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Original article

The ability of zinc to inhibit the sporulation and viability of *Clostridium sporogenes* and growth of other bacteria

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Summary The objective of this study was to investigate the influence of zinc on the sporulation and viability of *Clostridium sporogenes* and on the growth of other bacteria. When 0.5% $ZnCl_2$ was added to a sporulation medium, it completely inhibited *C. sporogenes* (PA 3679) sporulation for up to 3 weeks. At concentrations of 0.5% and 1.0%, $ZnCl_2$ not only completely inactivated the vegetative cell viability (>7.0 Log reduction) but also significantly reduced the spore viability (<2.1 Log reduction) of *C. sporogenes*. Taken together, it was concluded that zinc blocks *C. sporogenes* sporulation by damaging (or killing) vegetative cells and probably by interfering with the biosynthesis of spore components. In addition to the inhibitory effect on the sporulation and viability of *C. sporogenes*, $ZnCl_2$ was found to have a broad antimicrobial spectrum against all Gram-positive and Gram-negative spoilage and pathogenic bacteria tested. The minimal inhibitory concentration for inhibiting the bacteria ranged between 3.7 and 7.4 mM. Therefore, we expect that this compound or a combination thereof has a potential as a surface-cleaning agent or disinfectant.

Keywords Antimicrobial spectrum, *Clostridium sporogenes,* minimal inhibitory concentration, pathogenic bacteria, spoilage bacteria, spore, sporulation, vegetative cell, zinc.

Introduction

In the past years, researchers have proposed different ways to control spore-forming bacteria, including food spoilage and pathogenic species of *Clostridium* and *Bacillus*, which involve the use of chemical and physical decontamination techniques, antimicrobials and combinations of treatments. *Clostridium sporogenes*, one of the important food-spoilage clostridial species, is putrefactive and spore-forming anaerobe and is involved in the spoilage of canned foods. Moreover, as *C. sporogenes* (especially PA 3679 strain) is the only possible *Clostridium* surrogate for *C. botulinum* (IFT, 2000), this bacterium has successfully served as a nonpathogenic surrogate for investigating effective strategies for inhibiting *C. botulinum* in various studies, for example, thermal (Guan *et al*., 2003; McGlynn *et al*., 2003) and chemical inhibition (Perigo *et al*., 1967; Chen & Hotchkiss, 1993).

The influences of cations on the sporulation and heat resistance of spore-forming bacteria have been studied extensively for past decades (Murrell, 1969; Marquis & Shin, 1994). Most of the previous studies on cations have been concerned with their stimulating effects on sporulation and/or heat resistance of bacterial spores, for example, calcium (Murrell, 1969), manganese (Charney *et al*., 1951; Roberts & Hitchins, 1969), iron (Sugiyama, 1951) and potassium (Wakisaka *et al*., 1982). In contrast, fewer attempts have been made with cations to prevent spore-forming pathogenic and spoilage organisms, regardless of their spores and vegetative cells. In our previous study where we examined the effects of cations and anions on *C. sporogenes* sporulation, we observed that unlike calcium, a few cations tested caused the poor sporulation of *C. sporogenes* (Mah *et al*., 2008). These observations led us to extend our research efforts to other cations affecting the viability and/or sporulation of *C. sporogenes*. As the

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outcome of this endeavour, we observed that, surprisingly, a compound $ZnCl_2$ completely abolished the sporulation of *C. sporogenes* PA 3679 in the present study, which is mainly related to its bacteriocidal activity against clostridial vegetative cells.

Zinc has been reported to possess antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *S. hyicus*, *Salmonella* sp., *Enterococcus faecalis*, *E. faecium* and oral anaerobes, including *Streptococcus mutans*, *Fusobacterium nucleatun* and *Prevotella intermedia* (McCarthy *et al*., 1992; Aarestrup & Hasman, 2004; Sheng *et al*., 2005; Burguera-Pascu *et al*., 2007). However, little information is available regarding the effect of zinc on either sporulation or viability of spore-forming bacteria. Therefore, we report here that zinc has a markedly detrimental effect on the sporulation of *C. sporogenes* via reducing the viability of both vegetative cells and spores and has a broad antimicrobial spectrum against various bacteria.

Materials and methods

Microorganisms

The original stock of *C. sporogenes* PA 3679 spores was obtained from the Center for Technical Assistance of the former National Food Processors Association (NFPA, Dublin, CA, USA). This strain is currently available from the American Type Culture Collection (ATCC 7955; ATCC, Manassas, VA, USA). The obtained spore suspension was divided into cryogenic sterile vials (Fisher Scientific, Pittsburgh, PA, USA) and kept in a freezer (−20 °C) until use.

The original stocks of other bacteria, including *Listeria monocytogenes* KCTC 3710, *S. aureus* KCTC 1916, *B. cereus* KCTC 1012, *B. subtilis* KCTC 1021, *S.* Typhimurium KCTC 1925, *Pseudomonas aeruginosa* KCTC 1750, *Enterobacter aerogenes* KCTC 2190 and *E. coli* KCTC 1039, were obtained from the Korean Collection for Type Culture (KCTC, Daejeon, Korea). The strains maintained in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) containing glycerol (15%, v/v) were divided into cryogenic sterile vials (glycerol stock) and kept in a freezer (−70 °C) until use.

Preparation of *Clostridium sporogenes* vegetative cell culture

To prepare a pure culture of vegetative cells in stationary phase, a multiple-stage inoculation procedure was employed (Uehara *et al*., 1965). The procedure utilised tryptone–peptone–glucose–yeast extract (TPGY) medium consisted of 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) in 1 L of distilled water (USFDA, 1998). Ten millilitres of TPGY broth was inoculated with 10-μL 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 followed by incubation under the same condition. After 2 days of incubation, the vegetative cell culture was immediately subjected to either the preparation of spore suspension described in the following section or the treatment with $ZnCl_2$ (if mentioned specifically, $MnCl_2$ or $FeCl_2$,) described in a later section. The concentration of vegetative cells in the culture was approximately 10^7 CFU mL^{-1}.

Preparation of *Clostridium sporogenes* spore suspension

To obtain a large amount of vegetative cells to sporulate, 100 mL of the vegetative cell culture prepared above was transferred into 1 L of TPGY broth and the flask was incubated anaerobically, using an anaerobic chamber as described previously, for 2 days. 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 and then resuspended in 100 mL of a sporulation medium (the composition of the medium was described right below; this also served as a vegetative cell suspension for small-scale sporulation procedure when resuspended in a sporulation medium with no calcium carbonate, see the following section).

To induce the sporulation of vegetative cells, the resuspended vegetative cell pellet was added to 900 mL of a sporulation medium, giving a total volume of 1 L. The sporulation medium consisted of 60 g tryptone, 1 g dextrose, 1 g sodium thioglycolate (all from Difco) and 5 g calcium carbonate (J. T. Baker Chemical Co., Phillipsburg, NJ, USA) in 1 L of distilled water, and the pH was adjusted to 5.0, which was described by Uehara *et al*. (1965), modified by Duncan & Foster (1968) and then further modified by adjusting initial pH to 5.0 and supplementing with calcium carbonate as a sporulation-stimulating material based on our previous observations (Mah *et al*., 2008). After incubation for 10 days at 32 °C in an anaerobic chamber, the spore crop prepared from vegetative cells was washed by centrifugation as mentioned previously and resuspended in an 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), which served as a spore suspension. The spore suspension prepared was found to contain around 5×10^6 CFU mL^{-1} of spores and kept in a freezer (−20 °C) until subjected to the treatment with $ZnCl_2$.

Determination of effect of zinc on sporulation

To simultaneously determine the effects of multiple compounds on sporulation, we employed a small-scale sporulation procedure described in our previous study (Mah *et al.*, 2008). Briefly, 1 mL of the vegetative cell suspension (vegetative cell pellet resuspended in a sporulation medium prepared with no supplements) prepared earlier was added to 100 mL of a sporulation medium (pH 5.0) at a final concentration of about 10^5 CFU mL^{-1} and then incubated anaerobically at 32 °C for up to 4 weeks. The sporulation medium used (control with no supplements) was supplemented with $ZnCl_2$ (alternatively, $CaCO_3$ or $CaCl_2$, sporulation stimulators, served as positive controls; $MnCl_2$ or $FeCl_2$, sporulation inhibitors; all purchased from Sigma-Aldrich Co., St. Louis, MO, USA) at a level of 0.5% (w/v) prior to use, based on observations in our previous study (Mah *et al.*, 2008). Test samples were taken at 1-week intervals for the determination of viable counts of spores.

Determination of effect of zinc on viability

To determine the effect of zinc on viability, the vegetative cell culture and spore suspension prepared in the previous sections were divided into different groups: control with no treatments and samples treated with $ZnCl_2$ (if mentioned specifically, $MnCl_2$ or $FeCl_2$) at different concentrations up to 1.0% (w/v). All the samples were stored in a 35 °C incubator for given time intervals (Figs 2 and 3) and drawn for the determination of viable counts of vegetative cells or spores.

Enumeration of spores and vegetative cells

The spore suspension sample (or culture in sporulation medium) was placed in a 90 °C water bath for 10 min to activate spores and to inactivate vegetative cells. After heat-shock treatment, the sample was cooled in crushed ice water and 10-fold serially diluted in sterile 0.1% peptone water (w/v). One millilitre of each dilution was pour-plated onto TPGY medium solidified with 1.5% agar (w/v). The TPGY medium was autoclaved at 121 °C for 15 min and held in a 45 °C water bath prior to use. Solidified plates were incubated anaerobically for 3 days at 32 °C. The colonies of viable spores were manually counted after 72 h of incubation, and the numbers of spores were calculated from dual plating. The total number of vegetative cells was determined by the same procedure except for heating at 90 °C.

Determination of antimicrobial activity

To determine the antimicrobial spectrum of zinc, agar diffusion test (Mah *et al.*, 2001) was carried out. Briefly, an aliquot of glycerol stock from each strain was activated twice in TSB medium for 24 h at 37 °C. The discs (diameter of 8 mm) containing $ZnCl_2$ at different concentrations (0.125%, 0.25%, 0.5% and 1.0%, w/v) were placed onto an agar medium inoculated with 0.1 mL of a 24-h culture of each strain. The plate was incubated at 37 °C for 24 h, and the diameter of inhibition halo was measured.

Determination of inhibitory effect of zinc on bacterial growth

To estimate the minimal inhibitory concentration (MIC) of $ZnCl_2$, the growth curves of spoilage and pathogenic bacteria in TSB containing twofold serial dilutions of the compound were monitored by measuring the optical density at 600 nm of bacterial cultures in a Bioscreen apparatus (Labsystems, Helsinki, Finland). Briefly, each well of the plate was filled with 340 μL of fresh TSB containing $ZnCl_2$ at a given concentrations (0.05%, 0.1%, 0.5% or 1.0%, w/v) and inoculated with 10 μL of a 24-h TSB culture of each strain. Access of oxygen to the culture was minimised by closing the gap between the cover and the bottom part of the plate with adhesive tape. The plate was then incubated at 37 °C for 48 h with continuous shaking and absorbance reading was taken at 600 nm every 3 h.

Statistical analysis

The data were presented as means and standard deviations of triplicate samples. The significance of differences was determined by one-way analysis of variance (ANOVA) with Tukey's pairwise comparison module (unless mentioned specifically) 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.

Results

Effects of zinc and other compounds on sporulation of *Clostridium sporogenes*

We compared distinct patterns of sporulation induced by different minerals (0.5% level), as compiled in Fig. 1. In detail, the viable numbers of spores produced in a control with no supplements increased slightly, but significantly ($P < 0.05$), to about 2 Log CFU mL^{-1} after 3 weeks of sporulation induction and thereafter remained relatively constant. As expected, the addition of sporulation-stimulating calcium salts, either $CaCl_2$ or $CaCO_3$ (Mah *et al.*, 2008), caused remarkable increases ($P < 0.05$) in spore numbers when compared with the control with no supplements, and the numbers reached to almost 5 Log CFU mL^{-1} after 4 weeks, respectively. It is noteworthy that the addition of $CaCO_3$ resulted in

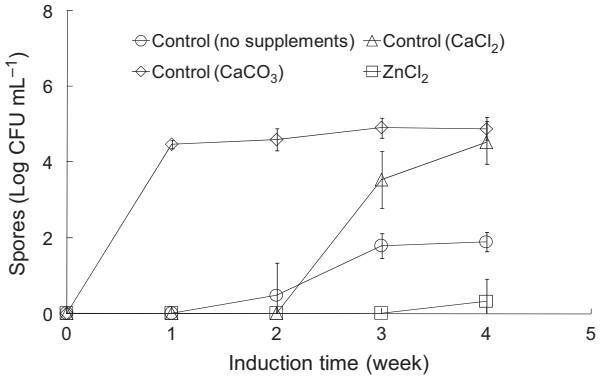

Figure 1 Distinct patterns of sporulation of *Clostridium sporogenes* vegetative cells exposed to zinc and sporulation-stimulating calcium salts. The respective mineral was added to a sporulation medium at a final concentration of 0.5% (w/v), and a basal medium with no addition of mineral served as a control. The vegetative cells (a final concentration of 10^5 CFU mL^{-1}) to sporulate in media supplemented with calcium salts served as positive controls. Sporulation was induced anaerobically at 32 °C. Error bars indicate standard deviations calculated from triplicates.

significantly faster sporulation ($P < 0.05$) than that of $CaCl_2$ during the first 3 weeks, which is in strong agreement with our previous report (Mah *et al.*, 2008). In contrast, $ZnCl_2$ completely abolished sporulation during the first 3 weeks after onset of sporulation. Likewise, after 4 weeks of induction, no sporulation was detected in two of three replicates, which was not significantly different ($P > 0.05$) from those observed during the first 3 weeks.

Effect of zinc on viability of *Clostridium sporogenes* vegetative cells

The results presented in Fig. 1 led us to investigate the effect of a series of chloride cations ($ZnCl_2$, $MnCl_2$ and $FeCl_2$) on clostridial sporulation. As shown in Table 1, when a culture of *C. sporogenes* vegetative cells was subjected to the treatment with either $ZnCl_2$ or other compounds, $MnCl_2$ and $FeCl_2$, at the same concentrations of 0.5% (37 mM zinc, 40 mM manganese, 39 mM iron), only $ZnCl_2$ caused a complete elimination of the viability within a week; however, $MnCl_2$ and $FeCl_2$ led to a similar and even rather enhanced ($P < 0.05$) viability of the vegetative cells, respectively, as compared with control (with no treatments).

To further investigate the responses of *C. sporogenes* vegetative cells to zinc treatment, we examined changes in viable numbers of the vegetative cells (initial level, 10^7 CFU mL^{-1}) exposed to $ZnCl_2$ at different concentrations. As shown in Fig. 2, 0.5% and 1.0% $ZnCl_2$ immediately (the first observation at 1 h after onset of treatment) led to an almost complete and complete loss,

Table 1 Comparison of the effect of zinc with other cations on the viability of *Clostridium sporogenes* vegetative cells

	Survivors (Log CFU mL^{-1})[a]	
Treatments	**Onset**	**1 week**
Control	5.94 ± 0.16	3.63 ± 0.03^B
$MnCl_2$	–	3.78 ± 0.31^B
$ZnCl_2$	–	0.00 ± 0.00^C
$FeCl_2$	–	4.82 ± 0.22^A

[a]Data were taken as a mean ± standard deviation calculated from triplicates. Values in the same column that are not followed by the same letter are significantly different ($P < 0.05$).
The respective mineral was added to a fresh 2-day culture of vegetative cells (10^6 CFU mL^{-1}) at a final concentration of 0.5% (w/v) and a culture with no addition of mineral served as a control.

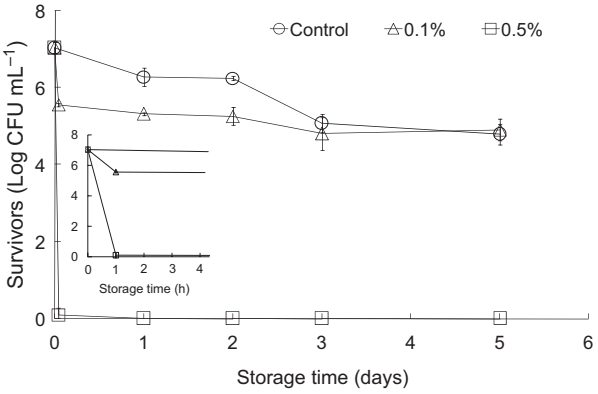

Figure 2 Effect of zinc on the viability of *Clostridium sporogenes* vegetative cells. $ZnCl_2$ was added to a fresh 2-day culture of vegetative cells (10^7 CFU mL^{-1}) at a given concentration and a culture with no addition served as a control. The cultures treated were stored at 35 °C. Error bars indicate standard deviations calculated from triplicates. The inset shows a magnification of the plot during the first 4 h after onset of treatment. No data for treatment with 1.0% $ZnCl_2$ are shown because the viability of vegetative cells was completely eliminated during the first hour of treatment.

respectively, of the viability of *C. sporogenes* vegetative cells. Likewise, the treatment with 0.1% $ZnCl_2$ resulted in a 1.6 Log reduction in the viable number of *C. sporogenes* vegetative cells during the first hour after the treatment; however, although the viable number decreased constantly thereafter and reached a minimum at day 3 ($P < 0.05$), the reduction rate tended to become slow as the test period continued. Furthermore, there appeared no significant differences ($P > 0.05$) in the reduced viable numbers between the treated and untreated vegetative cells (control) from day 3 to day 5. These results indicate that zinc has an ability to markedly damage (or kill) *C. sporogenes* vegetative

cells, which obviously leads to the severe sporulation injury.

Effect of zinc on viability of *Clostridium sporogenes* spores

We also examined changes in the viable numbers of the spores (initial level, 5×10^6 CFU mL^{-1}) exposed to $ZnCl_2$ at different concentrations. As shown in Fig. 3, it turned out that zinc reduced the viability of *C. sporogenes* spores throughout a 4-week test period. At concentrations of 0.5% and 1.0%, $ZnCl_2$ treatments immediately (the first observation at 1 h after onset of treatment) led to a significant reduction ($P < 0.05$) in the viability of *C. sporogenes* spores, causing 0.7 Log and 1.5 Log reductions in viable spore numbers, respectively. However, it was subsequently observed that while the viable number of spores treated with 1.0% $ZnCl_2$ remained relatively constant throughout the 4-week test period ($P > 0.05$) and reached a minimum (approximately 4×10^4 CFU mL^{-1}; 2.1 Log reduction) at week 4 ($P < 0.05$), that of spores treated with 0.5% $ZnCl_2$ increased slightly again at week 2 through week 4 ($P > 0.05$; $P < 0.05$ of Fisher's tests). Furthermore, the increased viable number of spores steadily returned towards the control level ($P > 0.05$ at weeks 1, 2 and 4), as the test period continued. These results imply that zinc does not eliminate spore viability but prevents the germination of spores. Regardless of this, it seems clear that the effect of zinc on the spore viability increases as the concentration increases. Taken together, these results indicate that zinc has an inhibitory effect on the viability not only of vegetative cells but also of spores of *C. sporogenes*.

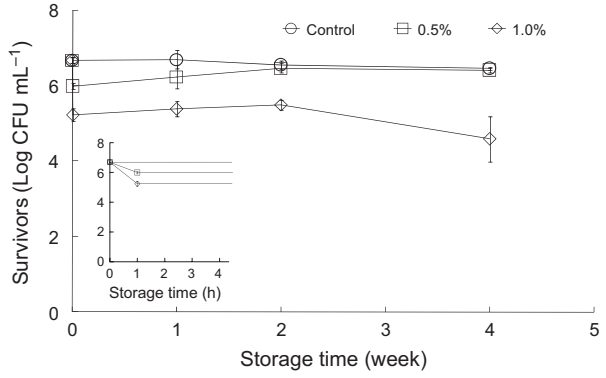

Figure 3 Effect of zinc on the viability of *Clostridium sporogenes* spores. $ZnCl_2$ was added to a spore suspension (5×10^6 CFU mL^{-1}) at a given concentration and a suspension with no addition served as a control. The suspensions were stored at 35 °C. Error bars indicate standard deviations calculated from triplicates. The inset shows a magnification of the plot during the first 4 hours after onset of treatment. Data for treatment with 0.1% $ZnCl_2$ are not shown because the treated spores revealed no significant differences in the viable numbers as compared with control throughout the test period.

Antimicrobial activity of zinc against various bacteria

We examined whether zinc has an antimicrobial activity against various bacteria. As shown in Table 2, the $ZnCl_2$ at the concentration over 0.5% showed antimicrobial activity against Gram-positive bacteria, including *L. monocytogenes*, *B. cereus* and *B. subtilis*, and Gram-negative bacteria, including *S.* Typhimurium, *P. aeruginosa*, *E. aerogenes* and *E. coli*, according to the results of the agar diffusion test. Only *S. aureus* was inhibited by $ZnCl_2$ at 0.25% level.

To estimate the MIC of $ZnCl_2$ for the bacteria, the growth curves of the strains exposed to different concentrations of $ZnCl_2$ were monitored and compared, as shown in Figs 4 and 5. The MIC for simultaneously inhibiting the eight bacteria ranged between 0.05% (3.7 mM) and 0.1% (7.4 mM). The addition of 0.5% $ZnCl_2$ caused a similar inhibition pattern with that at 1.0% $ZnCl_2$, though a larger deviation (data not shown). In detail, among the bacteria tested, *L. monocytogenes* was the most sensitive to the inhibitory effect of $ZnCl_2$ treatment. There was no log phase observed throughout 2 days of the incubation period (Fig. 4a). *Pseudomonas aeruginosa* seemed to be the most tolerant to the $ZnCl_2$ treatment among the bacteria tested. At the level of 0.05% $ZnCl_2$, no significant ($P > 0.05$) growth inhibition was detected throughout the incubation period. Furthermore, the addition of 0.1% $ZnCl_2$ resulted in slightly, but not significantly, inhibited growth of *P. aeruginosa* (Fig. 5b). The other bacteria tested showed a similar susceptibility pattern to each concentration of $ZnCl_2$ (Figs 4 and 5).

Table 2 Antimicrobial activity of zinc on various bacteria

	Concentration of $ZnCl_2$[a]			
Microorganisms	**1%**	**0.5%**	**0.25%**	**0.125%**
Listeria monocytogenes	14[b]	11	–[c]	–
Staphylococcus aureus	16	13	11	–
Bacillus cereus	14	13	–	–
Bacillus subtilis	12	10	–	–
Salmonella Typhimurium	11	09	–	–
Pseudomonas aeruginosa	15	13	–	–
Enterobacter aerogenes	11	10	–	–
Escherichia coli	15	11	–	–

[a]Discs (diameter, 8 mm) containing $ZnCl_2$ solution at different concentrations (w/v) were placed onto an agar medium inoculated with 0.1 mL of a 24-h culture of each test microorganism.
[b]Diameter (mm) of inhibition halo. Each value is the mean calculated from triplicates; standard deviations are not shown as they were <1 mm in each case.
[c]No inhibition.

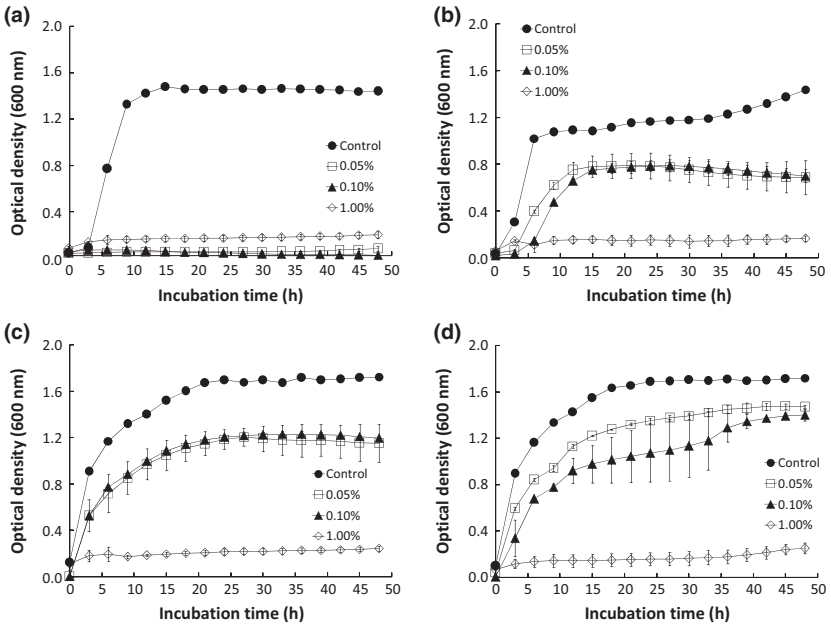

Figure 4 Inhibitory effect of zinc on the growth of Gram-positive bacteria. The growth curves of *Listeria monocytogenes* (a), *Staphylococcus aureus* (b), *Bacillus cereus* (c) and *Bacillus subtilis* (d) are shown. $ZnCl_2$ was added to tryptic soy broth at a given concentration (w/v) shown in the plot. Bacteria were grown in the broth at 37 °C using a Bioscreen apparatus. Bacterial growth was measured as the absorbance at 600 nm. Error bars indicate standard deviations calculated from triplicates.

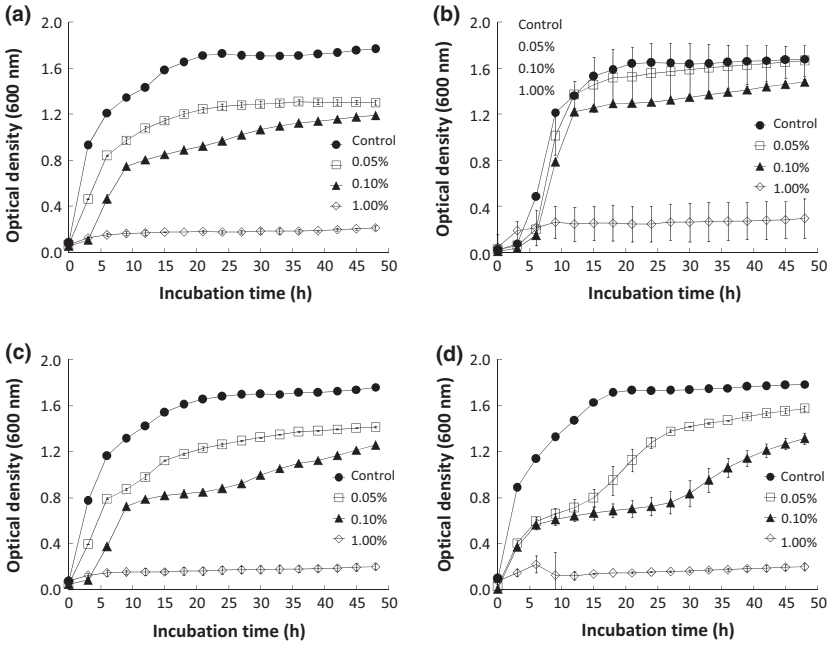

Figure 5 Inhibitory effect of zinc on the growth of Gram-negative bacteria. The growth curves of *Salmonella* Typhimurium (a), *Pseudomonas aeruginosa* (b), *Enterobacter aerogenes* (c) and *Escherichia coli* (d) are shown. $ZnCl_2$ was added to tryptic soy broth at a given concentration (w/v) shown in the plot. Bacteria were grown in the broth at 37 °C using a Bioscreen apparatus. Bacterial growth was measured as the absorbance at 600 nm. Error bars indicate standard deviations calculated from triplicates.

Discussion

Prior to the current study, the observation that zinc (up to 10 mM) can reduce the frequency of sporulation has only been reported for *B. thuringiensis* (Içgen *et al.*, 2002). On the other hand, Kihm *et al.* (1988) reported that the addition of zinc (0.5–1.0 mM) to a complex medium caused the stimulation of sporulation in several strains of *C. botulinum*. In the present study, consistent with the observation by Içgen *et al.* (2002), we observed that $ZnCl_2$ at concentrations of 0.5% (37 mM zinc) and above completely eliminated sporulation (for at least up to 3 weeks) and vegetative cell viability of *C. sporogenes*. When *C. sporogenes* vegetative cells and spores were exposed to the treatments with rather low concentrations (0.1% and 0.5%, respectively) of $ZnCl_2$, their viability was immediately and substantially reduced but gradually returned towards the control levels, as the test period progressed (Figs 2 and 3).

We tried to unveil the mechanism(s) by which zinc inhibits bacterial sporulation. In the present study, we observed that zinc abolishes the viability of *C. sporogenes* vegetative cells within 1 h. Therefore, one possible explanation for the inhibition of sporulation is the toxic effect on the viability of vegetative cells to sporulate, which is strongly supported by the fact that zinc has an antimicrobial spectrum not only against some Gram-positive and Gram-negative bacteria but also against a yeast (McCarthy *et al.*, 1992; Aarestrup & Hasman, 2004). Consistently, Galeano *et al.* (2003) reported that a formulation containing zinc inactivated vegetative cells of *B. anthracis*, *B. cereus* and *B. subtilis*. Apart from this, the authors also observed that their formulation had no inhibitory effect on spores of those species. In contrast, we observed the inhibitory action of $ZnCl_2$ on the viability of *C. sporogenes* spores. Moreover, the inhibitory effect increased as the concentration increased. To explain the inhibitory effect of zinc on *C. sporogenes* spores, it can be speculated that zinc somehow results in a serious interference with germination and subsequent growth processes. At present, this speculation is likely to be correct because zinc can complex with amino acids and peptides, important building blocks in the germination and growth processes, because of its affinity for both thiol and hydroxyl groups (Christianson, 1991). If the interference is a part of the means by which zinc affects the viability of *C. sporogenes* spores, it indicates that zinc may also further interfere with biosynthesis of spore components because those amino acids and peptides are also important building blocks in the sporulation processes. Another explanation is probably a breakdown of the spore structure caused by zinc; however, lack of knowledge makes it somewhat difficult to conclude that this is probable. It is expected that this issue can be clarified by an electron microscopic study of zinc-treated spore structure.

Zinc is considered to be relatively nontoxic to human beings (Fosmire, 1990), and the current US recommended dietary allowance is 11 mg day^{-1} for male adult and 8 mg day^{-1} for female adult (IOM, 2001). Thus, zinc has been suggested to be effective in mouth rinsing at concentrations of 0.1% to 1.0%, considering its antimicrobial property against oral anaerobes (Sheng *et al.*, 2005; Burguera-Pascu *et al.*, 2007). Moreover, zinc has been known to inhibit various pathogenic bacteria (McCarthy *et al.*, 1992; Aarestrup & Hasman, 2004). In the present study, we observed that the MICs of $ZnCl_2$ for *S. aureus*, *S.* Typhimurium and *E. coli* were lower than 0.05% (3.7 mM), which is in agreement with a previous report where the MICs of $ZnCl_2$ were determined to be 0.25 mM for *Staphylococcus* spp. and 2 mM for *Salmonella* sp., *Enterococcus* spp. and *E. coli*, respectively (Aarestrup & Hasman, 2004). Other bacteria tested in this study showed similar MICs (< 3.7 for *L. monocytogenes* to over 7.4 mM for *P. aeruginosa*). Considering the antimicrobial spectrum and safety of $ZnCl_2$, we expect that this compound or a combination thereof has a potential as a surface-cleaning agent and/or disinfectant to decontaminate *Clostridium* and other spoilage and pathogenic bacteria in/on facilities and equipments. Therefore, it would be interesting in future to determine the substantivity of zinc prior to use. It would also be important to see whether zinc is equally effective against cells attached to surfaces or spores within a biofilm. These approaches would be the key to practical applications of zinc in industry where there is a need to control those bacteria.

Conclusion

This study indicates that zinc has a deleterious effect on the viability of both vegetative cells and spores of *C. sporogenes*. This study also suggests that a solution containing $ZnCl_2$ alone or in combination would be useful to clean and disinfect facilities and/or equipments where there is a known or likely high prevalence of carriage of *Clostridium* and other bacteria, considering its wide antimicrobial spectrum and safety.

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