Applied and Environmental Microbiology, July 2005, p. 3556–3564 Vol. 71, No. 7
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.7.3556–3564.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

# Assessment of Heat Resistance of Bacterial Spores from Food Product Isolates by Fluorescence Monitoring of Dipicolinic Acid Release

Remco Kort,[1]* Andrea C. O'Brien,[1] Ivo H. M. van Stokkum,[2] Suus J. C. M. Oomes,[3] Wim Crielaard,[1] Klaas J. Hellingwerf,[1] and Stanley Brul[1,3]

*Laboratory for Microbiology, Swammerdam Institute for Life Sciences, University of Amsterdam,[1] and Department of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit,[2] Amsterdam, and Department of Microbiological Control, Unilever Research and Development, Vlaardingen,[3] The Netherlands*

Received 1 September 2004/Accepted 17 January 2005

**This study is aimed at the development and application of a convenient and rapid optical assay to monitor the wet-heat resistance of bacterial endospores occurring in food samples. We tested the feasibility of measuring the release of the abundant spore component dipicolinic acid (DPA) as a probe for heat inactivation. Spores were isolated from the laboratory type strain *Bacillus subtilis* 168 and from two food product isolates, *Bacillus subtilis* A163 and *Bacillus sporothermodurans* IC4. Spores from the lab strain appeared much less heat resistant than those from the two food product isolates. The decimal reduction times (*D* values) for spores from strains 168, A163, and IC4 recovered on Trypticase soy agar were 1.4, 0.7, and 0.3 min at 105°C, 120°C, and 131°C, respectively. The estimated *Z* values were 6.3°C, 6.1°C, and 9.7°C, respectively. The extent of DPA release from the three spore crops was monitored as a function of incubation time and temperature. DPA concentrations were determined by measuring the emission at 545 nm of the fluorescent terbium-DPA complex in a microtiter plate fluorometer. We defined spore heat resistance as the critical DPA release temperature ($T_c$), the temperature at which half the DPA content has been released within a fixed incubation time. We found $T_c$ values for spores from *Bacillus* strains 168, A163, and IC4 of 108°C, 121°C, and 131°C, respectively. On the basis of these observations, we developed a quantitative model that describes the time and temperature dependence of the experimentally determined extent of DPA release and spore inactivation. The model predicts a DPA release rate profile for each inactivated spore. In addition, it uncovers remarkable differences in the values for the temperature dependence parameters for the rate of spore inactivation, DPA release duration, and DPA release delay.**

Bacterial spores are common contaminants of food products, and their outgrowth may cause food spoilage or food-borne illness. They are extremely resistant to heat and other preservation treatments in comparison to vegetative cells. The inactivation of spores requires high temperatures and long heating times, which are costly and detrimental to the nutritional and organoleptic quality of most food products. To minimize the required heat treatment, there is an urgent need in the food industry for tailored preservation procedures, based on models that accurately predict the presence of viable cells at every step of the food production process (6, 41). To assess the required heat inactivation procedure for the most resistant cell type, the bacterial spore, we isolated and classified two food contaminants and developed a rapid and sensitive screening method to determine the heat resistance of their spores.

Under nutrient-limited conditions, vegetative cells of *Bacillus* species undergo the cell differentiation process of sporulation (17, 42). The resulting spores are metabolically dormant and show, besides resistance to heat, resistance to other potentially lethal treatments that include radiation, high pressure, chemicals, and desiccation. Although spore dormancy and associated resistance are very stable—spores may survive over hundreds and even millions of years (46)—these properties are lost within minutes during the process of germination, which is triggered by the presence of nutrients (31). There is a considerable amount of information on the factors that modulate the heat resistance of spores, but the exact nature of the damage that actually kills the spore is still obscure. Heat resistance factors include the protection of spore DNA by small-acid soluble proteins, the accumulation of divalent cations, such as $Ca^{2+}$ and $Mn^{2+}$, and the dehydration of the spore core (40). In addition, there is a role in heat resistance for dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA), to which the divalent cations are chelated in the core of the spore.

Dipicolinic acid was first identified in bacterial spores by Powell (36). This compound has been identified exclusively in bacterial spores and is involved in their dormancy, wet-heat resistance, and germination. The synthesis of DPA occurs in a sporulating cell in one step from dihydroxydipicolinic acid, an intermediate in lysine biosynthesis. DPA is transported from the mother cell compartment over the outer and inner membranes of the forespore. The proteins involved in DPA transport are most probably encoded by the *spoVA* operon (43); the DPA synthase is encoded by the two genes of the *spoVF* operon (11). Mutations in the *spoVF* locus show significantly increased spore core water content and decreased heat resistance (30). The addition of exogenous DPA to sporulating cells of these mutants rescues the heat resistance of their spores (2).

* Corresponding author. Mailing address: Laboratory for Microbiology, Room C-809, Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands. Phone: 31 (0) 20 525 7062. Fax: 31 (0) 20 525 7056. E-mail: rkort@science.uva.nl.

However, DPA is not indispensable for full heat resistance, as mutants generating DPA-less spores with restored heat resistance have been isolated (50). The DPA content of wild-type spores is approximately 10% of the dry weight of the spore, and DPA is usually present in a 1:1 molar ratio with $Ca^{2+}$. No clear correlation has been found between heat resistance and the total amount of DPA present in the core of wild-type spores. In contrast, many studies have shown that differences in the amount and type of cation strongly affect spore heat resistance (7, 25, 29, 40). Previously, an assay for the release of DPA during spore germination was developed using absorption of DPA in the UV region; this assay allowed the detection of DPA concentrations down to 0.5 μM (39). An alternative method for the detection of DPA was obtained by the use of the strongly enhanced fluorescence of the lanthanide ion $Tb^{3+}$ upon DPA binding. This fluorescent DPA assay was initially developed by Rosen et al. as a method for the detection of bacterial spores (37). The assay has been optimized by Hindle and Hall to a detection limit of 2 nM DPA, which corresponds to $10^4$ spores $ml^{-1}$ (18).

Release of DPA from bacterial spores occurs under a number of different conditions. First, DPA is excreted from spores during the first minute of germination, when nutrients bind to the germinant receptors. The release of DPA is one of the first events in the process of spore germination and occurs simultaneously with the release of cations, the uptake of water, and the loss of the phase-bright appearance of the spore (12, 15, 49). Second, DPA release occurs during the process of spore activation by a sublethal heat treatment that breaks spore dormancy and leads to an increase in the number of germinating spores. The fraction of DPA released from the spores during heat activation differs strongly among several published studies and depends in part on the nature of the heat treatment and the *Bacillus* species involved (4, 39). Third, DPA is released during wet-heat-induced spore inactivation, which is the focus of this study. The relationship between the release of DPA and the heat resistance of spores has been studied for spores from a number of *Bacillus* species, all showing that DPA release is slower than the loss of viability of the spores during heating (13, 47). Although the correlation between DPA release and the spore death rate was complex, in these studies higher rates of death were associated with higher rates of DPA release. A more recent study on heat-induced DPA release from *Bacillus stearothermophilus* spores showed that the rate of spore death has a higher temperature dependence than the rate of DPA release (24).

Isothermal inactivation curves of heat-treated microbial spores or vegetative cells are traditionally presented as linear, semilogarithmic plots, based on the assumption that cell death follows first-order kinetics. The negative reciprocal of the slope in these plots, known as the *D* value, or decimal reduction time (expressed in minutes), is a widely used measure for heat resistance. In addition, a susceptibility measure for changes in temperature, the *Z* value, is defined as the temperature difference (expressed in degrees Celsius) required for changing the *D* value by a factor of 10. The description of cell death in terms of first-order kinetics, often referred to as the mechanistic theory, is not always adequate, however, as a large number of inactivation curves show clear nonlinearity. The upward or downward concavity observed in such curves has been described by models that assume a more complicated or higher-order inactivation kinetics (44). Alternatively, the nonlinearity has been ascribed to phenotypic variation of cells that leads to a spectrum of heat resistances within a population (32). According to the latter models, the inactivation curve is shaped by a distribution function of resistances, such as the Prentice or Weibull distribution (22, 32). A particular case of frequently observed nonlinearity in semilogarithmic inactivation curves is a tailing deviation in which a minor subpopulation displays a much greater heat resistance than the rest of the population, mostly described by a biphasic exponential decay function (1, 10, 14, 20).

In this study we show that some of the inactivation curves of spores from *Bacillus* strains 168, A163, and IC4 have a clear tailing deviation, which is described by a model that includes biphasic inactivation kinetics and the traditional concepts of *D* and *Z* values. We demonstrate that the heat resistance properties of the major part of the spore population correlate with the temperature dependence of heat-induced DPA release. In addition, we developed a quantitative model to describe the relationship between the temperature dependence of the DPA release kinetics and spore inactivation. The high sensitivity, selectivity, and rapidity of the fluorescent DPA assay are of crucial importance to a direct assessment of the heat resistance of spores occurring in food samples.

(Part of this work was presented at the 104th General Meeting of the American Society for Microbiology, New Orleans, LA, 23 to 27 May 2004, and at the European Spores Conference, Smolenice Castle, Slovakia, 17 to 20 June 2004.)

TABLE 1. Strains used in this study

| Strain | Source | Reference(s) |
|---|---|---|
| *Bacillus subtilis* 168 | Bacillus Genetic Stock Center | 8 |
| *Bacillus subtilis* A163 | Unilever R&D, Vlaardingen, The Netherlands | 9, 29; this study |
| *Bacillus sporothermodurans* IC4 | Unilever R&D, Vlaardingen, The Netherlands | 34; this study |

## MATERIALS AND METHODS

**Classification of bacterial strains.** The two sporulating food product isolates used in this study, A163 and IC4, were classified by comparative analysis of their 16S rRNA genes. The 16S rRNA genes were amplified by PCR with primers 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′. Both DNA strands of the purified PCR products were sequenced with a set of gene-specific primers. Database comparisons of the 16S rRNA gene sequence of strain IC4 indicated high sequence similarity to *Bacillus sporothermodurans* (99.8%), *Bacillus oleronius* (97.9%), and *Bacillus smithii* (96.3%). In order to confirm the classification at the species level, DNA-DNA hybridizations were carried out. Strain IC4 showed 85% hybridization to *B. sporothermodurans*. The 16S rRNA gene sequence of strain A163 indicated 99.9% sequence similarity to both *Bacillus subtilis* subsp. *subtilis* and *Bacillus subtilis* subsp. *spizizenii*. Subsequent DNA-DNA hybridizations showed 84% and 64% hybridization to *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, respectively. As a DNA-DNA hybridization of 70% is generally accepted as the lower limit for species delineation (48), strain IC4 can be classified as *B. sporothermodurans* and strain A163 as *B. subtilis* (Table 1).

**Culture conditions and isolation of endospores.** *B. subtilis* cultures of 400 ml were grown in 2-liter shake flasks at 200 rpm at 37°C. A defined, 3-[*N*-morpholino]propanesulfonic acid (MOPS)-buffered growth medium, initially described by Neidhardt et al. (26), and with a number of modifications (16, 19), was used. This medium, referred to below as MOPS medium, contains 1.32 mM $K_2HPO_4$, 0.4 mM $MgCl_2$, 0.276 mM $K_2SO_4$, 0.01 mM $FeSO_4$, 0.14 mM $CaCl_2$, 80 mM

MOPS, 4 mM Tricine, 10 mM glucose, 10 mM $NH_4Cl$, 3 nM $(NH_4)_6Mo_7O_{24}$, 0.4 μM $H_3BO_3$, 30 nM $CoCl_2$, 10 nM $CuSO_4$, 10 nM $ZnSO_4$, 0.1 mM $MnCl_2$, and, if required, 50 μg/ml tryptophan. The pH of the medium was adjusted to 7.6 with KOH. Spores were harvested after 4 days of incubation at 37°C and purified by the water-washing method (28). The purity of spore samples was confirmed visually by microscopic inspection. The number of spores in the cell-free spore suspensions was determined with a hemocytometer (Bürker-Türk, Marienfeld, Germany), followed by dilution to concentrations ranging from $0.5 \times 10^8$ to $5 \times 10^8$ spores per ml and storage in sterile distilled water at −80°C.

**Heat inactivation and counting of spores.** The wet-heat inactivation of spores was carried out using the screw-cap tube method of Kooiman (23). A spore suspension ($0.5 \times 10^8$ to $5 \times 10^8$ spores per ml) of 200 μl was injected with a Hamilton syringe into a preheated (15 min of equilibration) metal screw-cap tube containing 9.8 ml of inactivation medium. The inactivation medium was either sterile Trypticase soy broth or sterile physiological salt solution. Control experiments did not show significant differences in the rate of spore inactivation between these two media (data not shown). Heating was carried out with the metal tubes completely immersed in a glycerol bath. Sampling after a desired incubation time was done through immediate transfer of a tube to ice water. Spore suspensions were diluted 10 times and counted using a hemocytometer. Ten randomly selected squares were counted, with a surface area of 0.0025 $mm^2$ and a depth of 0.01 mm each. Heat inactivation of spores was determined by the loss of their ability to germinate and to form colonies (i.e., viability counts). Dilution series of spore suspensions were prepared in 0.1% peptone–0.85% NaCl and added to Trypticase soy agar pour plates. The number of colonies was counted after 4 days of incubation at 37°C. All heat inactivation experiments and viability counts were carried out in duplicate.

**Monitoring of DPA release.** Fluorescence monitoring of DPA release upon heat inactivation of spores is based on the enhancement of the fluorescence emission of the terbium ion ($Tb^{3+}$) upon binding to DPA (37). Recently, buffer conditions and DPA extraction procedures for the fluorescence monitoring assay have been further improved (18, 21, 33). The DPA assay is not sensitive to other cellular material in the spore sample, because DPA is not present in vegetative cells. Fluorescence emission spectra were recorded from 475 to 600 nm with an Aminco-Bowman series 2 luminescence spectrometer, with excitation at 270 nm and sensitivity at 565 V. The DPA (Aldrich Chemical Co.) concentration used for measuring the $(DPA\text{-}Tb)^+$ emission spectrum was 100 μM in 100 μM terbium(III) chloride hexahydrate (Aldrich Chemical Co.), 20 mM Tris buffer, pH 7.5. The experimental settings for the calibration curve were identical to those for all further DPA measurements, which were carried out in a 96-well plate in a Spectra Max Gemini XS microplate fluorometer. The photomultiplier was set to medium sensitivity, the dichroic cutoff filter to 420 nm, and excitation to 270 nm; emission was monitored at 545 nm. The detection limit (DL) of the assay was determined by the size and noise level of the background signal, expressed as the relative standard deviation of the background (RSDB), and by the sensitivity of the technique (the slope of the calibration curve). By convention the DL is defined as $0.03 \times \text{RSDB} \times \text{BEC}$, where BEC is the background equivalent concentration, i.e., the concentration intercept on the calibration curve (5). The release of DPA was monitored after variable periods of the heat treatment described above. Heat-treated spore suspensions in 0.9% NaCl were cleared from insoluble material by centrifugation. Samples (100 μl) were transferred to 200-μl wells of a 96-well microtiter plate containing 100 μl of a freshly prepared solution of 20 μM $TbCl_3$ in 400 mM sodium acetate buffer, pH 5.0. Two independent experiments were carried out for each data point, and all measurements were done in duplicate. DPA concentrations were determined using a calibration curve of DPA dissolved in 0.9% NaCl solution.

## RESULTS AND DISCUSSION

**Heat inactivation of *Bacillus* spores.** Spore suspensions from the *B. subtilis* laboratory strain 168 and the food product isolates *B. subtilis* A163 and *B. sporothermodurans* IC4 were incubated at the temperature ranges 98°C to 111°C, 111°C to 120°C, and 121°C to 131°C, respectively. The viability of spores was monitored over incubation periods of 20 min for strains 168 and A163 and 7 min for strain IC4. The plots in Fig. 1 indicate large differences in the heat resistance of spores from the three *Bacillus* strains in the order 168 < A163 < IC4 (Table 2). All inactivation plots can be described by a biphasic exponential decay function that includes a second term to describe the inactivation of a subpopulation of more heat-resistant spores:

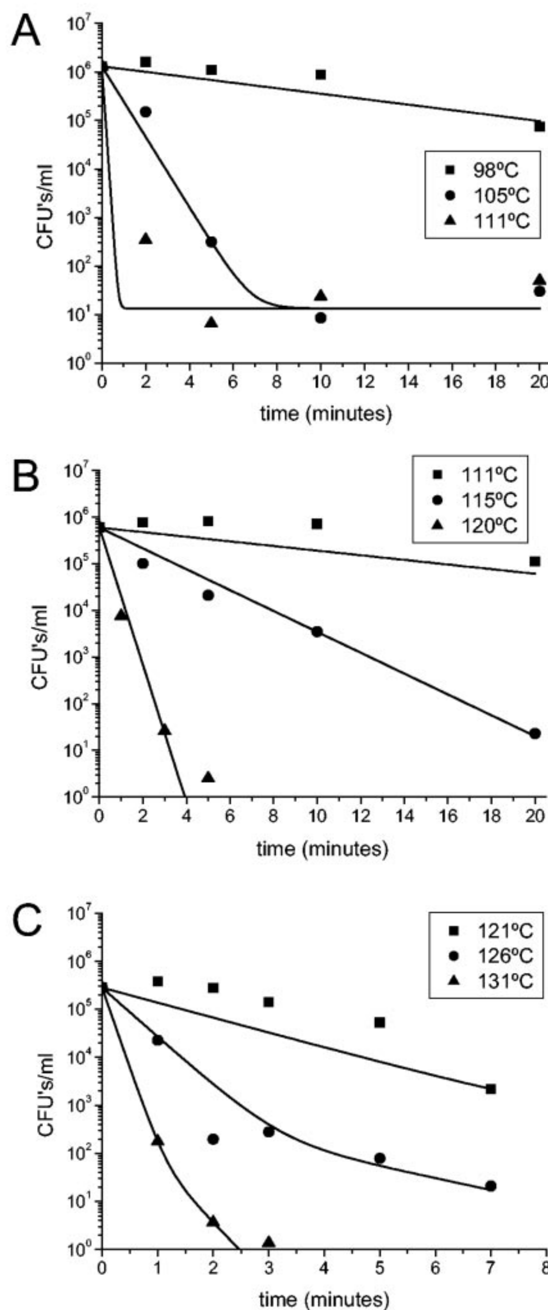


FIG. 1. Wet-heat inactivation of *Bacillus* spores. All data points in each of the three curves have been fitted with a constant *Z* value. The lines represent the fit with equations 1 and 3. Fit parameters are shown in Table 2. (A) Heat inactivation of spores from strain 168 (cultured in modified MOPS medium with 1.5 mM $Ca^{2+}$). (B) Heat inactivation of spores from strain A163 (cultured in MOPS medium). (C) Heat inactivation of spores from strain IC4 (cultured in modified MOPS medium with 1.5 mM $Ca^{2+}$).

$$N(t,T) = N_0 10^{-t/D(T)} + N_r 10^{-t/D_r(T)} \quad (1)$$

where *N* is the number of viable spores at time *t* and temper-

TABLE 2. Wet-heat inactivation and DPA release parameters for spores from *Bacillus* strains 168, A163, and IC4[a]

| Parameter | Value for strain: | | | Equation | Figure(s) |
|---|---|---|---|---|---|
| | 168 | A163 | IC4 | | |
| $D_{98}$ (min) | 18 | | | 3 | 1A |
| $D_{105}$ (min) | 1.4 | | | 3 | 1A |
| $D_{111}$ (min) | 0.2 | 22 | | 3 | 1A, 1B |
| $D_{115}$ (min) | | 2.6 | | 3 | 1B |
| $D_{120}$ (min) | | 0.7 | | 3 | 1B |
| $D_{121}$ (min) | | | 3.2 (22) | 3 | 1C |
| $D_{126}$ (min) | | | 1.0 (4.1) | 3 | 1C |
| $D_{131}$ (min) | | | 0.3 (0.8) | 3 | 1C |
| $N_0$ | $1.3 \times 10^6$ | $5.7 \times 10^5$ | $2.8 \times 10^5$ | 1 | 1A, 1B, 1C |
| $N_r$ | 13 | 0 | 890 | 1 | 1A, 1B, 1C |
| $Z$ (°C) | 6.3 | 6.1 | 9.7 (7.3) | 3 | 1A, 1B, 1C |
| $A_1$ (%) | 14 | 23 | 9.5 | 4 | 3 |
| $\Delta T$ (°C) | 1.5 | 2.5 | 2.7 | 4 | 3 |
| $T_c$ (°C) | 108 | 121 | 131 | 4 | 3 |

[a] The $D$ and $Z$ values, as well as the parameters $N_0$ and $N_r$ for spores from strains 168, A163, and IC4, result from fitting equations 1 and 3 to the data points in Fig. 1 (fits shown). For comparison the DPA release parameters of the sigmoidal fits (equation 4) shown in Fig. 2 for spores from the same strains have been indicated in the lower part of the table. The $D$ value is the decimal reduction value with the inactivation temperature in the subscript; $N_r$ is the heat-resistant rest population; $N_0$ plus $N_r$ equals the number of viable spores at time zero; $A_1$ is the minimum percentage of DPA released, and $A_2$ the maximum, fixed at 100%; $T_c$ is the center at $(A_1 + A_2)/2$ and $\Delta T$ the associated temperature range. The $D_r$ and $Z_r$ values of the heat-resistant fraction $N_r$ of IC4 spores are given in parentheses. It should be noted that the uncertainty in the $D_{r_{121}}$, $D_{r131}$, and $Z_r$ values is very large.

ature $T$, $N_r$ is the heat-resistant subpopulation of spores, and $N_0$ plus $N_r$ is the total number of viable spores at time zero. The $D$ value is the decimal reduction value of the major spore population, and the $D_r$ value is the reduction value of the heat-resistant subpopulation (Table 2). The $D_r$ value is also known in the literature as $D_2$ (10) or the "tail $D$ value" (20). In addition, a $Z$ value is defined as the number of degrees Celsius required to change the $D$ value by a factor of 10:

$$Z = (T_1 - T_0)/\log(D_0/D_1) \quad (2)$$

Alternatively, we have

$$D(T) = D(T_0)10^{-(T-T_0)/Z} \quad (3)$$

Accordingly, the substitution of the $D(T)$ and $D_r$(T) values from equation 3 in equation 1 shows how the number of viable spores depends on the $Z$ and $Z_r$ values. We used the resulting equation to fit all data points in each of the three graphs in Fig. 1. The heat inactivation plots of spores from strain 168 in Fig. 1A and those obtained under slightly different experimental conditions (29) reproducibly indicate that a fraction of the spore population, approximately 10 to 100 spores or 0.001 to 0.01%, is not inactivated within 20 min of incubation at 105°C and 111°C (Table 2). This tailing deviation results from the presence of a heat-resistant subpopulation of the spores, which is represented by the parameter $N_r$ in equation 1. From the fact that $N_r$ is (almost) constant in time in Fig. 1A, it follows that the $D_r$ value cannot be precisely estimated, because it is an extremely large number with high uncertainty. Higher inactivation temperatures and longer inactivation times are necessary for a better assessment of the heat resistance properties of the heat-resistant subpopulation $N_r$. We used the heat-resistant fraction of strain 168 spores as an inoculum to start a new culture and harvested the spores generated after 4 days for the reassessment of their heat resistance properties (data not shown). We did not find any significant differences in the heat resistance properties compared to those presented in Fig. 1A, in agreement with the concept of a heat-resistant subpopulation resulting from phenotypic, and not genotypic, variations (32). Since the data point at 2 min overshoots the fit at 111°C for the inactivation of strain 168 spores, the steepness of the initial phase of this plot was confirmed in a separate inactivation experiment. The results obtained indicated that more than 99.9% of the strain 168 spores were inactivated within 1 min of incubation (data not shown). Spores from the food product isolate A163 do not show a clear heat-resistant subpopulation, and their inactivation matches the mechanistic concept of ideal, first-order kinetics (10). The heat inactivation kinetics of strain IC4 spores are clearly nonlinear. Application of the biphasic kinetic model as in equation 1 for all three inactivation temperatures in Fig. 1C results in a heat-resistant subpopulation of approximately $10^3$ spores, or 0.3% of the spores (Table 2). The $Z$ value parameters were converged to 6.3°C, 6.1°C, and 9.7°C for spores from strains 168, A163, and IC4, respectively. In addition, the estimated $Z_r$ value of the heat-resistant fraction of IC4 spores was 7.3°C (Table 2). These values are in agreement with those commonly observed for *Bacillus* spores, which usually vary between 5.5°C and 10°C (38).

It is evident that the data points at the lowest inactivation temperature in all three graphs in Fig. 1 (indicated by the squares) overshoot the fit by the kinetic model. This can be explained by the notion that at the lower inactivation temperatures the effect of spore activation is most pronounced. Sublethal heat treatments can activate spores, leading to an increase in the germination efficiency and thus to an underestimation of the number of spores inactivated by the heat treatment. The number of germinating spores constitutes 60% of the spores of the laboratory strain 168, a fact evident from comparisons between the total number of spores counted in the hemocytometer and the CFU in the absence of a heat treatment. For the food product isolate A163, the fraction of viable spores under the same conditions is only 7%, while for the extremely heat resistant isolate IC4, an even lower germination efficiency, 3%, was observed. The germination efficien-

cies for 168, A163, and IC4 change to 74%, 10%, and 4% after incubations of 2 min at 98°C, 5 min at 111°C, and 1 min at 121°C, respectively. The effect of heat-induced activation was not included in our kinetic model. Even then, it is still unlikely that we will describe 100% of the spore population. We assume that the spores that do not germinate on the Trypticase soy agar plates are superdormant and show the same inactivation kinetics as spores that germinate within 4 days of incubation. We cannot exclude the possibility that part of the spore population that does not germinate is actually not superdormant but dead. Discrimination between superdormant and dead spores has been raised before as an interesting topic of research (27) and can possibly be assessed by (a combination of) chemical and enzymatic treatments, gamma irradiation, or high pressure to facilitate spore germination.

Although more-extensive studies need to be done, the striking differences in germination efficiency between heat-resistant strains may imply that a gain in the heat resistance of spores compromises the spore germination efficiency. The low germination efficiency of spores from strains A163 and IC4 did not come as a surprise. The fact that these spores from these strains have been isolated from a heat-treated food product indicates that these robust, heat-resistant spores did not germinate well, even under nutrient-rich conditions. Recent experiments in our laboratory indicate that the relatively high heat resistance of spores is not always maintained after germination and culturing under nutrient-rich conditions (A. C. O'Brien and R. Kort, unpublished observations). In line with this concept, spores from the laboratory strain 168, which has been selected for efficient germination for many generations, may have become more sensitive to heat inactivation over time.

**Fluorescence monitoring of DPA release from spores.** We hypothesized that monitoring of the release of spore constituents during a heat treatment can be used as a rapid and sensitive method to determine the heat resistance of spores. This method will overcome the need for the laborious and time-consuming determination of viability counts. Obvious indicators for heat-injured or leaky spores are $Ca^{2+}$ ions and DPA, since a major fraction of the dry weight of the spore consists of the Ca–DPA complex. Because we could not get reproducible results from the fluorescence monitoring of $Ca^{2+}$ release from spores upon heat treatment by use of a $Ca^{2+}$-binding fluorescent dye (data not shown), we decided to monitor DPA release by a fluorescent assay that uses the enhanced fluorescence of ions of the lanthanide metal terbium ($Tb^{3+}$) upon complex formation with DPA (18, 37). Excitation of the $(Tb\text{-}DPA)^{+}$ complex at 270 nm results in a typical emission spectrum with peaks at 490, 545, and 584 nm, as presented in Fig. 2A. The calibration curve for DPA concentrations from 0 to 10 μM in a 96-well microtiter plate of 200 μl is presented in Fig. 2B. The DL of the assay was derived from this curve with an RSDB of 4.5% and a BEC of 110 nM (see Materials and Methods). Accordingly, the DL equals 15 nM, which is equivalent to 3 pmol of DPA, or the contents of approximately $7 \times 10^3$ spores per well in the microtiter plate. Thus, a minimal spore concentration of $7 \times 10^4$ spores $ml^{-1}$ is required to measure DPA release under the assay conditions used here. The linear dynamic range of the assay is almost 3 orders of magnitude, from approximately 15 nM to approximately 10 μM (data not shown). At concentrations higher than 10 μM, the presence of nonchelated $Tb^{3+}$ becomes limiting. This problem can be avoided by the use of higher concentrations of $TbCl_3$ in the buffer, but this will lower the detection limit (see also reference 18).

The fluorescence emission is monitored throughout this work at the peak of the highest relative intensity at 545 nm. Just as during the process of germination, during heat inactivation DPA is most probably released from spores as a complex of $Ca^{2+}$ and DPA in a 1:1 ratio. The equilibrium constant for the formation of Ca-DPA is $10^{4.4}$, while that for $(Tb\text{-}DPA)^{+}$ is $10^{8.7}$, indicating that with a sufficient excess of $Tb^{3+}$ (10 μM), the assay is tolerant of micromolar concentrations of $Ca^{2+}$ (18). The maximal amount of DPA release from spores of strain 168 in this study corresponds to a DPA concentration of $4.3 \pm 0.2$ μM. This means that 0.043 μmol is present in $10^8$ spores, thus $0.43 \pm 0.02$ fmol per spore, only marginally higher than the 0.365 fmol per spore reported previously (18). We found approximately twice the amount of DPA per spore for strains A163 and IC4: $0.85 \pm 0.04$ fmol and $0.82 \pm 0.03$ fmol, respectively. Several studies on the correlation between DPA content per spore and heat resistance were carried out in the past, but no clear correlation was found (25). For spores from the three strains that we have studied, this correlation is not clear, either; heat-resistant spores from the two food product isolates contain approximately twice the amount of DPA as spores from strain 168, but there is no greater amount of DPA per spore in the extremely heat resistant spores from the IC4 strain. A correlation is generally observed between wet-heat resistance and the water content of the spore protoplasts, as determined by buoyant density sedimentation experiments (3). However, the spore protoplast water contents of the three spore crops used in this study did not show this correlation (data not shown). The latter result confirms the results of previous sedimentation experiments showing that for extremely heat resistant spores with $D_{100}$ values of >100 min, such as those used in this study, the protoplast water content no longer correlates with spore heat resistance (3).

**Temperature dependence of DPA release: a novel probe for heat resistance.** In order to test the feasibility of using the DPA release profiles as a probe for heat resistance of bacterial spores, we determined these profiles in the temperature window from 90°C to 160°C for the three different *Bacillus* spore batches with variable heat resistance (Table 2; Fig. 3). The incubation time of 1.5 min was chosen arbitrarily; it is the time point at which approximately all DPA has been released by spores from strain 168 at 111°C (Fig. 4A). The DPA release profiles determined for the three spore batches all show a sigmoidal shape with a center that shifts to a higher temperature as the heat resistance of the spore batch increases (Fig. 3). The DPA release can be fitted as a function of temperature with equation 4:

$$DPA(T) = (A_1 - A_2)/\{1 + \exp[(T - T_c)/\Delta T]\} + A_2 \quad (4)$$

where $A_1$ is the minimum value, $A_2$ is the maximum value (set to 100%), $T_c$ is the critical DPA release temperature [the center at $(A_1 + A_2)/2$], and $\Delta T$ is the associated temperature range (see Table 2). We found $T_c$ values for spores from strains 168, A163, and IC4 of 108°C, 121°C, and 131°C, respectively.

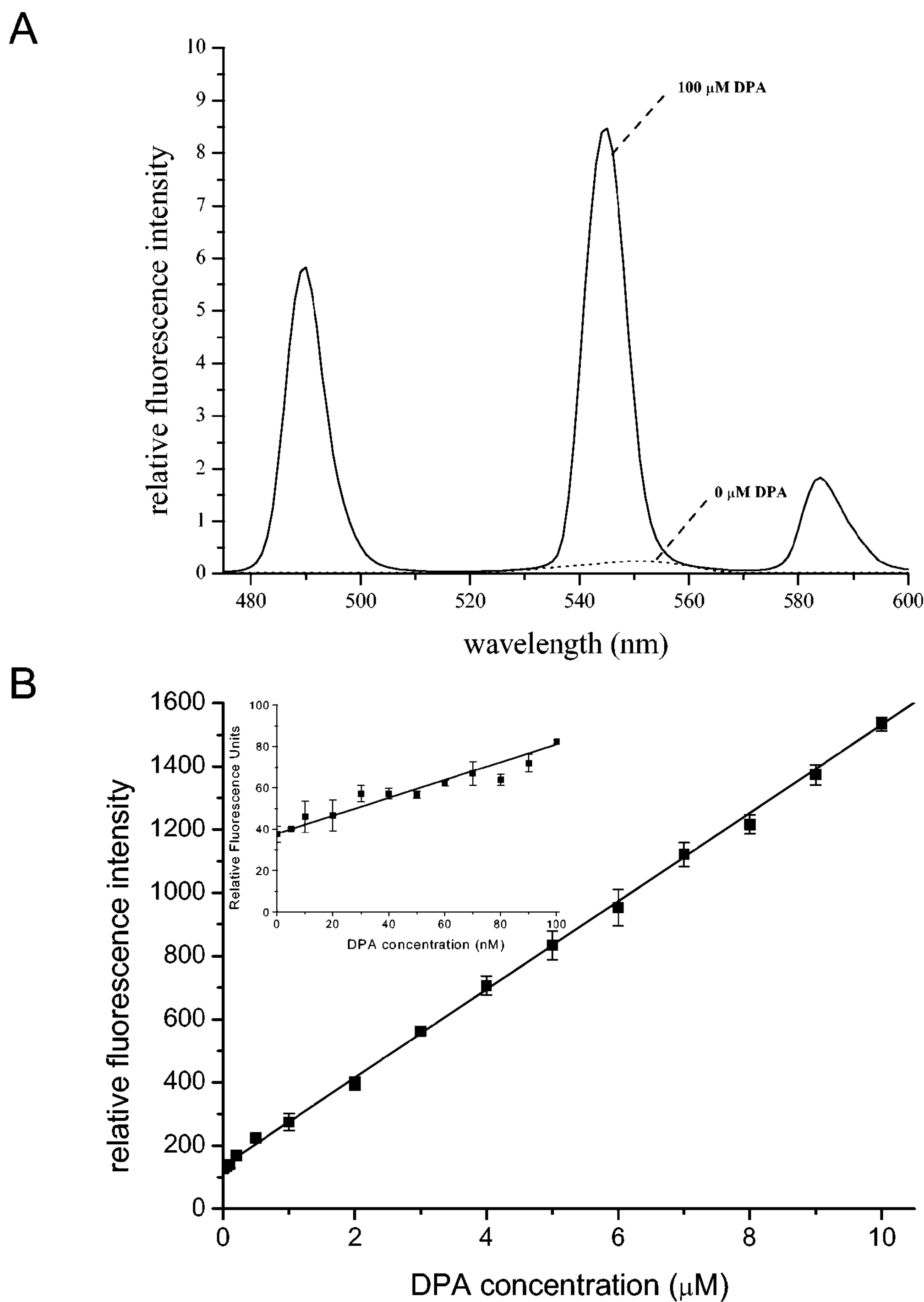


FIG. 2. Fluorescence emission of the $(Tb\text{-}DPA)^{+}$ complex. (A) Fluorescence emission spectra were recorded from 475 to 600 nm with an Aminco-Bowman series 2 luminescence spectrometer, with excitation at 270 nm and sensitivity at 565 V. The dipicolinic acid concentrations used for the emission spectra were 0 (dashed line) and 100 (solid line) μM in 100 μM terbium(III) chloride, 20 mM Tris buffer, pH 7.5. (B) Calibration curve for DPA concentrations from 0 to 10 μM monitored at 545 nm in a microplate fluorometer. The inset for DPA concentrations from 0 to 100 nM was used to determine the detection limit of 15 nM (see the text for more details). The experimental settings for the calibration curve were identical to those used for all further DPA measurements (see Materials and Methods).

This clearly establishes that a relatively high $T_c$ value is indicative of a relatively high heat resistance of the spore crop. We calculated the $D$ values for the 168, A163, and IC4 spores at the critical DPA release temperature for the major fraction in all three spore batches by using the parameters in Table 2 and equation 2. The $D$ value at the critical DPA release temperature is 0.4 ± 0.1 min for all three spore crops tested. This makes it very clear that the $T_c$ values are associated with a

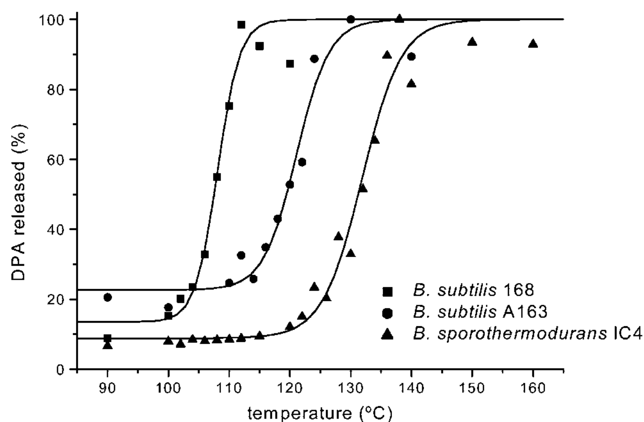


FIG. 3. Heat-induced dipicolinic acid release by *Bacillus* spores. DPA release is expressed as the percentage of the total content in spores from strains 168 (squares), A163 (circles), and IC4 (triangles). Spore suspensions were incubated for 1.5 min at the indicated temperatures, followed by determination of the amount of released DPA by 545-nm fluorescence emission measurements at 270-nm excitation of the $(Tb\text{-}DPA)^+$ complex in a microplate fluorometer.

relatively high spore killing rate, with $D$ values on the subminute time scale.

In addition to striking differences in $T_c$ values for the three spore crops, we found a steeper DPA release curve for the spores from strain 168 ($\Delta T = 1.5°C$) than for the two more heat resistant spore batches ($\Delta T = 2.5°C$ and 2.7°C for spores from strains A163 and IC14, respectively [Table 2]). This indicates a higher susceptibility for DPA release of 168 spores to changes in the incubation temperature. However, if we compare the $\Delta T$ values with $Z$ values, we cannot establish yet whether there is a correlation (Table 2). Spore inactivation and DPA release studies on a larger number of spore crops are required to substantiate the correlation between the $\Delta T$ and $Z$ values.

The plots in Fig. 3 show very clearly that this rapid fluorescence assay can provide a good measure of the heat resistance of bacterial spores with $T_c$ as the heat resistance parameter. However, the identification of minor heat-resistant fractions in a spore population (Fig. 1A) is not possible with this DPA assay. For this latter purpose, agar pour plates are required. As a next step in the analysis of DPA release kinetics, we looked for mechanisms that could underlie the sigmoidal behavior of the three plots in Fig. 3. We developed a simple, kinetic model for DPA release and investigated the important parameters involved.

**Kinetic model for DPA release.** As a starting point for our kinetic model, we experimentally determined the DPA release kinetics for the *B. subtilis* 168 spores at three different temperatures (Fig. 4A). Interestingly, while the killing of spores is well described by a first-order exponential-decay function (equation 1; Fig. 1), the heat-induced release of DPA cannot be described by an exponential-rise function of the same order (Fig. 4A). Apparently, the heat inactivation of a spore does not instantaneously result in the release of its DPA content, and factors other than DPA release are involved in heat inactivation. The delay in DPA release may result from the slow disintegration of structures within the spore, which may require longer incubation times and higher temperatures than spore inactivation. The disintegration of spore structures has recently been observed by scanning electron microscopy, showing that autoclaved spores have a wrinkled appearance resulting from a loss of internal volume (35).

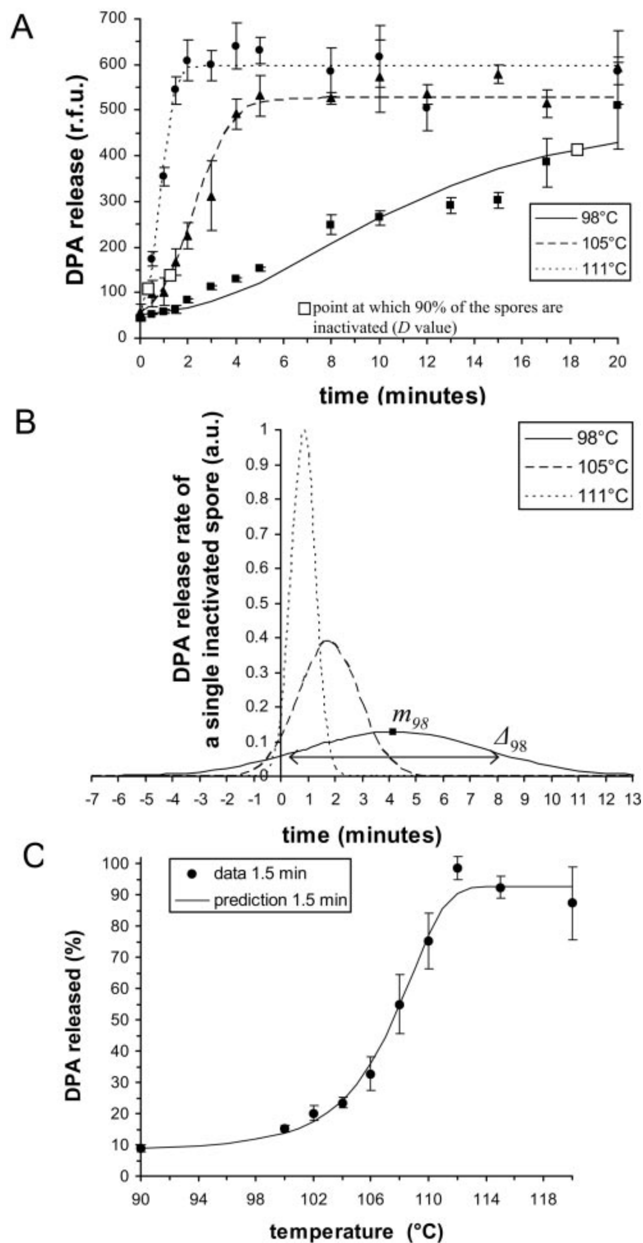


FIG. 4. Kinetics of DPA release. (A) DPA release (expressed in relative fluorescence units) in spores isolated from the laboratory strain 168 during heat inactivation at 98°C (squares), 105°C (triangles), and 111°C (circles). Lines indicate DPA release as described by the model (equation 8). (B) DPA release rate profiles (expressed in arbitrary units) of an inactivated spore of *B. subtilis* 168 at 98°C (solid line), 105°C (dashed line), and 111°C (dotted line), as calculated from the proposed model (equation 5). The black square indicates $m_{98}$, the DPA release delay parameter at 98°C (expressed in minutes), and the double-headed arrow indicates $\Delta_{98}$, the DPA release duration parameter at 98°C (expressed in minutes). (C) Measured (circles) (see also Fig. 3) and model-predicted (equation 9) (solid line) DPA release of spores from *B. subtilis* 168 during a 1.5-min incubation period.

The time points at which 90% of the spores have been inactivated (equal to the $D$ values), as indicated in Fig. 4A, show a remarkable difference among DPA release patterns as a function of temperature. At 98°C most of the DPA has been released at this time point, while at 105°C and 111°C only a minor fraction of the DPA has been released from the spores (Fig. 4A). If we assume a homogenous spore population, then each inactivated spore will give rise to a DPA release profile $p(t,T)$, of which we model the release rate with a Gaussian shape, with parameters $m$ (delay) and $\Delta$ (release duration):

$$p(t,T) = \exp\{[-\ln(2)][2\{t - m(T)\}/\Delta(T)]^2\}/\Delta(T) \quad (5)$$

The temperature dependence of the delay and duration parameters is described by equations 6 and 7:

$$m(T) = m(T_0)10^{-(T-T_0)/Z_m} \quad (6)$$

$$\Delta(T) = \Delta(T_0)10^{-(T-T_0)/Z_\Delta} \quad (7)$$

If we neglect the heat-resistant subpopulation, the time and temperature dependence of the DPA release is now described by the convolution of $N(t,T)$ with $p(t,T)$:

$$\mathrm{DPA}(t,T) = F_2\{[1 - 10^{-t/D(T)}]*p(t,T)]\} + F_1 \quad (8)$$

where * is the symbol for convolution, $F_1$ is the minimum value, and $F_1$ plus $F_2$ is the maximum value for the fluorescence of the DPA released, as measured by the emission of the $(\mathrm{DPA\text{-}Tb})^+$ complex. The convolution integral results in an analytical expression (see, e.g., reference 45). Thus, the curve $\mathrm{DPA}(t,T)$ measured at three temperatures can be well described with a small number of parameters (Fig. 4A). The profiles of the rate of DPA release during inactivation at 98°C, 105°C, and 111°C are plotted in Fig. 4B. The profiles are described by the $Z$ parameters $Z_\Delta$ and $Z_m$, which indicate the temperature sensitivity for the DPA release duration ($Z_\Delta$) and the DPA release delay ($Z_m$). If we now compare the three $Z$ values, we see that the parameter most affected by the temperature is the spore inactivation rate (only 6.3°C is required to change this rate by a factor of 10), followed by the DPA release duration (15°C) and finally the DPA release delay (19°C). We can interpret this behavior by presuming two different mechanisms that contribute to DPA release: (i) equilibration between unbound and bound DPA or Ca–DPA in the spore and (ii) the destruction of a spore structure(s) that limits the release of unbound DPA from the spore. The latter factor, which inhibits efflux of unbound DPA and is determined by the delay parameter $Z_m$, is less temperature dependent and accordingly shows a higher activation energy barrier than the conversion from bound to unbound DPA in the core of the spore.

We tested if our model based on the experimental data in Fig. 4A and 1A was able to predict the sigmoidal release of DPA as found in Fig. 3A. The prediction is shown in Fig. 4C.

At a fixed time, equation 8 can be written as

$$\mathrm{DPA}(t = t_{\mathrm{fix}},T) = A_1 + (A_2 - A_1)\{[1 - 10^{-t_{\mathrm{fix}}/D(T)}]*p(t_{\mathrm{fix}},T)\} \quad (9)$$

with $A_1$ and $A_2$ as the minimal and maximal values for the percentage of DPA released as in equation 4. The estimated parameters are collated in Table 3. These equations represent a coherent description of the time and temperature dependence of CFU and DPA for the laboratory strain 168, as can be judged from Fig. 4. In conclusion, we describe here a simple method that is—in contrast to the previous laborious methods, which require viability counts of dilution series—suitable for screening a large number of spore batches for the initial assessment of their heat resistance. The critical DPA release temperature as an alternative measure for spore heat resistance and the highly sensitive, selective, and rapid DPA assay used here form a potentially powerful combination for a direct assessment of the heat resistance of spores occurring in food samples.

TABLE 3. Model parameters for heat-induced DPA release by *B. subtilis* 168 spores

| Parameter[a] | Value | Equation | Figure |
|---|---|---|---|
| $D_{98}$ (min) | 18 | 3 | 1A |
| $m_{98}$ (min) | 4.2 | 6 | 1A |
| $\Delta_{98}$ (min) | 7.9 | 7 | 4B |
| $Z$ (°C) | 6.3 | 3 | 1A |
| $Z_m$ (°C) | 18.5 | 6 | 4B |
| $Z_\Delta$ (°C) | 14.6 | 7 | 4B |
| $N_0$ | $1.3 \times 10^6$ | 1 | 1A |
| $N_r$ | 13 | 1 | 1A |
| $F_1$ (AU) | 50 | 8 | 4A |
| $F_{2_{98}}$ (AU) | 450 | 8 | 4A |
| $F_{2_{105}}$ (AU) | 480 | 8 | 4A |
| $F_{2_{111}}$ (AU) | 550 | 8 | 4A |
| $A_1$ (%) | 8 | 9 | 4C |
| $A_2$ (%) | 84 | 9 | 4C |

[a] The parameters are defined as follows: $D_{98}$, the $D$ value at 98°C; $m_{98}$, the DPA release delay parameter at 98°C (min); $\Delta_{98}$, the DPA release duration parameter at 98°C (expressed in minutes); $Z$, the temperature change that is required to change the $D$ value by a factor of 10 (expressed in degrees Celsius); $Z_m$, the temperature change that is required to change the $m$ value by a factor of 10 (expressed in degrees Celsius); $Z_\Delta$, the temperature change that is required to change the $\Delta$ value by a factor of 10 (expressed in degrees Celsius); $N_0$, the number of viable spores at time zero minus the rest population $N_r$; $N_r$, the heat-resistant rest population of the spores; $F_1$, the minimum DPA concentration as measured by the emission of the $\mathrm{Tb(DPA)}^+$ complex (expressed in relative fluorescence or arbitrary units [AU]); $F_2$, the maximum DPA concentration (arbitrary units) with the temperature (in degrees Celsius) in subscript; $A_1$, the minimum DPA release (expressed as a percentage); $A_2$, the maximum DPA release (expressed as a percentage).

## ACKNOWLEDGMENTS

We thank Jody Hehenkamp for assistance with the heat inactivation of IC4 spores, Jan Smelt for stimulating discussions, and Peter Setlow for critical reading of the manuscript.

R.K. and A.C.O. were supported by grant ABC-5587 from NWO-STW. Work on strain classifications and spore viability counts was supported by a grant to Unilever from the Dutch Ministry of Economic Affairs, the Ministry of Education, Culture, and Science, and the Ministry of Housing, Spatial Planning, and the Environment.

## REFERENCES

1. **Ababouch, L. H., L. Grimit, R. Eddafry, and F. F. Busta.** 1995. Thermal inactivation kinetics of *Bacillus subtilis* spores suspended in buffer and in oils. J. Appl. Bacteriol. **78:**669–676.
2. **Balassa, G., P. Milhaud, E. Raulet, M. T. Silva, and J. C. Sousa.** 1979. A *Bacillus subtilis* mutant requiring dipicolinic acid for the development of heat-resistant spores. J. Gen. Microbiol. **110:**365–379.
3. **Beaman, T. C., and P. Gerhardt.** 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl. Environ. Microbiol. **52:**1242–1246.
4. **Beaman, T. C., H. S. Pankratz, and P. Gerhardt.** 1988. Heat shock affects permeability and resistance of *Bacillus stearothermophilus* spores. Appl. Environ. Microbiol. **54:**2515–2520.
5. **Boumans, P. W. J. M., R. Payling, D. G. Jones, and A. Bengtson.** 1997. Glow discharge optical emission spectrometry, p. 440–451. John Wiley & Sons, Chichester, United Kingdom.

6. **Brul, S., P. Coote, S. Oomes, F. Mensonides, K. Hellingwerf, and F. Klis.** 2002. Physiological actions of preservative agents: prospective of use of modern microbiological techniques in assessing microbial behaviour in food preservation. Int. J. Food Microbiol. **79:**55–64.
7. **Brul, S., F. M. Klis, S. J. C. M. Oomes, R. C. Montijn, F. H. J. Schuren, P. Coote, and K. J. Hellingwerf.** 2002. Detailed process design based on genomics of survivors of food preservation processes. Trends Food Sci. Technol. **15:**325–333.
8. **Burkholder, P. R., and N. H. Giles.** 1947. Induced biochemical mutations in *Bacillus subtilis*. Am. J. Bot. **34:**345.
9. **Cazemier, A. E., S. F. Wagenaars, and P. F. ter Steeg.** 2001. Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli. J. Appl. Microbiol. **90:**761–770.
10. **Cerf, O.** 1977. Tailing of survival curves of bacterial spores. J. Appl. Bacteriol. **42:**1–19.
11. **Daniel, R. A., and J. Errington.** 1993. Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in *Bacillus subtilis*. J. Mol. Biol. **232:**468–483.
12. **Dring, G. J., and G. W. Gould.** 1971. Sequence of events during rapid germination of spores of *Bacillus cereus*. J. Gen. Microbiol. **65:**101–104.
13. **El-Bisi, H. W. L., R. V. Lechowich, M. Amaha, and Z. J. Ordal.** 1962. Chemical events during death of bacterial endospores by moist heat. J. Food Sci. **27:**219–231.
14. **Fujikawa, H., and T. Itoh.** 1996. Tailing of thermal inactivation curve of *Aspergillus niger* spores. Appl. Environ. Microbiol. **62:**3745–3749.
15. **Gould, G. W., and G. J. Dring.** 1972. Biochemical mechanisms of spore germination, p. 401–408. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
16. **Hageman, J. H., G. W. Shankweiler, P. R. Wall, K. Franich, G. W. McCowan, S. M. Cauble, J. Grajeda, and C. Quinones.** 1984. Single, chemically defined sporulation medium for *Bacillus subtilis*: growth, sporulation, and extracellular protease production. J. Bacteriol. **160:**438–441.
17. **Hilbert, D. W., and P. J. Piggot.** 2004. Compartmentalization of gene expression during *Bacillus subtilis* spore formation. Microbiol. Mol. Biol. Rev. **68:**234–262.
18. **Hindle, A. A., and E. A. Hall.** 1999. Dipicolinic acid (DPA) assay revisited and appraised for spore detection. Analyst **124:**1599–1604.
19. **Hu, P., T. Leighton, G. Ishkhanova, and S. Kustu.** 1999. Sensing of nitrogen limitation by *Bacillus subtilis*: comparison to enteric bacteria. J. Bacteriol. **181:**5042–5050.
20. **Humpheson, L., M. R. Adams, W. A. Anderson, and M. B. Cole.** 1998. Biphasic thermal inactivation kinetics in *Salmonella enteritidis* PT4. Appl. Environ. Microbiol. **64:**459–464.
21. **Jones, G., II, and V. I. Vullev.** 2002. Medium effects on the photophysical properties of terbium(III) complexes with pyridine-2,6-dicarboxylate. Photochem. Photobiol. Sci **1:**925–933.
22. **Kilsby, D. C., K. W. Davies, P. J. McClure, C. Adair, and W. A. Anderson.** 2000. Bacterial thermal death kinetics based on probability distributions: the heat destruction of *Clostridium botulinum* and *Salmonella* Bedford. J. Food Prot. **63:**1197–1203.
23. **Kooiman, W. J.** 1973. The screw cap tube technique: a new and accurate technique for the determination of the wet heat resistance of bacterial spores, p. 87–92. *In* A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spore research. Academic Press, London, United Kingdom.
24. **Mallidis, C. G., and J. S. Scholefield.** 1985. The release of dipicolinic acid during heating and its relation to the heat destruction of *Bacillus stearothermophilus* spores. J. Appl. Bacteriol. **59:**479–486.
25. **Murrel, W. G., and A. D. Warth.** 1965. Composition and heat resistance of bacterial spores, p. 1–24. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Washington, D.C.
26. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
27. **Newsome, R.** 2003. Dormant microbes: research needs. Food Technol. **57:**38–42.
28. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination and outgrowth, p. 391–450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, United Kingdom.
29. **Oomes, S. J. C. M., and S. Brul.** 2004. The effect of metal ions commonly present in food on gene expression of sporulating *Bacillus subtilis* cells in relation to spore wet heat resistance. Innovat. Food Sci. Emerg. Technol. **5:**307–316.
30. **Paidhungat, M., B. Setlow, A. Driks, and P. Setlow.** 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. **182:**5505–5512.
31. **Paidhungat, M., and P. Setlow.** 2002. Spore germination and outgrowth, p. 537–548. *In* A. L. Sonenshein, J. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C.
32. **Peleg, M., and M. B. Cole.** 1998. Reinterpretation of microbial survival curves. Crit. Rev. Food Sci. Nutr. **38:**353–380.
33. **Pellegrino, P. M., N. F. Fell, and J. B. Gillespie.** 2002. Enhanced spore detection using dipicolinate extraction techniques. Anal. Chim. Acta **455:**167–177.
34. **Periago, P. M., A. Van Zuijlen, P. S. Fernandez, P. M. Klapwijk, P. F. Ter Steeg, M. G. Corradini, and M. Peleg.** 2004. Estimation of the non-isothermal inactivation patterns of *Bacillus sporothermodurans* IC4 spores in soups from their isothermal survival data. Int. J. Food Microbiol. **95:**205–218.
35. **Perkins, D. L., C. R. Lovell, B. V. Bronk, B. Setlow, P. Setlow, and M. L. Myrick.** 2004. Effects of autoclaving on bacterial endospores studied by Fourier transform infrared microspectroscopy. Appl. Spectrosc. **58:**749–753.
36. **Powell, J. F.** 1953. Isolation of dipicolinic acid (pyridine-2,6-dicarboxylic acid) from spores of *Bacillus megaterium*. Biochem. J. **54:**210–211.
37. **Rosen, D. L., C. Sharpless, and L. B. McGown.** 1997. Bacterial spore detection and determination by use of terbium dipicolinate photoluminescence. Anal. Chem. **69:**1082–1085.
38. **Russell, A. D.** 2003. Lethal effects of heat on bacterial physiology and structure. Sci. Prog. **86:**115–137.
39. **Scott, I. R., and D. J. Ellar.** 1978. Study of calcium dipicolinate release during bacterial spore germination by using a new, sensitive assay for dipicolinate. J. Bacteriol. **135:**133–137.
40. **Setlow, P., and E. A. Johnson.** 2001. Spores and their significance, p. 33–70. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
41. **Smelt, J. P., J. C. Hellemons, P. C. Wouters, and S. J. van Gerwen.** 2002. Physiological and mathematical aspects in setting criteria for decontamination of foods by physical means. Int. J. Food Microbiol. **78:**57–77.
42. **Stragier, P., and R. Losick.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30:**297–341.
43. **Tovar-Rojo, F., M. Chander, B. Setlow, and P. Setlow.** 2002. The products of the *spoVA* operon are involved in dipicolinic acid uptake into developing spores of *Bacillus subtilis*. J. Bacteriol. **184:**584–587.
44. **van Gerwen, S. J., and M. H. Zwietering.** 1998. Growth and inactivation models to be used in quantitative risk assessments. J. Food Prot. **61:**1541–1549.
45. **van Stokkum, I. H. M., D. S. Larsen, and R. van Grondelle.** 2004. Global and target analysis of time-resolved spectra. Biochim. Biophys. Acta **1657:**82–104. (Erratum, **1658:**262.)
46. **Vreeland, R. H., W. D. Rosenzweig, and D. W. Powers.** 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature **407:**897–900.
47. **Walker, H. W., and J. R. Matches.** 1965. Release of cellular constituents during heat inactivation of endospores of aerobic bacilli. J. Food Sci. **30:**1029–1036.
48. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, L. Krichevsky, L. H. Moore, W. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463–464.
49. **Woese, C., and H. J. Morowitz.** 1958. Kinetics of the release of dipicolinic acid from spores of *Bacillus subtilis*. J. Bacteriol. **76:**81–83.
50. **Zytkovic, T. H., and H. O. Halvorson.** 1972. Some characteristics of dipicolinic acid-less mutant spores of *Bacillus cereus*, *Bacillus megaterium*, and *Bacillus subtilis*, p. 49–52. *In* H. O. Halvorson, R. Hanson, and L. L. Campell (ed.), Spores V. American Society for Microbiology, Washington, D.C.