Comparison of Pressure and Heat Resistance of Clostridium botulinum and Other Endospores in Mashed Carrots

DIRK MARGOSCH, MATTHIAS A. EHRMANN, MICHAEL G. GÄNZLE,* AND RUDI F. VOGEL

TU München, Lehrstuhl Technische Mikrobiologie, Weihenstephaner Steig 16, D-85350 Freising, Germany

ABSTRACT

Inactivation of bacterial endospores in food requires a combination of pressure and moderate heat. Endospore resistance of seven Clostridium botulinum strains was compared with those of Bacillus spp. (B. cereus, B. subtilis, B. licheniformis, B. smithii, B. amyloliquefaciens) and Thermotoga aerabacterium thermosaccharolyticum with respect to pressure (600 to 800 MPa) and temperature (80 to 116°C) treatments in mashed carrots. A large variation was observed in the pressure resistance of C. botulinum spores. Their reduction after treatments with 600 MPa at 80°C for 1 s ranged from more than 5.5 log units to no reduction. Spores of the proteolytic C. botulinum TMW 2.357 exhibited a greater resistance to pressure than spores from all other bacteria examined, with the exception of B. amyloliquefaciens. Heat resistance of spores did not correlate with pressure resistance, either within strains of C. botulinum or when C. botulinum spores were compared with spores of T. thermosaccharolyticum. A quantitative release of dipicolinic acid was observed from C. botulinum spores on combined pressure and temperature treatments only after inactivation of more than 99.999% of the spores. Thus, dipicolinic acid is released by a physicochemical rather than a physiological process. The resistance of spores to combined physiological and temperature treatments correlated with their ability to retain dipicolinic acid. B. amyloliquefaciens, a mesophilic organism that forms highly pressure-resistant spores is proposed as a nonpathogenic target organism for high-pressure process development.

High hydrostatic pressure technology in food preservation offers the potential to inactivate microorganisms and enzymes while altering the flavor and nutrient content of food to a lesser extent than conventional heat treatments (5, 41). Furthermore, pressure treatment as a preservation method offers the possibility of reducing the energy requirement needed for food processing.

Pressure treatment at ambient temperature from 200 to 800 MPa is effective at eliminating vegetative bacteria (41, 45); however, bacterial spores are not inactivated by pressure treatment at ambient temperature (40). Endospores of the genera Bacillus and Clostridium tolerate a pressure over 1,000 MPa at 25°C (41). Inactivation of bacterial endospores requires the combination of pressure and moderate heat (26), and the efficacy of pressure treatment is enhanced by low pH (46, 50), in the presence of nisin (37, 46) or argon (11) and by oscillatory compression procedures (13, 18–20). Resistance of bacterial spores to pressure treatment is also influenced by environmental factors prevailing during sporulation (e.g., mineral content and temperature of the sporulation medium) (22, 27). It was suggested that resistance of spores to combined pressure and temperature treatments depends on their ability to retain dipicolinic acid (DPA) and on the heat resistance of DPA-free spores (27). The pressure resistance of vegetative cells strongly varies within strains of one species and does not correlate with heat resistance (3, 15). Comparable results were obtained with spores of Bacillus species (27, 30).

* Author for correspondence. Tel: +49 8161 713204; Fax: +49 8161 713327; E-mail: michael.gaenzle@wzw.tum.de.

Until now, studies on the effect of pressure on endospores were carried out with an almost exclusive focus on spores of Bacillus sp., namely Bacillus subtilis, and Clostridium sporogenes, and only few reports on the pressure resistance of Clostridium botulinum spores are available. C. botulinum has a ubiquitous occurrence in soil or in sediments of lakes and forms seven types of neurotoxins differing in their serological specificities. Strains of C. botulinum are classified into four groups according to physiological differences and the type of toxin formed. Strains of group I (proteolytic strains forming heat-resistant spores) and II (nonproteolytic, psychrotrophic strains forming spores with a much lower heat resistance) and toxins of type A, B, E, and F are involved in human botulism (9). In low-acid food (pH > 4.5), spores of C. botulinum can germinate and produce neurotoxin. To ensure the safety of low-acid canned food, processes are employed that attain a 12-decimal reduction of the heat-resistant spores of C. botulinum. Reddy and others (35, 36) evaluated the effects of pressure in combination with moderate heat on spores of four C. botulinum strains. Spores of heat-sensitive C. botulinum type E were less pressure resistant than spores of heat-resistant C. botulinum type A, and spore counts of the latter were not reduced by more than 3 log units following treatment at 827 MPa and 75°C. Sizer et al. (44) emphasized the need for a suitable target organism with a pressure resistance higher than that of C. botulinum, which has not been identified to date.

It was the aim of this study was to determine the pressure resistance of toxigenic C. botulinum and Bacillus cereus, as well as of the food spoilers B. subtilis, Bacillus...
TABLE 1. Strains used and their origins

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain designation and origin</th>
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<tbody>
<tr>
<td>B. licheniformis</td>
<td>TMW 2.492, pasteurized carrots&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>TMW 2.485, pasteurized carrots&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. smithii</td>
<td>TMW 2.487, pasteurized carrot juice&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. amyloliquefaciens</td>
<td>TMW 2.479, Fad 82, ropy bread&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. cereus</td>
<td>TMW 2.383&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T. thermosaccharolyticum</td>
<td>TMW 2.299, dung&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type A, proteolytic</td>
<td>TMW 2.356, REB 1750&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type B, proteolytic</td>
<td>TMW 2.357, REB 89&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type F, proteolytic</td>
<td>TMW 2.358, REB 1072&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type B, proteolytic</td>
<td>TMW 2.359&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type A, proteolytic</td>
<td>ATCC 19397, no. 83&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type B, nonproteolytic</td>
<td>ATCC 25765, no. 156&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. botulinum type B, nonproteolytic</td>
<td>TMW 2.518, no. 160, ham&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> TMW, strain collection, Technische Mikrobiologie Weihenstephan, this study.
<sup>b</sup> Röcken and Spicher (1993) strain reclassified as B. amyloliquefaciens on the basis of the 16S rRNA sequence and randomly amplified polymorphic DNA patterns (data not shown).
<sup>c</sup> Institut für medizinische Mikrobiologie und Infektionsepidemiologie, Leipzig, Germany.
<sup>d</sup> Frauenhofer-Institut für Verfahrenstechnik und Verpackung, Freising, Germany.
<sup>e</sup> Lehrstuhl für Hygiene und echnologie der Lebensmittel tierischen Ursprungs, München, Germany.

**lichenumformis, Bacillus amyloliquefaciens, Bacillus smithii, and Thermoanaerobacterium thermosaccharolyticum, and to identify a nonpathogenic, nontoxic strain that forms spores with a higher resistance to pressure than C. botulinum or B. cereus for use as a target strain for process development. Various sporulation conditions and two nonproteolytic and five proteolytic strains of C. botulinum were employed to determine the variation in pressure resistance within this species, and the pressure resistance of selected strains was compared with their heat resistance. Mashed carrots were used as a food model system. Furthermore, the ability of C. botulinum spores to retain DPA after pressure treatment was investigated and compared with the inactivation level.**

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and preparation of spore suspensions.** The bacterial strains used in this study and their origin are shown in Table 1. All bacilli were grown aerobically in ST1 broth (Merck, Darmstadt, Germany) at 60°C (B. smithii) or 30°C (other bacilli). T. thermosaccharolyticum was grown anaerobically in C. thermosaccharolyticum broth (medium 61 (2)) at 60°C, and strains of C. botulinum were grown anaerobically in RCM broth (Merck) at 30°C. Spores were prepared by plating aliquots of 0.1 ml from fresh overnight cultures on agar plates at the temperatures noted above. All Bacillus strains were grown aerobically on ST1 agar supplemented with 10 mg/liter MnSO₄·H₂O. T. thermosaccharolyticum was grown anaerobically on Caldicellulosiruptor agar (medium 640 (2)), and strains of C. botulinum were grown anaerobically on ST1, RCM, egg meat (BD Difco, Heidelberg, Germany), or WSH agar (1 liter of soil extract prepared according to Gams et al. (14), 20 g of meat extract, 3 g of yeast extract, 0.5 g of cysteine-HCl, 5 g of CaCO₃, 1.5 fresh egg whites, with pH adjusted to 7.0). The agar plates were incubated 5 days for the bacilli and 10 days for the other bacteria. Preparations showed between 90 and 99% bright phase spores by phase-contrast microscopy. Spores were collected from the plates by flooding the surface of the culture with 10-ml aliquots of cold sterile distilled water. After harvesting, the spore suspensions were washed four times by centrifugation at 5,000 rpm for 15 min at 5°C, resuspended in sterile distilled water, and stored at −80°C until use. Between the second and third wash cycle, the suspensions were pasteurized at 80°C for 10 min.

**Determination of cell counts.** Cell counts of the Bacillus strains were determined on ST1 agar. Appropriate dilutions were plated with a spiral plater (IUL, Königswinter, Germany), and plates were incubated aerobically for 36 h at 30 or 60°C, as specified in the previous section. Cell counts of C. botulinum and T. thermosaccharolyticum were determined in RCM agar and C. thermosaccharolyticum agar, respectively. Appropriate dilutions were pipetted into petri dishes and mixed with the respective agar, and plates were incubated anaerobically for 36 h at 30 or 60°C, as specified.

**Pressure treatment of spores.** Heat-sterilized mashed carrots (pH 5.15), used as the pressurization medium, were obtained in a local supermarket. Alternatively, Tris-His buffer (THB; 10 mM Tris-HCl, 20 mM histidine-HCl) adjusted to pH 4.0, 5.15, or 6.0 was used. The pressurization media were inoculated with spores to a spore count of 2.0 × 10⁶ to 4.5 × 10⁹ spores per ml and transferred to 2-ml Eppendorf reaction tubes, sealed with silicon stops (avoiding enclosure of air), and stored on ice until treatment. The samples were pressurized at 80°C and 600 MPa or 800 MPa by high-pressure autoclaves with 30-ml internal volume (Dunze Hochdrucktechnik, Hamburg, Germany) and ethanol-rhizinus oil (80:20) as pressure transmission fluid. The temperature of the pressure cell was maintained by a thermostat jacket connected to a water bath (Haake GH, Karlsruhe, Germany) and the internal temperature was monitored by a Pt100 thermocouple in the autoclave. The samples were preheated at 80°C for 10 min in the pressure vessel and the compression and decompression rates were 2 MPa/s (standard conditions) or 6 MPa/s where indicated. During compression, the temperature in the samples rose by approximately 20 or 36°C, respectively, and decreased to 80°C within 10 min (see “Effect of temperature on pressure inactivation and DPA release of C. botulinum spores”). After decompression, the sample tubes were cooled and stored on ice until determination.
TABLE 2. Effect of the sporulation medium on heat (5 min at 100°C in THB) and pressure (16 min at 600 MPa and 80°C in mashed carrots) resistance of C. botulinum TMW 2.357

<table>
<thead>
<tr>
<th>Medium</th>
<th>Pressure treatment</th>
<th>Temperature treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSH</td>
<td>–1.2 ± 0.0</td>
<td>–2.9 ± 0.4</td>
</tr>
<tr>
<td>ST1</td>
<td>–3.1 ± 0.2</td>
<td>–6.0 ± 0.5</td>
</tr>
<tr>
<td>RCM</td>
<td>–3.5 ± 0.2</td>
<td>–6.3 ± 0.4</td>
</tr>
</tbody>
</table>

* Log spore counts (N) are depicted relative to the spore counts of untreated samples (N₀).

FIGURE 1. Log spore counts (N) of C. botulinum spores after treatment at 600 MPa and 80°C in mashed carrots. Spore counts are depicted relative to the spore counts of untreated samples (N₀). TMW 2.357 (■), TMW 2.356 (●), TMW 2.359 (▲), TMW 2.358 (○), ATCC 19397 (▲), ATCC 25765 (dotted line), TMW 2.518 (dashed line). Data shown are means of two independent experiments, and error bars indicate standard deviations. Lines dropping below the x-axis indicate spore counts below the detection limit, log(N/N₀) = –5.5.

RESULTS

Development of a sporulation medium for C. botulinum strains. To obtain spores from each of the seven strains used in this work, three sporulation media were evaluated. Strains TMW 2.356, 2.358, and 2.359 did not form spores on STI or RCM agar. To obtain a medium supporting spore formation of all strains, the WSH medium was formulated. With this medium, spores could be obtained from all seven strains of C. botulinum.

Because the sporulation medium can strongly influence the heat resistance of Bacillus spores (4), we determined the resistance of C. botulinum TMW 2.357 spores obtained from these three different media (Table 2). Spores obtained from cultures on WSH medium were more resistant to treatments with wet heat or pressure compared with spores obtained from STI and RCM media. Likewise, C. botulinum TMW 2.359 spores obtained from WSH medium were more resistant to treatments with wet heat or pressure compared with spores obtained from egg-meat medium. The difference in spore counts was approximately one order of magnitude (data not shown). In this work, all further studies were performed with spores obtained from WSH-grown cultures (i.e., with the most resistant spores obtainable).

Variation in resistance of C. botulinum strains to heat and pressure. All seven strains of C. botulinum were used to determine the resistance of their spores to combined pressure and temperature treatments. Spores obtained on WSH medium were subjected to treatments in mashed carrots at 600 MPa and 80°C, and spore inactivation was monitored over a period of 64 min (Fig. 1). Great differences in the pressure resistance of these seven strains were observed. The two nonproteolytic strains formed pressure-sensitive spores, and spore counts were reduced by more than 5.5 orders of magnitude within 1 s of holding time at pressure. In comparison, the spore counts of the proteolytic strain ATCC 19397 were reduced by more than five orders of magnitude after 12 min, and spores of the strain TMW 2.357 were inactivated by less than three orders of magnitude after 60 min of holding time at pressure.

The pressure resistance of the five proteolytic strains of C. botulinum was compared with their resistance to wet heat (100°C, Table 3). Strains of C. botulinum differed greatly in their resistance to heat; however, heat resistance did not correlate with pressure resistance. C. botulinum TMW 2.359 was the most heat resistant strain and strain TMW 2.357 was the most pressure resistant strain.

Effect of acidity on pressure inactivation and DPA release of C. botulinum spores. Low pH values were reported to accelerate the pressure-induced inactivation of bacterial endospores (37). Because resistance of spores to combined pressure and temperature treatments correlates...
TABLE 3. Comparison of heat (10 min at 0.1 MPa and 100°C) and pressure (16 min at 600 MPa and 80°C) resistance of WSH spores of proteolytic strains of C. botulinum in mashed carrots

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pressure treatment</th>
<th>Temperature treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMW 2.356</td>
<td>−2.6 ± 0.4</td>
<td>−2.3 ± 0.0</td>
</tr>
<tr>
<td>TMW 2.357</td>
<td>−1.2 ± 0.0</td>
<td>−3.6 ± 0.0</td>
</tr>
<tr>
<td>TMW 2.358</td>
<td>−4.1 ± 0.2</td>
<td>−5.6 ± 0.3</td>
</tr>
<tr>
<td>TMW 2.359</td>
<td>−2.6 ± 0.5</td>
<td>−0.5 ± 0.1</td>
</tr>
<tr>
<td>ATCC 19397</td>
<td>−7.2 ± 0.2</td>
<td>−4.4 ± 0.1</td>
</tr>
</tbody>
</table>

* Log spore counts (N) are depicted relative to the spore counts of untreated samples (N₀).

with their ability to retain DPA (27), we furthermore determined the effect of pH on the pressure-induced release of DPA from C. botulinum endospores. Inactivation and release of DPA was monitored following pressure treatments at 800 MPa and 80°C and at pH values of 4.0, 5.15, or 6.0. Experiments were performed in THB to obtain a pressure-independent buffer system and because compounds from the carrots interfered with the quantification of DPA. Release of DPA from the spores is compared with the decrease of spore counts in Figure 2. A decrease in pH from 6.0 to 5.15 did not affect inactivation of C. botulinum TMW 2.357. When the pH was further decreased to 4.0, an accelerated inactivation of the spores was observed.

A release of DPA from the spores was observed after pressure treatment. Treatment at low pH resulted in an increased release of DPA. After 1 h at pressure, the release of DPA was 77, 67, and 35% at pH 4.0, 5.15, and 6.0, respectively. To determine whether a short pressure pulse could generate DPA-free, heat-sensitive spores of C. botulinum, TMW 2.357 spores treated for 2 min at 800 MPa and pH 4.0, 5.15, and 6.0 released only 47, 33, and 21%, respectively, of their DPA corresponding to 2.4 ± 0.4, 1.4 ± 0.4, and 0.9 ± 0.1 log reduction of spore counts, respectively, and these spores remained heat resistant (Fig. 2).

**Effect of temperature on pressure inactivation and DPA release of C. botulinum spores.** Temperature is an important factor in the control of pressure-induced inactivation of bacterial endospores (27, 36). To affect temperature during pressure inactivation at 800 MPa, WSH C. botulinum TMW 2.357 spores were subjected to pressure treatment in mashed carrots at compression rates of 2 and 6 MPa/s. Starting at 80°C, temperature in the mashed carrots rose to 100 and 116°C following compression with 2 MPa/s and 6 MPa/s, respectively (Fig. 3A). After reaching the maximum pressure level, temperature decreased rapidly by conduction to the pressure vessel. This transient difference in temperature strongly accelerated spore inactivation. The processing time required for a spore count reduction of 5.5 log units decreased from over 70 min (compression with 2 MPa/s) to 10 min (compression at 6 MPa/s). To compare inactivation kinetics with DPA release, the experiment was repeated with THB as the pressurization medium (Fig. 3B). The temperature profile during treatments in THB did not differ from those treatment in mashed carrots (data not shown). As observed during treatments in mashed carrots, the transient increase in temperature resulted in a strongly accelerated inactivation. This accelerated inactivation was reflected by an accelerated release of DPA in treatments with a compression rate of 6 MPa/s. A quantitative release of DPA was observed after 64 min of holding time at pressure, whereas spore counts already were reduced below the detection limit after 4 min.

The inactivation of C. botulinum spores in THB was slightly delayed compared with treatments in carrots. The difference was 1 log unit or less. The pH of the THB buffer was set to match the pH of mashed carrots at ambient pressure. Because the pKₐ values of Tris and histidine are much less dependent on pressure than the main buffering com-

![Figure 2. Effect of continuous pressurization or pressure pulse treatment at 80°C in Tris-His buffer (THB) on spore counts of C. botulinum TMW 2.357, as well as on the release of dipicolinic acid (DPA) from spores. Experiments were performed at pH 6.0 (A), pH 5.15 (B), and pH 4.0 (C). Spore counts are indicated by circles (●, ○), and the DPA release relative to the initial DPA content of the spores is indicated by squares (■). Closed symbols indicate the respective results of continuous pressurization at 800 MPa, and open symbols indicate pressure pulse treatment (i.e., 800 MPa for 2 min at 80°C) followed by incubation at 0.1 MPa and 80°C. Data shown are means of duplicate or triplicate independent experiments, and error bars indicate standard deviations. Lines dropping below the x-axis indicate spore counts below the detection limit, log(N/N₀) = −6.0.](http://example.com/figure2.png)
FIGURE 3. Effect of the compression and decompression rates of 2 or 6 MPa/s on inactivation of spores of C. botulinum TMW 2.357 in mashed carrots (A) and Tris-His buffer (THB; B). Furthermore, the figure shows (panel A) the temperature during pressure treatments in either mashed carrots or THB and (panel B) release of dipicolinic acid (DPA) after treatments in THB. Spore counts are indicated by circles (●, 2 MPa/s; ○, 6 MPa/s), and the DPA release relative to the initial DPA content of the spores is indicated by squares (■, 2 MPa/s; □, 6 MPa/s). The solid and dashed lines indicate the sample temperature during treatments with 2 and 6 MPa/s compression and decompression rates, respectively. Data shown are means of duplicate or triplicate independent experiments, and error bars indicate standard deviations. Lines dropping below the x-axis indicate spore counts below the detection limit, log(N/N₀) = −5.1.

FIGURE 4. Log spore counts of Bacillus subtilis TMW 2.485 (dotted line), B. licheniformis TMW 2.492 (dashed line), B. amyloliquefaciens TMW 2.479 (●), B. smithii TMW 2.487 (○), T. thermosaccharolyticum TMW 2.299 (■), and C. botulinum type B TMW 2.357 (□) after treatment with 800 MPa at 80°C in mashed carrots. Data shown are means of two, three, four, or five independent experiments, and error bars indicate standard deviations. Lines dropping below the x-axis indicate spore counts below the detection limit, log(N/N₀) = −6.0.

components in carrots, carboxylic acids, and phosphates, this discrepancy can be attributed to differences in pH during pressure treatment.

Comparison of the pressure resistance of spores from C. botulinum with other bacterial endospores. To compare the pressure resistance of C. botulinum spores with that of other bacterial endospores, spores of strain TMW 2.357 obtained from cultures on WSH medium and spores of B. cereus TMW 2.383, B. subtilis TMW 2.485, B. licheniformis TMW 492, B. smithii TMW 2.487, B. amyloliquefaciens TMW 2.479, and T. thermosaccharolyticum TMW 2.299 were subjected to pressure treatment at 800 MPa and 80°C in mashed carrots. B. amyloliquefaciens TMW 2.479 spores were more resistant to pressure and temperature treatments than spores from 20 other bacilli (this work, 27). After treatment for 64 min at 800 MPa and 70°C, spore counts of this strain were only reduced by 2.1 ± 0.2 log units. C. botulinum TMW 2.357 spores were more resistant to pressure than the spores of six other strains of C. botulinum (Fig. 4). Moreover, C. botulinum TMW 2.357 spores obtained from WSH medium were more resistant to heat or pressure than spores obtained from other culture media. T. thermosaccharolyticum TMW 2.299 was selected because its heat resistance generally exceeds that of C. botulinum by more than 10-fold (24), and the spores of strain TMW 2.299 used in this work withstood treatments of 0.1 MPa and 100°C for 10 min without reduction of viable cell counts. Previous work on the inactivation of strain B. licheniformis TMW 2.492 by pressure and temperature at 200 to 800 MPa and 60 to 80°C (27) has shown that it exhibited a higher resistance compared with other strains of B. subtilis for which data on their pressure resistance is available (12, 13, 21, 22, 27, 28, 46), with the exception of two strains of B. subtilis isolated from rye bread (27). The inactivation kinetics of these strains are displayed in Figure 4. Only B. amyloliquefaciens TMW 2.479 spores exhibited a higher resistance to pressure and temperature treatments than C. botulinum TMW 2.357. Other strains, including T. thermosaccharolyticum TMW 2.299, were less resistant to pressure. Spore counts of B. subtilis, B. licheniformis, and B. cereus were reduced below the detection limit following one compression and decompression cycle without holding time at pressure (Fig. 4; data not shown).

DISCUSSION

The resistance of C. botulinum spores to combined pressure and temperature treatments at various pH values was determined. Strains were selected to obtain a worst-case scenario by choosing the most resistant types of spores under specific sporulation conditions from the most pressure-resistant strain. The pressure resistance was compared with heat resistance. To provide a rationale for C. botulinum
resistance of spores to pressure, DPA release from spores after pressure treatment was determined. Comparison of C. botulinum spore resistance with that of spores from other bacteria relevant to preservation of low-acid foods provides a first step toward the identification of a suitable target organism for the development and evaluation of industrial high-pressure processes.

The comparison of resistance of spores from seven strains of C. botulinum to heat and to combined pressure and temperature treatments has shown a strong effect of sporulation conditions on the heat or pressure resistance of C. botulinum endospores. This result corroborates previous observations obtained with spores of B. subtilis \((4, 22, 27)\). The resistance of spores to physical treatments was increased particularly by the use of soil extract. Moreover, only a medium containing soil extract supported sporulation by all strains employed in this study. The effect of soil extract on spore pressure resistance is possibly mediated by the content of metal ions in soil as divalent cations are known to affect heat and pressure resistance of spores \((4, 22, 27)\). Because endospores present in food are likely to originate from soil, these types of spores are relevant in food processing.

A high variation of pressure and heat resistance within various spores of strains of C. botulinum was observed. Remarkably, the \(D_{120^\circ C}\)-value for thermal inactivation of C. botulinum TMW 2.359 was determined as 7.2 min, which exceeds \(D\)-values for other C. botulinum strains by a factor of 6 \((48)\). Spores from 18 B. subtilis and B. amyloliquefaciens strains also exhibited a high variation in pressure resistance \((27)\). Taken together, these results highlight the need to study a large number of strains to provide reliable data on the inactivation of spores in pressure and temperature processes for food preservation.

In accordance with the studies of Reddy and others \((35, 36)\), we observed that proteolytic strains were substantially more pressure resistant than nonproteolytic strains. It is difficult to compare the resistance of spores of proteolytic C. botulinum strains used in our work with that of C. botulinum strains BS-A and 62-A \((36)\) because the temperature profiles during processing differ strongly and different suspension media were used. However, the resistance of WSH-derived spores of C. botulinum strain TMW 2.357 (this work) can be considered to be higher compared with strains BS-A and 62-A \((36)\). Spore counts of C. botulinum strains BS-A and 62-A were reduced in phosphate buffer by 2 \(\pm\) 0.6 and 3 \(\pm\) 0.6 log units after treatment for 20 min at 827 MPa, an average temperature of about 75°C, and a maximum temperature of 92°C. Strain TMW 2.357 was reduced in THB by 2.4 \(\pm\) 0.1 log units after treatment for 23 min at a pressure of 800 MPa, an average temperature of 87.0°C, and a maximum temperature of 100°C. It must be taken into account that spore counts of pressure-treated samples obtained by plating techniques are underestimated compared with spore counts obtained by most probable number techniques and long incubation times \((27)\).

In agreement with literature data for spores of C. botulinum and other bacteria, the inactivation of spores observed here was strongly enhanced with an increase of temperature or pressure \((27, 36, 39)\). In contrast to most other spores, a reduction of the C. botulinum spore count by more than 5 log units is attained only at pressure and temperature levels exceeding 600 MPa and 100°C. We furthermore observed a decrease of spore pressure resistance when the pH was decreased from 5.15 to 4.0 in a pressure-independent buffer system. Likewise, the pressure-induced inactivation of Bacillus coagulans spores was independent of pH 5.0 to 7.0, whereas a further reduction to pH 4.0 accelerated the spore-inactivating effect of the pressure treatment \((37)\). The pH value of food is a function of pressure, and in aqueous systems buffered with phosphates or carboxylic acids, the pH is depressed by 1.0 pH unit with compression from 0.1 to 300 MPa \((29)\). Therefore, pH 4.5 or less occurs during pressure treatments even in foods with a pH above 4.5 at ambient pressure.

Pressure treatment opens channels of B. subtilis spores, permitting the release of DPA \((32)\). Following pressure treatment at ambient temperature, this release of DPA results in an activation of the germination pathway \((7, 32, 49)\). However, the inactivation of B. subtilis and B. licheniformis spores by pressure processing at temperatures of more than 70°C is achieved by a two-stage mechanism that does not involve spore germination \((27)\). First, pressure causes release of DPA from spores and concomitant loss of heat resistance. Second, the DPA-free spores are inactivated by heat independent of the pressure level. In accordance with this model, a short pressure pulse generated DPA-free B. subtilis and B. licheniformis viable spores, which lost their heat resistance \((27)\). In this study, the pressure-induced inactivation of C. botulinum spores was determined at various levels of pH and temperature and compared with the release of DPA from the spores. Generally, pressure and temperature treatments resulted in a partial release of DPA, and a quantitative release of DPA from spores was observed only after treatment resulting in a reduction of spore counts by more than 5 log units. Compared with treatments at 800 MPa, 80°C, and pH 5.15, the release of DPA from spores was enhanced when the pH was reduced to 4.0 or when the temperature during treatment was increased. Likewise, the inactivation of spores by low pH is caused by a drastic change in the spore permeability barrier, which leads to the loss of DPA and a concomitant hydration of the core \((42)\). These findings support the results of Margosch et al. \((27)\) that pressure inactivation of bacterial endospores by combined pressure and temperature treatments does not involve spore germination and that the release of DPA during pressure and temperature treatments is a physicochemical process. C. botulinum TMW 2.357 spores released their DPA much more slowly than B. subtilis and B. licheniformis spores \((this\ study, 27)\). Remarkably, spores of the highly pressure resistant strain B. amylo liquefaciens TMW 2.479 also retained DPA during pressure treatment, and remained heat resistant following a short pressure pulse \((27)\). Therefore, a possible explanation for the high resistance of B. amylo liquefaciens and C. botulinum to pressure is the property of their spores to retain DPA during pressure treatments.
Proposal for a suitable target or surrogate strain for pressure and temperature processing of foods. Development and assessment of high-pressure food processes require a target or surrogate strain that should have a higher resistance to pressure compared with other food spoilage organisms (target strain for spoilage) and organisms relevant for food safety, especially *C. botulinum* (surrogate strain) (44). Moreover, to be suitable for use with pilot plant and industrial-scale equipment, the organism should be nontoxic and nonpathogenic and should not require specific equipment or growth media for cultivation and handling.

This study has clearly shown that heat resistance of various species does not relate to their high pressure resistance. As expected from literature data, *T. thermosaccharolyticum* spores were more resistant to wet heat than spores of other strains, including *C. botulinum* and *B. amyloliquefaciens* (this work, 24, 38). In contrast, *B. amyloliquefaciens* and *C. botulinum* exhibited a much higher resistance to pressure compared with *T. thermosaccharolyticum*. Likewise, the most pressure-resistant strain of *C. botulinum*, strain TMW 2.357, exhibited only a intermediate heat resistance compared with other *C. botulinum* strains (this work). Thus, heat or pressure resistance of spores correlated neither within strains of *C. botulinum* nor in comparison with spores of *T. thermosaccharolyticum* TMW 2.299. This observation is in general agreement with previous studies performed with other bacteria (27, 30). It is most noteworthy that spores of proteolytic strains of *C. botulinum* are among the most pressure-resistant bacterial endospores identified so far.

Therefore, the target organisms used to design and control thermal processing in food production are unsuitable as target or surrogate organisms in high-pressure processes. On the basis of the data currently available, we suggest *B. amyloliquefaciens* TMW 2.479 as such a target organism for high-pressure and high-temperature processing of low-acid foods. This strain is a mesophilic, aerobic, nonpathogenic, and nontoxicogenic microorganism growing on standard laboratory media. It was previously isolated from spoiled food (38) and exhibits a higher resistance to combined heat and pressure treatments than spores from *C. botulinum* and spores from other organisms for which literature data is available (*Alicyclobacillus acidoterrestris*, *Bacillus anthracis*, *B. cereus*, *B. coagulans*, *B. licheniformis*, *B. smithii*, *B. subtilis*, *C. botulinum*, *C. sporogenes*, *Geobacillus stearothermophilus*, and *T. thermosaccharolyticum* (this study, 1, 6, 8, 11–13, 16–22, 25, 27, 28, 31, 33–37, 39, 43, 46, 47, 49)). However, the 12D concept was established on the basis of heat resistance data from 109 strains of *C. botulinum* (10). Because only nine strains of *C. botulinum* and only a limited number of strains (<50) of other spore formers have been evaluated with respect to their pressure resistance, additional strains also need to be evaluated to allow the establishment of criteria for high-pressure processes in food production.

Our results support the hypothesis of Margosch et al. (27) that the resistance of spores to combined pressure and temperature treatments depends on their ability to retain DPA. Combined pressure and temperature treatments effectively reduced *C. botulinum* spore counts by more than 5.5 log units within 2 min of holding time at pressure and temperature levels above 600 MPa and 100°C, respectively. Therefore, pressure processing is a suitable technique to destroy *C. botulinum* spores in food at reduced temperatures while helping to retain aroma compounds and functional ingredients of foods—even sterilized foods. A target organism for the pressure processing of low-acid foods is suggested (*B. amyloliquefaciens* TMW 2.479), although additional pressure–death time data for a larger number of strains is required to establish target or surrogate organisms. The heat resistance of the *C. botulinum* TMW 2.359 spores exceed by far the heat resistance of *C. botulinum* strains on which the 12D concept was originally based. Thus, a closer look at the safety of novel food processing techniques might enable a fruitful revision of safety concepts for established (thermal) processes.

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**REFERENCES**


In the article “Comparison of Pressure and Heat Resistance of Clostridium botulinum and Other Endospores in Mashed Carrots,” by Margosch, Ehrmann, GaÈnzle, and Vogel, that appeared in the Journal of Food Protection 67(11):2530–2537, the $D_{120^\circ C}$-value of C. botulinum TMW 2.359 was given as 7.2 min. However, the value as determined in the experiments was 1.2 min.