APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2004, p. 635–637 Vol. 70, No. 1
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.1.635–637.2004

Combined Effects of High Hydrostatic Pressure and Temperature for Inactivation of *Bacillus anthracis* Spores

Cécile Cléry-Barraud,[1] Agnès Gaubert,[2] Patrick Masson,[1] and Dominique Vidal[2]*

Département de Biologie des Agents Transmissibles[2] and Département de Toxicologie,[1] Centre de Recherches du Service de Santé des Armées, 38702 La Tronche Cedex, France

Received 2 May 2003/Accepted 10 October 2003

Spores of *Bacillus anthracis* are known to be extremely resistant to heat treatment, irradiation, desiccation, and disinfectants. To determine inactivation kinetics of spores by high pressure, *B. anthracis* spores of a Sterne strain-derived mutant deficient in the production of the toxin components (strain RP42) were exposed to pressures ranging from 280 to 500 MPa for 10 min to 6 h, combined with temperatures ranging from 20 to 75°C. The combination of heat and pressure resulted in complete destruction of *B. anthracis* spores, with a *D* value (exposure time for 90% inactivation of the spore population) of approximately 4 min after pressurization at 500 MPa and 75°C, compared to 160 min at 500 MPa and 20°C and 348 min at atmospheric pressure (0.1 MPa) and 75°C. The use of high pressure for spore inactivation represents a considerable improvement over other available methods of spore inactivation and could be of interest for antigenic spore preparation.

Bacillus anthracis is a spore-forming gram-positive bacillus that causes anthrax. This zoonosis mostly affects herbivores. Occasionally humans contract the disease via the cutaneous route or via inhalation of aerosolized spores. The risk of biological warfare and bioterrorism is of increasing concern for governmental authorities, especially since the dissemination of spores through letters sent through the postal system. This caused 22 cases of human anthrax, with 5 fatal cases of inhalation anthrax (5). Consequently, research on anthrax prophylaxis and decontamination has been intensified. In this context, improved anthrax vaccine efficiency and spore inactivation methods are needed for safe antigenic preparation (2).

Bacterial spores can survive in the environment for an extraordinarily long time; they are in a dormant state and resist physical and chemical stress. Several techniques, alone or combined, have already been tested for the inactivation of *Bacillus* spores: heat, acid pHs, nisin, ultrasonic waves, irradiation, and high pressure (4, 11, 17). High-pressure technology has been used since 1990 in the food industry for preservation and food processing. High hydrostatic pressure has proven to be efficient for destruction of vegetative bacteria, viruses, and yeasts, with bacterial spores being more resistant to pressure (6, 9). Due to a renewed interest in methods of *B. anthracis* spore inactivation, UV resistance was recently revisited (10), but high hydrostatic pressure had never been applied to the inactivation of *B. anthracis* spores. The aim of this study was to evaluate the efficacy of high-pressure treatment, alone and in association with heat, on the inactivation of spores of *B. anthracis* strain RP42, derived from the Sterne strain. *B. anthracis* Sterne is an attenuated nonencapsulated variant. This strain does not have the pXO2 plasmid, which carries the capsule genes of the vegetative bacterium. The RP42 strain contains the pXO1 plasmid from which the genes for edema factor and lethal factor production were deleted. This avirulent strain is a valuable model for spore inactivation studies because only the plasmid virulence genes of the vegetative form are affected, whereas all chromosomal genes responsible for sporulation and germination are present. Thus, spore components and spore resistance of strain RP42 are assumed to be identical to those of the wild strain (14).

Bacterial strain and spore preparation. *B. anthracis* strain RP42 was used. This strain, kindly supplied by Michèle Mock (Institut Pasteur, Paris), is an unencapsulated and detoxified strain (14). This strain does not produce either edema factor or lethal factor; this was achieved by selective inactivation of the *cya* and *lef* genes carried by pXO1 (coding for edema factor and lethal factor, respectively) by insertion of antibiotic resistance cassettes. RP42 is avirulent for mice and totally devoid of toxic effect.

Sporulation was carried out in Roux bottles of agar (12.5 g liter^{-1}), supplemented with yeast extract (1 g liter^{-1}), meat extract (5 g liter^{-1}) and $MnSO_4$, H_2O (0.05 g liter^{-1}) in sterile distilled water. Roux bottles were inoculated with a young culture (6 h at 32°C), which was grown in nutrient broth and incubated for 8 days at 30°C. After that time, 90% sporulation was reached, as assessed by phase-contrast microscopy. Spores were collected by flooding the agar surface with 10 ml of sterile distilled water. After collection, spores were washed four times by centrifugation (5,000 × *g*, 15 min), suspended in sterile distilled water, and purified. The initial population of spores (N_0) (atmospheric pressure and room temperature) varied from 10^7 to 10^9 CFU ml^{-1}. Spore inactivation was expressed as $\log(N_t/N_0)$ versus pressurization time, where N_t is the number of CFU in pressure- and temperature-treated samples after time *t*.

High-hydrostatic-pressure treatment. The hydrostatic high-pressure equipment is composed of a thermostatic pressure vessel (Sofop, Olemps, France), a manual pump (Autoclave S.A., Nogent sur Oise, France), and a connection system. Experiments were performed at various pressures (280, 400, and 500 MPa) and temperatures (20, 45, and 75°C), with exposure

* Corresponding author. Mailing address: Département de Biologie des Agents Transmissibles, CRSSA, BP 87, F-38702 La Tronche Cédex, France. Phone: 33 (0) 4 76 63 69 19. Fax: 33 (0) 4 76 63 69 17. E-mail: domvidal@crssa.net.

times ranging from 10 to 360 min. Pressurization time does not include the come-up and the pressure release. The fluid used for pressure transmission was sterile distilled water. One hundred microliters of the spore suspension was transferred to a PCR Thermowell tube, and the volume was brought to 300 μl with sterile distilled water, with care taken to avoid enclosed air bubbles. Two samples containing the spore suspension were put into the pressure vessel, already thermally equilibrated. Results are expressed as the averages of six independent determinations of N_t.

Pressurized samples were serially diluted from 10^2- to 10^7-fold, and control samples were diluted to 10^7-fold. After treatment, enumeration of viable spores in both control samples and pressure-treated samples was done by plating the suspension on germination agar followed by incubation for 48 h at 30°C. The composition of the germination medium, tryptic soy agar from Difco Laboratories (Le Pont de Claix, France), was Tryptone peptone from pancreatic digestion (15 g liter^{-1}), Saytone peptone from soy papaya digestion (5 g liter^{-1}), NaCl (5 g liter^{-1}), and agar (15 g liter^{-1}) in sterile distilled water.

Results and discussion. Typical pressure-temperature inactivation curves of *B. anthracis* spores are given in Fig. 1. At 20°C, plots of $\log(N_t/N_0)$ versus time were linear whatever the pressure, indicating that pressure inactivation of spores follows a simple first-order kinetics as for inactivation at atmospheric pressure and 75°C (data not shown). The slope of the plots is the rate constant of inactivation, $-1/D$ where D is the time of pressurization required for inactivation of 90% of the spore population. Table 1 summarizes the D values. No inactivation of spores was observed at atmospheric pressure and 20 or 45°C up to 360 min of exposure. At 45 and 75°C, the kinetics of pressure inactivation of *B. anthracis* were biphasic and can be described by at least two exponential terms. A fast inactivation phase was followed by a slower process. At 45 and 75°C, only D values of the initial part of the curve were estimated because of lack of data and low sensitivity of the spore enumeration technique in the range 0 to 5 CFU for accurate estimation of the second part of the inactivation curve. Okazaki et al. (12) pointed out that survival curves for spores of *B. subtilis* do not obey the first-order kinetics. Raso et al. (15) also showed that high-pressure inactivation of *Bacillus cereus* spores, a species very close to *B. anthracis*, follows a multiple exponential kinetics. It has been hypothesized that a spore population, called superdormant, resists at prolonged treatments under 500 MPa and 75°C. Superdormant spores would be those which remain ungerminated in a medium which caused the germination of the majority of a spore population (15). This may suggest heterogeneous pressure-induced delayed germination population (18). It may be also hypothesized that resistant spores were spontaneous mutants induced by pressure. Indeed, Ludwig et al. obtained a spontaneous mutant of a *B. thuringiensis* strain by a single pressure treatment (7). These hypotheses have to be tested.

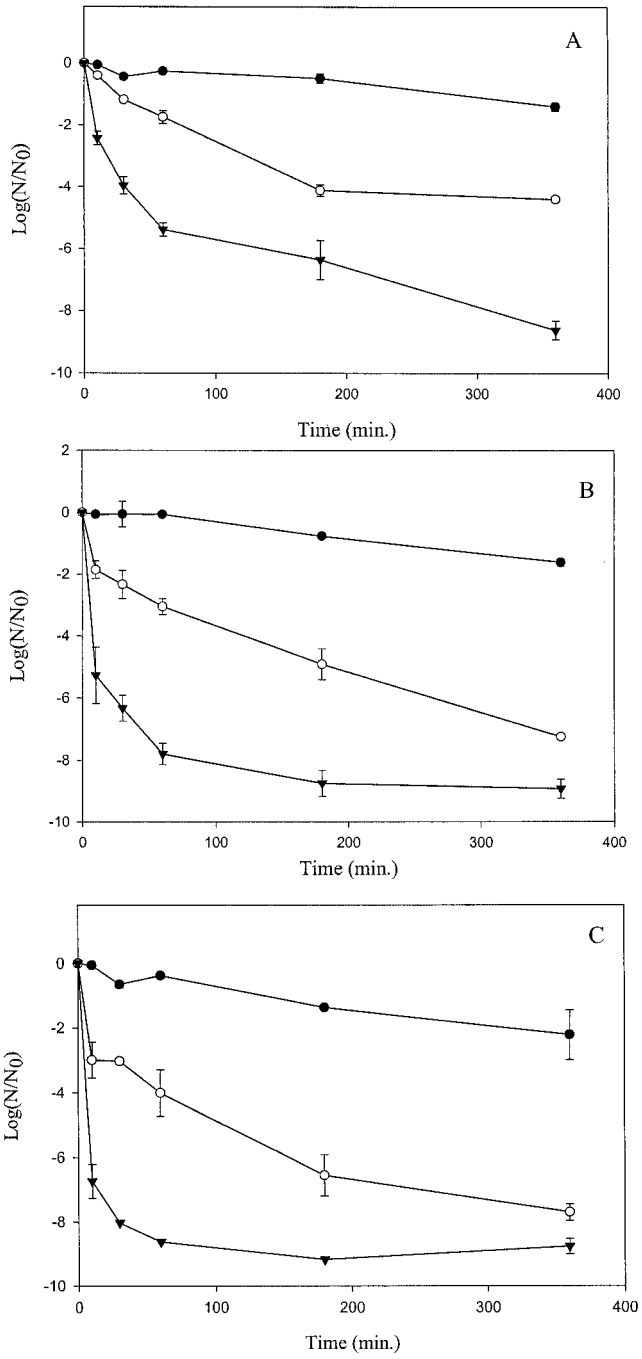

FIG. 1. Effect of temperature on spore inactivation at 280 MPa (A), 400 MPa (B), and 500 MPa (C). Shown are survival curves for pressure-treated *B. anthracis* spores at 20°C (●), 45°C (○), and 75°C (▼).

At a given temperature (20, 45, or 75°C) and after 360 min of treatment, high hydrostatic pressure increased the rate of spore inactivation. For example, at 45°C, the spore population was decreased by 4.4 log at 280 MPa, by 7.3 log at 400 MPa, and by 7.7 log at 500 MPa. In the same way, at 75°C, the spore population was decreased by 8.6 log at 280 MPa, 8.9 log at 400 MPa, and up to 9 log at 500 MPa. The association of high hydrostatic pressure with heat was synergistic. For example, the inactivation efficacy was better with a treatment at 280 MPa and 45°C than with a treatment at 500 MPa and 20°C.

High-pressure treatment exerts many effects on microorganisms arising from a single change in system volume. Pressure effects on microorganisms are complex and affect several biochemical processes, such as motility, substrate transport, cell

TABLE 1. Survival times for *B. anthracis* spores at various pressures and temperatures[a]

Pressure (MPa)	*D* (min) at:		
	20°C	45°C	75°C
0.1	No effect	No effect	348 ± 13
280	286 ± 34	35 ± 4	12 ± 2
400	218 ± 12	23 ± 2	9 ± 2
500	160 ± 59	19 ± 3	4 ± 2

[a] *D* value at 45 and 75°C were estimated only for the initial part of the inactivation curve, corresponding to a low-pressure-resistant spore population.

division, growth, DNA replication, translation, transcription, and viability (1). Bacterial spores are extremely resistant to pressure compared to vegetative forms (11). Spore inactivation by pressure is due to the induction of spore germination by pressure, with the germinated spores then being killed rapidly by pressure. The sporulation conditions, spore coat, relative impermeability of the spore core, presence of small acid-soluble proteins, and DNA repair are involved in spore resistance mechanisms depending on both the spore species and the physical-chemical perturbant (11). Sequential molecular mechanisms remain to be elucidated. However, *Bacillus* spores can be inactivated by hydrostatic pressure after induction of germination above a certain critical step related to the release of calcium and dipicolinic acid (DPA) (3). The DPA-calcium complex, involved in resistance to heat, electromagnetic radiations, and biocide chemical agents, plays a role in the protection of spore proteins against pressure-induced water penetration in the spore walls (11). The release of DPA begins under pressure of about 50 MPa and reaches a maximum at 110 MPa (6). Spores germinated under a pressure of 100 MPa become sensitive to UV and oxidizing agents. The molecular mechanism of the effect of high pressure on spores is linked to physicochemical modifications of proteins, changes in membrane permeability with rehydration of the spore, and flattening and lengthening of the spore. Moreover, it has been shown that spore germination in several species of *Bacillus* is induced by pressurization between 100 and 600 MPa (20, 21). The sensitivity of low-pressure (100-MPa)-germinated spores appears to be due to the degradation of α/β-type small acid-soluble proteins, which play an important role in the resistance of spores to extremely high pressure (600 MPa). Recently, it was shown that a pressure of 100 MPa induces spore germination by activation of the germinant receptors and a pressure of 550 MPa opens channels for the release of DPA from the spore core (13).

Our results confirm that the association of heat with pressure is very efficient for inactivation of *B. anthracis* spores, as has been shown for other *Bacillus* species spores (16). The high-pressure method represents a real improvement over other methods for the destruction of bacterial spores. In practice, sterility tests showed fluctuating values between 0 and 2 CFU even after 360 min of treatment. The populations of *B. cereus* and *B. anthracis* spores are likely heterogeneous, with different pressure resistances. Ludwig et al. (6) suggested the use of 1-min treatment cycles under pressure varying between 60 and 500 MPa at 60°C. Such a treatment is thought to favor germination of superdormant spores and thus their inactivation (19). Although implementation of the high-hydrostatic-pressure method is tricky and maintenance of equipment is costly, spore inactivation by pressure could be applied to antigenic preparation of spore-based anthrax vaccine (2, 8) as an alternative to the formaldehyde-inactivated spore technology.

We thank Françoise Desor and Didier Riou for excellent technical assistance.

This work was supported by Ministry of Defense grant 3-b/LR/EMA.

REFERENCES

1. **Bartlett, D. H.** 2002. Pressure effects on in vivo microbial processes. Biochim. Biophys. Acta **1595:**367–381.
2. **Brossier, F., M. Levy, and M. Mock.** 2002. Anthrax spores make an essential contribution to vaccine efficacy. Infect. Immun. **70:**661–664.
3. **Clouston, J. G., and P. A. Wills.** 1969. Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. J. Bacteriol. **97:** 684–690.
4. **Delacour, H., C. Cléry, P. Masson, and D. R. Vidal.** 2002. Inactivation des spores bactériennes par les hautes pressions hydrostatiques. Ann. Pharm. Fr. **60:**38–43.
5. **Inglesby, T. V., T. O'Toole, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. M. Friedlander, J. Gerberding, J. Hauer, J. Hughes, J. McDade, M. T. Osterholm, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2002. Anthrax as a biological weapon, 2002. Updated recommendations for management. JAMA **287:**2236–2252.
6. **Ludwig, H., G. van Almsick, and B. Sojka.** 1996. High pressure inactivation of micro-organisms, p. 237–244. *In* R. Hayashi and C. Balny (ed.), High pressure bioscience and biotechnology. Elsevier, Amsterdam, The Netherlands.
7. **Ludwig, H., G. K. G. Werner, E. Schattman, and M. Schauer.** 2002. High pressure induced alterations in morphology and cell characteristics of the bacterium *Bacillus thuringiensis*, p. 303–310. *In* R. Hayashi (ed.), Trends in high pressure bioscience and biotechnology. Elsevier Science, Amsterdam, The Netherlands.
8. **Masson, P., and C. Tonello.** 2000. Potential application of high pressures in pharmaceutical science and medicine. High Pressure Res. **19:**223–231.
9. **Nakayama, A., Y. Yano, S. Kobayashi, M. Ischikawa, and K. Sakai.** 1996. Comparison of pressure resistance of spores of six *Bacillus* strains with their heat resistances. Appl. Environ. Microbiol. **62:**3897–3900.
10. **Nicholson, W. L., and B. Galeano.** 2003. UV resistance of *Bacillus anthracis* spores revisited: validation of *Bacillus subtilis* spores as UV surrogates for spores of *B. anthracis* Sterne. Appl. Environ. Microbiol. **69:**1327–1330.
11. **Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow.** 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. **64:**548–572.
12. **Okazaki, T., K. Kakugawa, S. Yamauchi, T. Yoneda, and K. Suzuki.** 1996. Combined effects of temperature and pressure on inactivation of heat resistant bacteria, p. 415–418. *In* R. Hayashi and C. Balny (ed.), High pressure bioscience and biotechnology. Elsevier, Amsterdam, The Netherlands.
13. **Paidhungat, M., B. Setlow, W. B. Daniels, D. Hoover, E. Parafragkou, and P. Setlow.** 2002. Mechanism of induction of germination of *Bacillus subtilis* spores by high pressure. Appl. Environ. Microbiol. **68:**3172–3175.
14. **Pézard, C., E. Duflot, and M. Mock.** 1993. Construction of *Bacillus anthracis* mutant strains producing a single toxin component. J. Gen. Microbiol. **139:** 2459–2463.
15. **Raso, J., G. Barbosa-Canovas, and B. G. Swanson.** 1998. Sporulation temperature affects initiation of germination and inactivation by high hydrostatic pressure of *Bacillus cereus*. J. Appl. Microbiol. **85:**17–24.
16. **Raso, J., A. Palop, R. Pagan, and S. Condon.** 1998. Inactivation of *Bacillus subtilis* spores by combining ultrasonic waves under pressure and mild heat treatment. J. Appl. Microbiol. **85:**849–854.
17. **Roberts, C. M., and D. G. Hoover.** 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J. Appl. Bacteriol. **81:**363–368.
18. **Sale, A. J. H., G. W. Gould, and W. A. Hamilton.** 1970. Inactivation of bacterial spores by hydrostatic pressure. J. Gen. Microbiol. **60:**323–334.
19. **Sojka, B., and H. Ludwig.** 1997. Effects of rapid pressure changes on the inactivation of *Bacillus subtilis* spores. Pharm. Ind. **59:**436–438.
20. **Wuytack, E. Y., S. Boven, and C. W. Michiels.** 1998. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Appl. Environ. Microbiol. **64:**3220–3224.
21. **Wuytack, E. Y., J. Soons, F. Poschet, and C. W. Michiels.** 2000. Comparative study of pressure and nutrient-induced germination of *Bacillus subtilis* spores. Appl. Environ. Microbiol. **66:**257–261.