International Journal of Food Microbiology 128 (2008) 385–389

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Effects of minerals on sporulation and heat resistance of *Clostridium sporogenes*

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ARTICLE INFO

Article history:
Received 2 May 2008
Received in revised form 11 September 2008
Accepted 1 October 2008

Keywords:
Clostridium sporogenes
Sporulation
Heat resistance
Mineral
Calcium carbonate

ABSTRACT

In this study, various mineral supplements, such as chloride salts ($CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$ and KCl) supplying cations and calcium salts ($CaCl_2$, $CaCO_3$, $CaSO_4$, $Ca(OH)_2$ and $CaHPO_4$) supplying anions, were tested if they could stimulate the sporulation of *Clostridium sporogenes*, a surrogate microorganism for *C. botulinum*. Of the cations tested, the addition of $CaCl_2$ showed a slightly, but not significantly, greater increase in spore levels within 3 weeks of incubation, compared to that of the other cations. The optimum concentration of $CaCl_2$ was 0.5%, which yielded nearly 10^4 CFU/ml of spores. Of the anions tested, $CaCO_3$ promoted sporulation within one week, which was the most effective compound for promoting rapid sporulation among the minerals tested. $CaSO_4$ produced a pattern of sporulation similar to that of $CaCl_2$. While $CaHPO_4$ resulted in the maximum production of spores after 4 weeks, $Ca(OH)_2$ failed to induce sporulation. With an optimized concentration of 0.5% $CaCO_3$, the spore yield was approximately 10^5 CFU/ml. The spores prepared in sporulation medium with $CaCO_3$ (pH 5.0) had slightly, but not significantly, higher *D* values than those produced with $CaCl_2$ (pH 5.0) at temperatures ranging from 113 to 121 °C. However, no significant differences were observed in *Z* values (both 10.76 °C). In a large scale spore production, $D_{121\ °C}$ values of the spore crops prepared with $CaCl_2$ and $CaCO_3$ and resuspended in phosphate buffer (pH 7.0) were found to be both 0.92 min. In conclusion, our data suggest that $CaCO_3$ is highly effective in reducing sporulation time as well as enhancing heat resistance.

1. Introduction

Clostridium sporogenes PA 3679 is a non-pathogenic, putrefactive, spore-forming anaerobe. Since *C. sporogenes* spores have a much higher *D* value than *C. botulinum* spores, it has been commonly used as a non-toxic surrogate microorganism to validate effectiveness of sterilization processes to inactivate *C. botulinum* (Guan et al., 2003; Ocio et al., 1994). Indeed, since *C. sporogenes* is the only possible surrogate for *C. botulinum*, belonging to the genus (USFDA, 2000), it is therefore of great importance to extensively investigate this surrogate microorganism.

At present, a simple and convenient sporulation medium developed in the mid-1960s (Duncan and Foster, 1968) is still used with minor modification for preparing *C. sporogenes* spores. However, it is time-consuming (more than 3 weeks) to induce sporulation of vegetative cells using this medium, which in turn makes it difficult to obtain a satisfactory yield of *C. sporogenes* spores. Nevertheless, there is no other alternative to reducing the time required for preparing spores. Meanwhile, the effects of various cations on sporulation and heat resistance have been investigated, targeting different spore-forming bacteria such as bacilli and clostridia, because the spores have been known to contain a large amount of cations (Chung et al., 1971; Marquis and Shin, 1994; Setlow, 2006). As the most important mineral component composing bacterial spores, calcium has been considered to be closely related to the heat resistance of spores (Marquis and Shin, 1994; Murrell, 1969). Furthermore, calcium forms a complex with DPA, which constitutes approximately 10% of the total spore mass and contributes to heat resistance through dehydration of protoplasts (de Vries, 2004; Murrell, 1969; Scully et al., 2002). Like calcium, manganese has been known not only to induce sporulation in the genus *Bacillus* such as *B. subtilis* (Charney et al., 1951), *B. megaterium* (Weinberg, 1964) and *B. coagulans* var. *thermoacidurans* (Amaha et al., 1956), but also to increase heat resistance of *B. coagulans* var. *thermoacidurans* spores (Roberts and Hitchins, 1969). Magnesium is essential for sporulation (Murrell, 1969), but not for heat resistance (Roberts and Hitchins, 1969). Moreover, magnesium decreases heat resistance of *Bacillus* species, interfering with formation of the Ca-DPA complex (Murrell, 1969). Potassium is also necessary to produce spores in several *Bacillus* species (Wakisaka et al., 1982), and iron is required for heat resistance of *C. botulinum* (Sugiyama, 1951).

Since the types and contents of minerals in spores can be easily changed, depending on the mineral composition of the sporulation medium (Slepecky and Foster, 1959), minerals should be considered one of the most important components when developing and optimizing sporulation media. Although studies on the functions of cations relating to bacterial spores have been well established in

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doi:10.1016/j.ijfoodmicro.2008.10.002

Bacillus species, few studies with *Clostridium* spores have been found to date. And besides, the effects of anions on sporulation are largely unknown. Therefore, this study was carried out to examine the effects of various minerals, in the form of either cations or anions, on sporulation and heat resistance of *C. sporogenes*.

2. Materials and methods

2.1. Microorganism

C. sporogenes PA 3679 spores were obtained from the Center for Technical Assistance of the former National Food Processors Association (NFPA, Dublin, CA, USA). The spore suspension was divided into cryogenic sterile vials (Fisher Scientific, Pittsburgh, PA, USA) and then kept in a freezer (−20 °C) until use. The initial concentration of the stock suspension was approximately 2×10^7 CFU/ml. The concentration was determined by the enumeration procedure described below.

2.2. Preparation of vegetative cell

To prepare a pure culture of vegetative cells in stationary phase, a multiple stage inoculation procedure was employed with minor modifications, as follows (Uehara et al., 1965). The procedure utilized TPGY medium containing 50 g of tryptone, 20 g of yeast extract, 5 g of peptone, 4 g of dextrose and 1 g of sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, MD, USA) in 1 l of distilled water (USFDA, 1998). Ten milliliters of TPGY broth was inoculated with 10 μl of the stock spore suspension and incubated for 2 days at 32 °C in an anaerobic chamber (Coy laboratory products Inc., Grass Lake, MI, USA) containing an atmosphere of 95% nitrogen and 5% hydrogen (Oxarc Inc., Spokane, WA, USA). Subsequently, 100 ml of TPGY broth was inoculated with 1 ml of the culture and followed by incubation under the same conditions. After 2 days of incubation, the culture was transferred into 1 l of TPGY broth and the flask was incubated anaerobically for 2 days. To obtain cleaned vegetative cells, the culture was washed three times by centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA) with a Fiberlite F14B rotor (Fiberlite Centrifuge, Inc., Santa Clara, CA, USA) at 15,000 ×*g* for 10 min at 4 °C, resuspended in 100 ml of M/15 Sörensen's phosphate buffer (Na_2HPO_4 5.675 g, KH_2PO_4 3.63 g in 1 l of distilled water, pH 7.0), and then kept in a refrigerator (4 °C) until use. The concentration of the vegetative cell suspension was approximately 10^8 CFU/ml.

2.3. Induction of sporulation

To investigate the effects of minerals on sporulation of vegetative cells, a sporulation medium described by Uehara et al. (1965) and modified by Duncan and Foster (1968) was employed and served as a basal sporulation medium for subsequent research. The basal medium consisted of 60 g of tryptone, 1 g of dextrose and 1 g of sodium thioglycolate in 1 l of distilled water, and the initial pH was adjusted to 5.0 with 1 N HCl prior to sterilization in order to induce sporulation (Lu, 2006).

For modifying the basal medium to obtain a satisfactory amount of spores and to reduce induction time of sporulation, various minerals were added as supplements to a basal sporulation medium, one at a time, at a level of 0.5%, based on our previous study (Lu, 2006). All minerals were of ACS grade, purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), regardless of their brands such as Sigma, Fluka and Riedel-de Haën, unless mentioned specifically. Five different minerals including $CaCl_2$ (J. T. Baker Chemical Co. Phillipsburg, NJ, USA), $MgCl_2$ (JT Baker), $MnCl_2$, $FeCl_2$ and KCl (JT Baker) were used to supply the cations and 5 different minerals including $CaCl_2$ (JT Baker), $CaCO_3$, $CaSO_4$, $Ca(OH)_2$ and $CaHPO_4$ to supply the anions. The $CaCl_2$ and $CaCO_3$ were also tested in different concentrations to determine the optimal concentration.

To induce sporulation of vegetative cells, 1 ml of the vegetative cell suspension was added to 100 ml of each sporulation medium at a final concentration of 10^6 CFU/ml, and then incubated anaerobically at 32 °C. Test samples were taken at one week intervals and kept in a refrigerator (4 °C) until use.

Large scale spore production was carried out by inoculating 2 l of sporulation medium, which was supplemented with a selected mineral and adjusted to pH 5.0, with 200 ml of vegetative cell suspension prepared as described above, at a final concentration of 7×10^7 CFU/ml. After one week incubation in a sporulation medium supplemented with $CaCO_3$ or 3 week incubation in a medium supplemented with $CaCl_2$ at 32 °C in an anaerobic chamber, spore crops were washed by the same procedure as for vegetative cells and resuspended in a final volume of 50 ml M/15 Sörensen's phosphate buffer, and then kept in a refrigerator (4 °C) until use. The concentrations of the spore suspensions prepared were approximately 10^6 CFU/ml.

2.4. Enumeration of spore and vegetative cell

The spore suspension sample was placed in a 90 °C water bath for 10 min to stimulate germination of the spores and to inactivate vegetative cells. After heat treatment, the suspension was cooled in a crushed ice water bath and 10-fold serially diluted in sterile 0.1% peptone water. One milliliter of each dilution was pour-plated onto TPGY medium solidified with 1.5% agar. The TPGY medium was autoclaved at 121 °C for 15 min and held in a 45 °C water bath prior to use. The plates were incubated anaerobically for 3 days at 32 °C. Colonies were manually counted after both 48 and 72 h of incubation and the numbers of spores were calculated from dual platings.

Additionally, the total number of vegetative cells was determined by the same procedure except for heating at 90 °C.

2.5. Evaluation of heat resistance

The *D* value (time required for a 10-fold reduction in viable spores) of *C. sporogenes* spores that were produced in a sporulation medium (pH 5.0) or that were prepared in large scale spore production, washed and resuspended in M/15 Sörensen's phosphate buffer (pH 7.0) was determined using the multiple-point method. Fifty microliters of test spore suspension (the concentration was 10^4 CFU/ml for spores produced in a sporulation medium, 10^7 CFU/ml for spores resuspended in phosphate buffer) was carefully injected into a glass capillary tube with an inner diameter of 1.8 mm and an outer diameter of 3 mm (Corning Inc., Corning, NY, USA) using a pipette and then the open ends of the tubes were heat sealed. The tubes were immersed completely in an oil bath (Thermo Electron Corporation, Waltham, MA, USA) and heated between 113 and 124 °C for different times. After heating, the tubes were removed from the oil bath, cooled immediately in a crushed ice water bath, and washed in 70% ethyl alcohol. Both ends of tubes were cut aseptically; suspension was flushed out with 2 ml of sterile 0.1% peptone water. The treated samples were then 10-fold serially diluted in sterile 0.1% peptone water.

Each serially diluted sample was pour-plated onto TPGY medium, incubated in an anaerobic chamber, and then the colonies were manually counted, as described previously. The number of survivors was calculated from triplicate determinations in independent experiments. Survivor curves were plotted to determine *D* value. *D* values were obtained by taking the reciprocal of the slope from linear regression of the survivor curves. The *Z* value (the change in temperature required for a 10-fold reduction of *D* value) was estimated by plotting the $\log_{10} D$ values versus heating temperatures and taking the reciprocal of the slope from linear regression.

2.6. Statistical analysis

All experiments were conducted in triplicate and the data were presented as means and standard deviations. The effects of minerals on sporulation and heat resistance were compared and analyzed

statistically. The significance of differences was determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison module of the Minitab statistical software, version 12.11 (Minitab Inc., State College, PA, USA) and differences with P values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. Effects of cations on sporulation

As shown in Table 1, the addition of $CaCl_2$ resulted in a slight but not significant increase in spore numbers after 2 weeks of incubation and the amount increased to near 10^4 CFU/ml after 3 weeks. Both $MgCl_2$ and KCl produced similar patterns of sporulation as $CaCl_2$, yielding more than 10^3 CFU/ml of spores after 3 weeks. Unlike other cations, however, addition of $MnCl_2$ and $FeCl_2$ resulted in poor sporulation, yielding less than 10 CFU/ml of spores (data not shown), which is in partial disagreement with other studies suggesting that manganese induces sporulation in the genus *Bacillus* (Amaha et al., 1956; Charney et al., 1951; Weinberg, 1964). Meanwhile, with most cultures, it was observed that the amount of vegetative cells in a culture decreased as incubation time progressed, whereas the spore numbers in the culture increased. It was also found that 4-week cultures contained the highest amount of spores and lowest amount of vegetative cells, both ranging from 10^4 to 10^5 CFU/ml. These results indicate that it requires more than 3 weeks to induce sporulation of vegetative cells in sporulation media supplemented with the cations tested.

Considering the yield of both spores and vegetative cells in medium supplemented with $CaCl_2$, it was decided to optimize the concentration of this mineral. As shown in Table 2, the addition of $CaCl_2$ at a final concentration ranging from 0.4% to 0.6% yielded more than 10^4 CFU/ml of spores after 4 week incubation. For further study, therefore, we chose a concentration of 0.5% $CaCl_2$ because the highest spore yield was observed at this concentration, although this yield was not significantly different from that of 0.4% or 0.6% $CaCl_2$, at least up to 3 week incubation.

Regarding cations, $CaCl_2$ has been known to enhance heat resistance of spores of various species in the genus *Bacillus* such as *B. subtilis* (Fleming and Ordal, 1964), *B. megaterium* (Levinson and Hyatt, 1964) and *B. cereus* (Bhothipaksa and Busta, 1978). Therefore, it was expected that the optimized concentration of $CaCl_2$ might also affect heat resistance of *C. sporogenes* spores. This is described in more detail later.

3.2. Effects of anions on sporulation

Obtaining a satisfactory yield of *Clostridium* spores by using $CaCl_2$ to supplement the sporulation medium was a time-consuming process. In an effort to reduce sporulation time, therefore, we tested the effects of anions on sporulation.

Of the calcium salts tested, the addition of $CaCO_3$ resulted in a remarkable increase ($P<0.05$) in spore numbers after one week of incubation, yielding nearly 10^5 CFU/ml, which was statistically significant, compared with that of the other calcium salts, as shown in Table 3. The spore yield was constant throughout the entire incubation period. Meanwhile, the amount of vegetative cells in the culture taken after one week was lower than that of other cultures

Table 1
Effect of cation on sporulation of *C. sporogenes* PA 3679

Minerals used[a]	Incubation time (week)	Vegetative cells[b] (log_{10} CFU/ml)	Spores[b] (log_{10} CFU/ml)
$CaCl_2$	1	7.18 ± 0.23^{AB}	0.00 ± 0.00^{A}
	2	6.64 ± 0.58^{ABC}	0.83 ± 0.75^{AB}
	3	4.90 ± 0.76^{DE}	3.89 ± 0.60^{C}
	4	5.22 ± 0.59^{CDE}	4.10 ± 0.65^{C}
$MgCl_2$	1	6.67 ± 0.43^{ABC}	0.00 ± 0.00^{A}
	2	6.85 ± 0.51^{AB}	0.00 ± 0.00^{A}
	3	5.42 ± 0.92^{BCDE}	3.22 ± 1.92^{C}
	4	4.61 ± 0.78^{E}	4.83 ± 0.15^{C}
KCl	1	7.18 ± 0.16^{A}	0.00 ± 0.00^{A}
	2	6.41 ± 0.15^{ABCD}	0.49 ± 0.85^{A}
	3	5.76 ± 0.54^{ABCDE}	3.02 ± 0.85^{BC}
	4	4.88 ± 0.06^{DE}	3.70 ± 0.71^{C}

[a] The respective mineral was added to a sporulation medium at a final concentration of 0.5%.
[b] Data were taken as a mean±standard deviation calculated from triplicates. Mean values in the same column that are not followed by the same letter are significantly different ($P<0.05$).

Table 2
Effect of concentration of $CaCl_2$ on sporulation of *C. sporogenes* PA 3679

Concentration (%)	Incubation time (week)	Vegetative cells[a] (log_{10} CFU/ml)	Spores[a] (log_{10} CFU/ml)
0.0[b]	1	6.93 ± 0.20^{A}	0.00 ± 0.00^{A}
	2	6.42 ± 0.71^{A}	0.00 ± 0.00^{A}
	3	6.25 ± 1.00^{A}	1.72 ± 2.98^{AB}
	4	6.11 ± 1.00^{A}	1.73 ± 3.00^{AB}
0.2	1	7.08 ± 0.17^{A}	0.00 ± 0.00^{A}
	2	6.84 ± 0.28^{A}	0.00 ± 0.00^{A}
	3	6.68 ± 0.39^{A}	0.00 ± 0.00^{A}
	4	5.94 ± 0.51^{A}	2.91 ± 2.52^{AB}
0.4	1	7.32 ± 0.24^{A}	0.00 ± 0.00^{A}
	2	7.32 ± 0.36^{A}	0.00 ± 0.00^{A}
	3	6.05 ± 0.53^{A}	2.84 ± 1.60^{AB}
	4	6.12 ± 1.14^{A}	4.37 ± 0.71^{B}
0.5	1	7.18 ± 0.23^{A}	0.00 ± 0.00^{A}
	2	6.64 ± 0.58^{A}	0.83 ± 0.75^{AB}
	3	4.90 ± 0.76^{A}	3.89 ± 0.60^{AB}
	4	5.22 ± 0.59^{A}	4.10 ± 0.65^{B}
0.6	1	7.38 ± 0.15^{A}	0.00 ± 0.00^{A}
	2	7.24 ± 0.40^{A}	0.00 ± 0.00^{A}
	3	5.39 ± 0.97^{A}	2.41 ± 2.13^{AB}
	4	4.67 ± 0.28^{A}	4.19 ± 0.78^{B}
0.8	1	4.79 ± 4.15^{A}	0.00 ± 0.00^{A}
	2	5.88 ± 2.60^{A}	0.83 ± 0.75^{AB}
	3	5.72 ± 0.85^{A}	1.51 ± 1.63^{AB}
	4	5.44 ± 1.20^{A}	3.12 ± 1.57^{AB}
1.0	1	5.97 ± 2.02^{A}	0.00 ± 0.00^{A}
	2	6.97 ± 0.49^{A}	0.00 ± 0.00^{A}
	3	6.46 ± 0.59^{A}	0.33 ± 0.58^{AB}
	4	6.14 ± 0.95^{A}	1.31 ± 2.27^{AB}

[a] Data were taken as a mean±standard deviation calculated from triplicates. Mean values in the same column that are not followed by the same letter are significantly different ($P<0.05$).
[b] A basal sporulation medium described by Uehara et al. (1965) and modified by Duncan and Foster (1968).

Table 3
Effect of anion on sporulation of *C. sporogenes* PA 3679

Minerals used[a]	Incubation time (week)	Vegetative cells[b] (log_{10} CFU/ml)	Spores[b] (log_{10} CFU/ml)
$CaCl_2$	1	7.18 ± 0.23^{AB}	0.00 ± 0.00^{A}
	2	6.64 ± 0.58^{ABCD}	0.83 ± 0.75^{AB}
	3	4.90 ± 0.76^{EFG}	3.89 ± 0.60^{C}
	4	5.22 ± 0.59^{DEFG}	4.10 ± 0.65^{C}
$CaCO_3$	1	6.56 ± 0.34^{ABCD}	4.70 ± 0.14^{C}
	2	4.46 ± 0.71^{EFG}	4.68 ± 0.29^{C}
	3	4.26 ± 0.54^{FG}	4.94 ± 0.45^{C}
	4	4.20 ± 0.38^{G}	4.88 ± 0.31^{C}
$CaSO_4$	1	7.21 ± 0.20^{ABC}	0.00 ± 0.00^{A}
	2	7.33 ± 0.25^{A}	0.00 ± 0.00^{A}
	3	5.76 ± 0.12^{BCDEF}	2.83 ± 1.63^{BC}
	4	5.34 ± 0.31^{DEFG}	4.60 ± 0.30^{C}
$CaHPO_4$	1	7.11 ± 0.22^{ABC}	0.00 ± 0.00^{A}
	2	5.94 ± 0.74^{ABCDE}	2.90 ± 2.52^{BC}
	3	5.10 ± 0.67^{DEFG}	5.26 ± 0.26^{C}
	4	4.90 ± 0.75^{EFG}	5.30 ± 0.04^{C}

[a] The respective calcium salt was added to a sporulation medium at a final concentration of 0.5%.
[b] Data were taken as a mean±standard deviation calculated from triplicates. Mean values in the same column that are not followed by the same letter are significantly different ($P<0.05$).

sampled the same day, decreasing significantly to approximately 10^4 CFU/ml after 2 weeks, and thereafter decreasing gradually. The addition of $CaSO_4$ and $CaHPO_4$ resulted in spores and vegetative cells with similar growth patterns to that of $CaCl_2$. Although when $CaHPO_4$ was used as supplement the spore level increased to 10^5 CFU/ml after 3 or more weeks, showing the highest spore yield, there were no statistically significant differences, compared with $CaCO_3$. Unlike other calcium salts, the addition of $Ca(OH)_2$ yielded less than 10 CFU/ml of spores due to poor sporulation (data not shown).

Considering the induction time of sporulation as well as the favorable yield of both spores and vegetative cells, it was decided to optimize the concentration of $CaCO_3$. As shown in Table 4, the addition of $CaCO_3$ caused statistically significant increases after one week of incubation, yielding a satisfactory amount of spores ranging from 10^3 to 10^5 CFU/ml. Based on the amount of spores, 0.5% was selected as the optimum concentration of $CaCO_3$, because the highest yield of spores, nearly 10^5 CFU/ml, was obtained at this concentration.

Thus, together with the observation that $CaCO_3$ promoted sporulation with the shortest sporulation time among all minerals tested, producing a sufficiently large amount of spores, it was established that one week incubation with 0.5% $CaCO_3$ is optimal for sporulation of *C. sporogenes*. It is worth noting that 0.2% $CaCO_3$ was also effective in promoting sporulation, although a somewhat smaller amount of spores was observed at this concentration. Regarding the effect of $CaCO_3$ on sporulation, Greene (1938) reported that excess $CaCO_3$ strongly triggered sporulation, which was likely due to its buffering effect. In our study, we observed that $CaCO_3$ is effective in reducing sporulation time, regardless of pH changes (with/without pH adjustment) and amount of $CaCO_3$ (with a range between 0.4% and 0.6%). We also observed that the cultures prepared with 0.5% $CaCO_3$ had slightly but significantly higher ultimate pH values (7.67±0.07) than did those prepared with 0.5% $CaCl_2$ (6.93±0.25) when sporulation was performed for an optimized period of time. Meanwhile, Levinson and Sevag (1953) observed that the absence of chloride repressed germination of *B. megaterium* (in the original report, the bacterium was described as *B. megatherium*). On the contrary, Hachisuka et al. (1956) stated that carbonate inhibited germination of *B. subtilis*, which is likely to be of advantage in maintaining spore dormancy at least. Collectively, therefore, it can be proposed that carbonate is more favorable to sporulation than chloride. To our knowledge, there have been no other reports found in literature addressing this issue; this is the first study describing the effects of anions, particularly carbonate, on sporulation time.

Table 4
Effect of concentration of $CaCO_3$ on sporulation of *C. sporogenes* PA 3679

Concentration (%)	Incubation time (week)	Vegetative cells[a] ($\log_{10}$ CFU/ml)	Spores[a] ($\log_{10}$ CFU/ml)
0.0[b]	1	6.93 ± 0.20^{A}	0.00 ± 0.00^{A}
	2	6.42 ± 0.71^{ABC}	0.00 ± 0.00^{A}
0.2	1	6.48 ± 0.37^{ABC}	3.29 ± 0.74^{B}
	2	4.80 ± 0.07^{BC}	3.79 ± 0.95^{B}
0.4	1	6.34 ± 0.44^{ABC}	3.67 ± 0.94^{B}
	2	4.83 ± 0.27^{BC}	4.16 ± 1.03^{B}
0.5	1	5.89 ± 0.50^{ABC}	4.79 ± 1.23^{B}
	2	4.74 ± 0.57^{BC}	4.60 ± 0.64^{B}
0.6	1	6.66 ± 1.29^{AB}	3.91 ± 1.02^{B}
	2	5.04 ± 1.12^{ABC}	4.68 ± 1.11^{B}
0.8	1	6.09 ± 0.52^{ABC}	3.83 ± 0.65^{B}
	2	5.49 ± 0.63^{ABC}	4.10 ± 0.73^{B}
1.0	1	5.58 ± 0.85^{ABC}	2.82 ± 1.61^{B}
	2	4.63 ± 0.23^{C}	3.42 ± 1.12^{B}

[a] Data were taken as a mean±standard deviation calculated from triplicates. Mean values in the same column that are not followed by the same letter are significantly different ($P<0.05$).

[b] A basal sporulation medium described by Uehara et al. (1965) and modified by Duncan and Foster (1968).

Table 5
D and *Z* values of *C. sporogenes* PA 3679 spores in sporulation media

Mineral used[a]	*D* value (min) at[b]					*Z* value (°C)[b]
	113 °C	115 °C	118 °C	121 °C	124 °C	
$CaCl_2$	1.24±0.55	0.58±0.22	0.33±0.20	0.21±0.13	0.15±0.01	10.76±2.70
$CaCO_3$	1.48±0.65	0.93±0.27	0.47±0.16	0.29±0.05	0.13±0.06	10.76±1.21

[a] The respective calcium salt was added to a sporulation medium at a final concentration of 0.5%.

[b] Values were taken as a mean±standard deviation calculated from triplicates.

3.3. Comparative determination of heat resistance

It has been well known that the calcium ion contributes to heat resistance of spores (Slepecky and Foster, 1959). Since $CaCl_2$ has also been suggested to be effective in enhancing heat resistance of *Bacillus* spores (Bhothipaksa and Busta, 1978; Fleming and Ordal, 1964; Levinson and Hyatt, 1964), the fact that the addition of $CaCO_3$ causes the most rapid sporulation of *C. sporogenes* vegetative cells led us to speculate that it may function not only in inducing sporulation but also in enhancing heat resistance. To test this speculation, *D* and *Z* values of the spores that had been produced by adding $CaCl_2$ and $CaCO_3$ to a sporulation medium were determined. As shown in Table 5, the spore crops showed no difference in *Z* values. Moreover, the spores prepared with $CaCO_3$ seemed to have slightly, but not significantly, higher *D* values than those produced with $CaCl_2$ at temperatures ranging from 113 to 121 °C. Therefore, it is worth noting that $CaCO_3$ was more effective in reducing sporulation time and conferred a similar level of heat resistance to spores, as compared to $CaCl_2$.

Regardless of supplement used, however, the cultures (both a one-week culture with 0.5% $CaCO_3$ and a 3-week culture with 0.5% $CaCl_2$) showed somewhat lower *D* values in all cases, compared to the value obtained from a 3-week spore crop in an M/15 Sörensen's phosphate buffer at pH 7.0 in a preliminary study (data not shown). Regarding the effect of pH on heat resistance, Cameron et al. (1980) described that *C. sporogenes* spores showed much greater heat resistance in the buffer. In addition, Pang et al. (1983) reported that heat resistance of *C. sporogenes* spores produced in an anaerobic fermentor was found to be maximal around neutral pH. Therefore, a one-week spore crop grown in medium supplemented with 0.5% $CaCO_3$ and a 3-week spore crop in medium supplemented with 0.5% $CaCl_2$ were prepared in large scale spore production, washed and resuspended in M/15 Sörensen's phosphate buffer according to the protocol as described in Materials and methods, and $D_{121\ °C}$ values of the spore crops were determined. As shown in Table 5 and Fig. 1, while the cultures suspended in

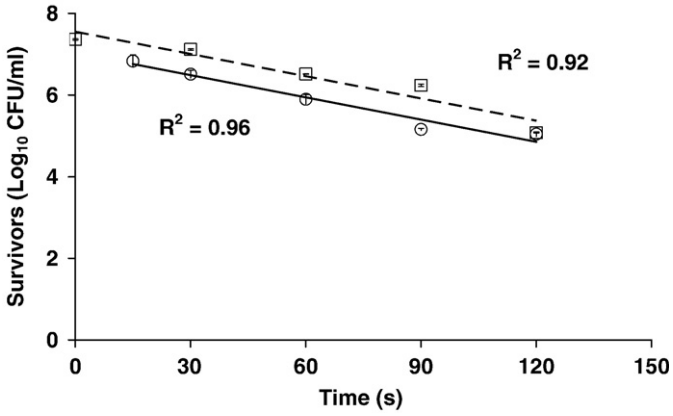

Fig. 1. Thermal survivor curves for *C. sporogenes* spores prepared in large scale spore production. The survivor curves for *C. sporogenes* spores prepared with $CaCO_3$ (○, solid line) and with $CaCl_2$ (□, dashed line) and resuspended in an M/15 Sörensen's phosphate buffer (pH 7.0) are presented, respectively. By taking the reciprocal of the slope from linear regression of the curve, it turned out that both spore crops had *D* values at 121 °C of 0.92 min. The bar indicates standard deviations calculated from triplicates.

sporulation medium supplemented with either $CaCl_2$ or with $CaCO_3$ in which sporulation of vegetative cells occurred had low $D_{121\ °C}$ values of 0.21 and 0.29 min, respectively, both spore crops resuspended in phosphate buffer exhibited considerably higher $D_{121\ °C}$ values of 0.92 min, regardless of the calcium salt used, which is in agreement with other studies discussed above. Although the mechanisms by which neutral pH influences heat resistance are less known, such noticeable differences in *D* values depending on the pH of the suspending solution in which spores were heated can be explained by a hypothesis that a higher pH may play a role in preventing a protonization of the spore cortex, and thereby contributing to heat resistance of spores. It is not discussed in depth herein, but has been reviewed by Palop et al. (1999). Henriques and Moran (2007) also reviewed the effect of pH and relative humidity on the permeability of coat and cortex that play an important role in spore resistance.

In this study, it has been demonstrated that most of the calcium salts tested stimulate sporulation of *C. sporogenes*. It is not surprising that calcium is a favorable mineral for sporulation because calcium dipicolinate has been known to be the most important molecular complex composing bacterial spores (Murrell, 1969). In *C. botulinum*, while zinc and manganese have been reported to repair thermal injuries of spores, iron and copper have been suggested to reduce heat resistance of the spores (Kihm et al., 1990). In contrast, Sugiyama (1951) observed that iron was required for heat resistance of *C. botulinum* spores. It was interesting to test whether these ions also affect heat resistance of *C. sporogenes* spores. In the present study, however, $MnCl_2$ and $FeCl_2$ resulted in a poor sporulation. It has also been described that both $MgCl_2$ and KCl can induce a similar level of sporulation as does $CaCl_2$ in this study. Although magnesium has been known to cause a decrease in heat resistance of *Bacillus* species (Murrell, 1969), little data is available regarding the effects of magnesium and potassium on heat resistance of *C. sporogenes* spores. Since these minerals were found to have no effect on reducing the induction time of sporulation in this study, however, it was of no interest to further pursue this issue. Regarding the degree and induction time of sporulation, it has been elucidated that while $CaCO_3$ not only stimulates sporulation as $CaCl_2$ does but also reduces sporulation time, $Ca(OH)_2$ represses sporulation, which implies that somehow anions may function in regulation of sporulation. However, the underlying mechanism by which $CaCO_3$ collaborates to promote sporulation is less clear. It will be a great challenge and interesting topic for researchers to unveil the underlying mechanisms. In conclusion, it is evident that $CaCO_3$ is the most reliable and suitable supplement for rapid sporulation of vegetative cells.

Acknowledgements

The authors acknowledge support by the USDA National Integrated Food Safety Initiative grant No. 2003-51110-02093 titled: Safety of foods processed using four alternative processing technologies and partial support from Washington State University Agriculture Research Center.

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