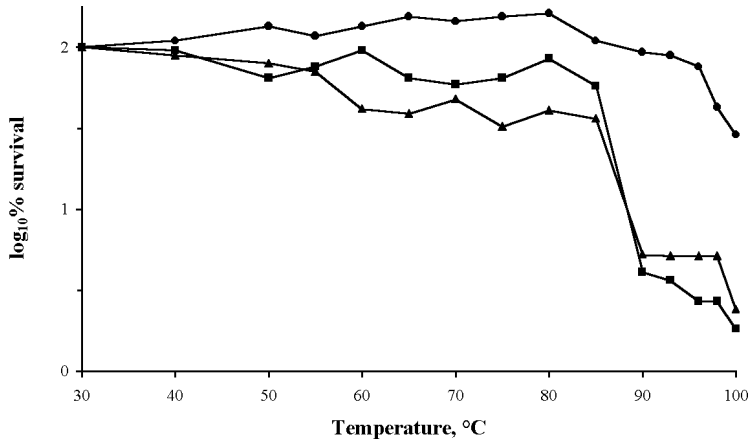

Fig. 5. Survival in untreated and HHP-treated spore suspensions in the course of a programmed elevation of temperature from 30 to 100 °C. ●: Untreated; ▲: at 300 MPa for 20 min treated; ■: at 600 MPa for 20 min treated

3. Conclusions

Results of our studies confirm earlier observations of other authors (CLOUSTON & WILLS, 1969; GOULD & SALE, 1970) on induction of germination of bacterial spores at HHP levels which results in a pasteurizing effect of various foods (HASSAN et al., 2002; MOHÁCSI-FARKAS et al., 2002). However, it was also shown in accordance with earlier observations (SALE et al., 1970) that a "superdormant" fraction of spores remained in their original resting state in spite of the pressurization levels applied. DSC thermograms taken with untreated and pressurized spore suspensions in comparison with thermogram of vegetative cells reflected the heat sensitizing effect of the pressure treatment due to the induction of spore germination. The molecular mechanism of the spore-germinative effect of pressure is not known and elucidation of the mechanism may help in choosing a more logical method for attacking the spores (GOULD, 1995). The bioluminescence testing was able to reflect initiation of metabolic activity parallel with the pressure-induced germination. The heat sensitive luciferase pool built in during sporulation seemed to withstand pressurization without inactivation. Intracellular enzymes may vary widely in their ability to withstand HHP and the barotolerance of any particular enzyme will depend on the composition of the system and temperature of treatment (SIMPSON & GILMOUR, 1997). The measurement of

bioluminescence indicated sensitively the characteristic changes of metabolic activity running parallel with the development of untreated spore populations and that of the survivors of the HHP treatment.

*

The authors thank the National Scientific Research Fund (OTKA) of Hungary for financial support (grant no. T 032715) and are grateful to Dr. P.J. HILL and the late Prof. G.S.A.B. STEWART, Dept. of Applied Biochemistry and Food Science, Univ. Nottingham, Loughborough, Leics., U.K. for providing them with the test organism.

References

CLOUSTON, J.G. & WILLS, P.A. (1969): Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. *J. Bacteriol., 97,* 684–690.

FARKAS, J., ANDRÁSSY, É., FORMANEK, Z. & MÉSZÁROS, L. (2002): Luminometric and differential scanning calorimetry (DSC) studies on heat- and radiation inactivation of *Bacillus subtilis luxAB* spores. *Acta microbiol. immunol. Hung., 49,* 155–164.

GOULD, G.W. (1995): The microbe as a high pressure target. -in: LEDWARD, D.A., JOHNSTON, D.E., ERNSHAW, R.G. & HASTINGS, A.P. (Eds) *High pressure processing of foods.* Nottingham University Press, Nottingham, pp. 27–35.

GOULD, G.W. & SALE, A.T.H. (1970): Initiation of germination of bacterial spores by hydrostatic pressure. *J. gen. Microbiol., 60,* 335–346.

HASSAN, Y., MÉSZÁROS, L., SIMON, A., TUBOLY, E., MOHÁCSI-FARKAS, CS. & FARKAS, J. (2002): Comparative studies on gamma radiation and high pressure induced effects on minced beef. *Acta Alimentaria*, *31,* 253–264.

HILL, P.J., REES, C.E.D., WINSON, M.K. & STEWART, G.S.A.B. (1993): Review: the application of *lux* genes. *Biotechnol. appl. Biochem., 17,* 3–14.

HILL, P.J., HALL, L., WINCOMBE, D.A., SOPER, C.J., SETLOW, P., WAITES, W.M., DENYER, S. & STEWART, G.S.A.B. (1994): Bioluminescence and spores as biological indicators of inimical processes. *J. appl. Bacteriol., Symp. Supplem., 76,* 129S–132S.

HOOVER, D.G. (2002): Microbial inactivation by high pressure. -in: JUJENA, V.K. & SOFOS, J.N. (Eds): *Control of foodborne microorganisms.* Marcel Dekker, Inc., New York, pp. 419–449.

JOHNSON, F.H. & ZOBELL, C.E. (1949): The retardation and thermal disinfection of *Bacillus subtilis* spores by hydrostatic pressure. *J. Bacteriol., 57,* 353–358.

MOHÁCSI-FARKAS, CS., KISKÓ, G., MÉSZÁROS, L. & FARKAS, J. (2002): Pasteurisation of tomato juice by high hydrostatic pressure treatment or by its combination with essential oils. *Acta Alimentaria, 31,* 243–252.

PRIEST, F.G. (1989): Isolation and identification of anaerobic endospore-forming bacteria. -in: HARWOOD, C.R. (Ed.) *Biotechnology handbooks 2: Bacillus.* Plenum Press, New York, pp. 27–56.

SALE, A.J.H., GOULD, G.W. & HAMILTON, W.A. (1970): Inactivation of bacterial spores by high hydrostatic pressure. *J. gen. Microbiol., 60,* 323–334.

SIMPSON, R.K. & GILMOUR, A. (1997): The effect of high hydrostatic pressure on the activity of intracellular enzymes of *Listeria monocytogenes. Lett. appl. Microbiol., 25,* 48–53.

TIMSON, W.J. & SHORT, A.J. (1965): Resistance of microorganisms to hydrostatic pressure. *Biotechnol. Bioengng, 7,* 139–159.

WANG, P.Z. & DOI, R.H. (1984): Overlapping promoters transcribed by *Bacillus subtilis* sigma-55 and sigma-37 RNA polymerase holoenzymes during growth and stationary phases. *J. biol. Chem., 259,* 8619–8625.

WUYTACK, E.E., BOVEN, S. & MICHIELS, C.W. (1998): Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. *Appl. environ. Microbiol., 64,* 3220–3224.