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ORIGINAL ARTICLE

Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity

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Abstract

Aims: To analyse the germination and its heterogeneity of individual spores of *Clostridium perfringens*.
Methods and Results: Germination of individual wild-type *Cl. perfringens* spores was followed by monitoring Ca-dipicolinic acid (CaDPA) release and by differential interference contrast (DIC) microscopy. Following the addition of KCl that acts via germinant receptors (GRs), there was a long variable lag period (T_{lag}) with slow release of *c.* 25% of CaDPA, then rapid release of remaining CaDPA in *c.* 2 min ($\Delta T_{release}$) and a parallel decrease in DIC image intensity, and a final decrease of *c.* 25% in DIC image intensity during spore cortex hydrolysis. Spores lacking the essential cortex-lytic enzyme (CLE) (*sleC* spores) exhibited the same features during GR-dependent germination, but with longer average T_{lag} values, and no decrease in DIC image intensity because of cortex hydrolysis after full CaDPA release. The T_{lag} of wild-type spores in KCl germination was increased significantly by lower germinant concentrations and suboptimal heat activation. Wild-type and *sleC* spores had identical average T_{lag} and $\Delta T_{release}$ values in dodecylamine germination that does not utilize GRs.
Conclusions: Most of these results were essentially identical to those reported for the germination of individual spores of *Bacillus* species. However, individual *sleC Cl. perfringens* spores germinated inefficiently with either KCl or exogenous CaDPA, in contrast to CLE-deficient *Bacillus* spores, indicating that germination of these species' spores is not completely identical.
Significance and Impact of the Study: This work provides information on the kinetic germination and its heterogeneity of individual spores of *Cl. perfringens*.

Introduction

Spores of bacteria of *Bacillus* and *Clostridium* species are dormant and extremely resistant to environmental stress factors (Setlow and Johnson 2007). These properties enable such spores to survive for extremely long periods in the environment and in the absence of nutrients. However, if nutrients return to these spores' environment, they can rapidly return to life in the processes of germination followed by outgrowth (Setlow 2003; Setlow and Johnson 2007; Paredes-Sabja *et al.* 2010). Many but not all of the triggers for spore germination, in particular nutrients and some monovalent cations, are sensed by proteins termed germinant receptors (GRs) located in spores' inner membrane. Signals transmitted by GRs then result in subsequent germination events, most notably the release of spores' large depot (*c.* 10% of spore dry wt) of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] and its chelated divalent cations, most commonly Ca^{2+} (CaDPA), followed by the hydrolysis of the spores' large peptidoglycan (PG) cortex. In addition to germinants that act via the GRs, there are also several types of germinants

that in *Bacillus* spores do not act via the GRs. These include exogenous CaDPA that acts by triggering the action of cortex-lytic enzymes (CLEs) that hydrolyse the spores' PG cortex leading to the release of endogenous CaDPA, and cationic surfactants such as dodecylamine that may trigger CaDPA release directly by acting on the spore's inner membrane.

An important feature of the germination of spores of both *Bacillus* and *Clostridium* species is that individual spores in populations initiate germination at very different times (Barker *et al.* 2005; Stringer *et al.* 2005; Chen *et al.* 2006; Webb *et al.* 2007; Zhang *et al.* 2010a; Wang *et al.* 2011). This property has significant applied implications, because dormant spores are vectors for much food spoilage and food borne disease, as well as notable human diseases caused by spores of both *Bacillus* (*Bacillus anthracis*, *Bacillus cereus*) and *Clostridium* (*Clostridium difficile*, *Clostridium perfringens*, *Clostridium botulinum*) species (Setlow and Johnson 2007). Because spore germination results in the loss of the dormant spore's extreme resistance, in theory, it would be possible to eliminate spores from foods or the environment by triggering their germination and then readily killing the now more sensitive germinated spores. However, this simple strategy has been rendered relatively ineffective by the heterogeneity in the timing of the germination of individual spores in populations. Indeed, this heterogeneity is so extreme that while the great majority of spores in population may germinate with nutrient germinants in 1–2 h, a small minority, often called superdormant spores, may not germinate for many hrs or even days (Ghosh and Setlow 2009). There is thus significant applied interest in the analysis of the heterogeneity in spore germination and its causes.

Analysis of heterogeneity in the germination of spore populations obviously requires that the behaviour of large numbers of individual spores be examined. A number of techniques have been developed for this purpose most notably using Raman spectroscopy to follow individual spores' CaDPA levels and using differential interference contrast (DIC) or phase contrast (PC) microscopy to follow the germination of thousands of individual spores simultaneously (Zhang *et al.* 2010b; Kong *et al.* 2011). This work has shown that following the addition of a nutrient germinant, CaDPA or dodecylamine to spores of *Bacillus* species, there is a lag period (T_{lag}) with generally only small and slow changes in DIC or PC intensity or CaDPA level followed by a rapid fall of *c.* 75% in these intensities in a period of 1–4 min. This latter event is because of the rapid release in a period termed $\Delta T_{release}$ of the great majority of the spores' CaDPA and its replacement by water, and a corresponding fall in the spore core's refractive index. Following CaDPA release, there is a further fall in DIC and PC intensity as the spore's PG cortex is hydrolysed by CLEs, allowing the spore core to expand with further water uptake and a further fall in the spore core refractive index. The main variable that describes spore germination heterogeneity is T_{lag}, which is extremely variable between individual spores in a genetically identical population. Factors that modulate T_{lag} values for spores of *Bacillus* species include heat activation, higher germinant concentrations and increased GR numbers, all of which shorten average T_{lag} values (Zhang *et al.* 2010a).

While the germination heterogeneity of spores of *Bacillus* species has been well studied, as has the process of germination of individual *Bacillus* spores, there has been much less work examining the germination and its heterogeneity of individual spores of *Clostridium* species. However, it is clear that germination of spores of *Clostridium* species is heterogenous (Billon *et al.* 1997; Barker *et al.* 2005; Stringer *et al.* 2005, 2009, 2011; Webb *et al.* 2007). Consequently in this work, we have used the methods developed to simultaneously examine the germination of thousands of individual spores of *Bacillus* species (Kong *et al.* 2011) to examine the germination of individual spores of *Cl. perfringens*. This work found that the process of germination of spores of this *Clostridium* species, as well as its heterogeneity, is generally quite similar to what has been found with spores of *Bacillus* species.

Materials and methods

Bacterial strains and spore preparation

Clostridium perfringens SM101 derivatives used in this study were as follows: (i) MRS101 (Δ*cpe*) (the wild-type strain) lacking the *Cl. perfringens* enterotoxin (CPE) (Sarker *et al.* 1999) and (ii) DPS121 (Δ*cpe* Δ*sleC*), a derivative of strain MRS101 lacking CPE and *Cl. perfringens* spores' only essential spore CLE SleC. The DPS121 strain was constructed by the introduction of the Δ*sleC* suicide vector (pDP66) into *Cl. perfringens* strain MRS101 by electroporation, and a chloramphenicol- and tetracycline-resistant Δ*cpe* Δ*sleC* mutant was isolated as described (Paredes-Sabja *et al.* 2009b). The *sleC* mutation was a deletion replacement generated by a double crossover. The identity of the Δ*cpe* Δ*sleC* strain DPS121 was confirmed by PCR and Southern blot analyses (data not shown).

Clostridium perfringens sporulating cultures were prepared as previously described (Paredes-Sabja *et al.* 2008, 2009b). Briefly, aliquots of an overnight fluid thioglycolate culture were inoculated into 10 ml of Duncan–Strong sporulation medium (Duncan and Strong 1968), followed by incubation for 24 h at 37°C, and the presence of spores was confirmed by PC microscopy. Large amounts of spores were prepared by scaling up the procedure, and spores were cleaned by repeated centrifugation and

washing with sterile distilled water at least ten times until spore suspensions were >99% free of sporulating cells, cell debris and germinated spores. Purified spores were suspended at a final optical density at 600 nm (OD_{600}) of *c.* 6 and stored at −20°C until use.

Spores of *Bacillus subtilis* PS533, a 168 strain carrying plasmid pUB110 conferring resistance to kanamycin (10 μg ml^{-1}) (17), were prepared at 37°C on 2× SG medium agar plates (Paidhungat *et al.* 2000) and purified as described (Nicholson and Setlow 1990).

Determination of DPA levels by Raman spectroscopy

The DPA levels in individual *Cl. perfringens* spores were determined by laser tweezers Raman spectroscopy (Huang *et al.* 2007). Dormant spores were suspended in 25 mmol l^{-1} Na-Hepes buffer (pH 7·4) at room temperature. An individual spore was captured with optical tweezers, its Raman spectrum was recorded and measurements were made on *c.* 100 individual spores. The spores' average CaDPA level was determined by the averaged intensity of their major CaDPA-specific Raman band at 1017 cm^{-1}, and this value was calibrated with a solution of 60 mmol l^{-1} CaDPA.

Spore germination

Clostridium perfringens spores were germinated in: (i) 25 mmol l^{-1} Na-Hepes buffer (pH 7·4) with various KCl concentrations at 30°C; (ii) 25 mmol l^{-1} Na-Hepes buffer (pH 7·4) with 0·8 mmol l^{-1} dodecylamine at 45°C; or (iii) 60 mmol l^{-1} CaDPA (made to pH 7·4 with NaOH) at 40°C. Except for dodecylamine germination, unless noted otherwise, spores in water were routinely heat activated prior to germination by a 10-min incubation at 80°C and subsequently cooled at 25°C for 5 min prior to germination experiments. This heat activation regimen gave optimal spore germination with KCl (see Results).

Monitoring spore germination – Method A

The germination of individual spores was monitored simultaneously by Raman spectroscopy and DIC microscopy as described (Zhang *et al.* 2010b).

Monitoring spore germination – Method B

The germination of 250–300 spores was also analysed simultaneously by DIC microscopy alone as described (Zhang *et al.* 2010b). Briefly, *c.* 1 μl of heat-activated spores (*c.* 10^8 spores ml^{-1} in water) was spread on the surface of a microscope coverslip that was quickly dried in a vacuum chamber at room temperature to fix the spores to the coverslip. The coverslip with the adhered spores was then mounted on and sealed to a DIC microscope sample holder kept at a constant temperature. The DIC microscope was set such that the polarizer and analyzer were crossed, making the DIC bias phase as zero. Preheated germinant/buffer solution was then added to the spores on the coverslip, and a digital CCD camera (16 bit; 1600 by 1200 pixels) was used to record the DIC images at intervals of 12 s for 60–90 min. These images were analysed with a computation program in MATLAB to locate each spore's position and to calculate the summed pixel intensity of an area of 40 × 40 pixels that covered the whole individual spore on the DIC image. The DIC image intensity of each individual spore was plotted as a function of the incubation time (with a resolution of 12 s), and the initial intensity at T_0 (the first DIC image recorded after the addition of the germinant) was normalized to 1 and the intensity at the end of measurements was normalized to zero. Invariably, the latter value had been constant for ≥10 min at the end of measurements. From the time-lapse DIC image intensity, we can determine the time of completion of the rapid fall of *c.* 75% in spore DIC image intensity, which is concomitant with the time of completion of spore CaDPA release, as confirmed by Raman spectroscopy (see Results). The parameters T_{lag}, $\Delta T_{release}$, T_{lys} and ΔT_{lys} were used to describe the CaDPA release and cortex hydrolysis kinetics during germination of individual spores. T_{lag} is the time between the mixing of spores with germinants and the initiation of the rapid release of most CaDPA, $T_{release}$ is the time of completion of rapid CaDPA release and $\Delta T_{release} = (T_{release} - T_{lag})$. T_{lys} is the time when spore cortex hydrolysis is completed as determined by the completion of the fall in wild-type spores' DIC image intensity, and $\Delta T_{lys} = (T_{lys} - T_{release})$. We also used a parameter, I_{lag}, which was defined as the intensity of a spore's DIC image at T_{lag}, to describe the germination of individual spores.

A spore that had completed CaDPA release as determined by spores' DIC image intensity was defined as a germinated spore. The degree of germination of multiple spores in response to different treatments was calculated by counting the number of germinated spores at various times and dividing by the number of dormant spores at the time of mixing of spores with germinants, with ≥250 individual spores analysed in each experiment.

Monitoring spore germination – Method C

The germination of multiple individual spores of *Cl. perfringens* MRS101 at 30°C and various KCl concentrations using spores (*c.* 0·5 μl of 2 × 10^9 spores ml^{-1}) attached to poly-L-lysine-coated coverslips mounted in 60-mm culture dishes with 25 mmol l^{-1} Na-Hepes buffer (pH 7·4)

(Zhang *et al.* 2010a). The germination of these spores was assessed by DIC microscopy as described (Zhang *et al.* 2010a) with *c.* 1000 individual spores monitored simultaneously at each KCl concentration. The starting time for these germinations (T_0) was defined as the time of addition of germinant, and imaging was generally started *c.* 5 min after T_0. Image J was used to count the total spores in each imaging field and to score spores that had germinated every 5 min as described (Zhang *et al.* 2010a).

Results

Raman spectra and average DPA levels of individual *Clostridium perfringens* spores

Previous work has found that bands because of CaDPA dominate the Raman spectra of individual spores of *Bacillus* species (Chen *et al.* 2006; Huang *et al.* 2007; Kong *et al.* 2011), and this was also the case for spores of *Cl. perfringens* MRS101 and DPS121 (Fig. 1). The average intensity of the CaDPA-specific 1017 cm^{-1} Raman band in *c.* 100 individual spores indicated that the CaDPA levels in spores of *Cl. perfringens* MRS101 and DPS121 were 377 ± 87 and 364 ± 105 attomol per spore, respectively, and these values were similar to those in *B. subtilis* spores (408 ± 53 attomol per spore).

Kinetics of germination of individual *Clostridium perfringens* spores

Given the dominant CaDPA-specific peak in the Raman spectrum of *Cl. perfringens* spores, it appeared likely that the kinetics of germination of individual *Cl. perfringens* MRS101 spores could easily be monitored by following either CaDPA release by Raman spectroscopy or spore refractility by DIC microscopy using methods developed for following the germination of individual spores of *Bacillus* species (Chen *et al.* 2006; Zhang *et al.* 2010b; Kong *et al.* 2011). The germinant for *Cl. perfringens* spores that we chose to use initially was KCl that acts via the GerK germinant receptor (Paredes-Sabja *et al.* 2008, 2009a), and we examined the germination of multiple individual MRS101 and DPS121 spores with 100 mmol l^{-1} KCl at either 30 or 40°C by DIC microscopy (Fig. 2). As expected, >90% of MRS101 spores germinated rapidly under these conditions, with germination slightly faster at 40°C, although at both temperatures most spores germinated within *c.* 20 min. A small percentage of these spores also germinated in the germination buffer alone (see below).

In contrast to the MRS101 spores, the KCl germination of the DPS121 spores that lacked the CLE SleC was slower and much less complete, with only *c.* 10% of these spores germinating in 90 min at either 30 or 40°C, and the germination of these spores at 40° was slightly slower than at 30°C. However, those DPS121 spores that did germinate with KCl still showed the variable T_{lag} period seen with the MRS101 spores, although the T_{lag} values were much longer for the DPS121 spores (Table 1) – and note that most DPS121 spores did not germinate under these conditions and thus must have T_{lag} values ≥90 min. Approximately 2·5% of the DPS121 spores also released

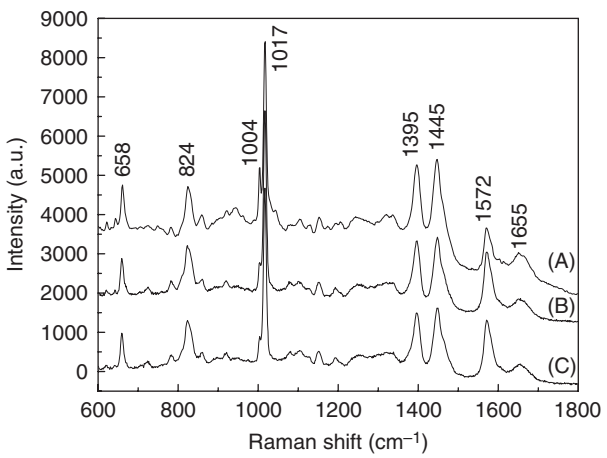

Figure 1 Average Raman spectra of spores of (A) *Bacillus subtilis* PS533 (wild-type); (B) *Clostridium perfringens* MRS101 (wild-type); and (C) *Cl. perfringens* DPS121 (*sleC*). The spectra were averaged from the spectra from *c.* 100 individual spores and the average Ca-dipicolinic acid levels were determined as described in Materials and methods.

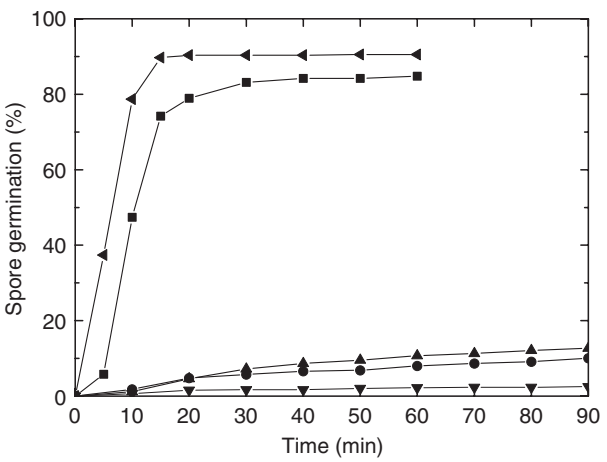

Figure 2 KCl germination of *Clostridium perfringens* MRS101 and DPS121 spores. *Clostridium perfringens* spores were germinated with 100 mmol l^{-1} KCl at 30 or 40°C, and germination was followed by Method B as described in Materials and methods. The symbols used are: ■, MRS101 spores at 30°C; ◀, MRS101 spores at 40°C; ▲, DPS121 spores at 30°C; ●, DPS121 spores at 40°C; and ▼, DPS121 spores in buffer only at 30°C.

Table 1 Mean values and standard deviations of germination parameters of *Clostridium perfringens* spores with KCl, CaDPA and dodecylamine*

Spores	Germinant and temperature	T_{lag} (min)	$T_{release}$ (min)	T_{lys} (min)	$\Delta T_{release}$ (min)	ΔT_{lys} (min)	I_{lag} (%)	Germination (%)
MRS101	100 mmol l^{-1} KCl, 40°C	3·9 ± 1·9	5·5 ± 1·8	7·0 ± 1·9	1·6 ± 0·6	1·5 ± 0·7	81·2 ± 10·7	90·3 (310)
	100 mmol l^{-1} KCl, 30°C	7·9 ± 3·6	10·0 ± 3·8	13·5 ± 4·7	2·0 ± 0·5	3·6 ± 2·1	81·6 ± 8·5	85·9 (285)
	10 mmol l^{-1} KCl, 30°C	11·9 ± 6·1	14·0 ± 6·0	16·2 ± 6·1	2·1 ± 0·9	2·2 ± 1·3	71·5 ± 7·3	85·3 (319)
	2 mmol l^{-1} KCl, 30°C	19·8 ± 11·1	21·5 ± 10·8	23·6 ± 11·0	1·7 ± 0·6	2·2 ± 1·2	73·6 ± 6·0	82·4 (331)
	0·5 mmol l^{-1} KCl, 30°C	30·9 ± 13·4	33·0 ± 13·3	34·8 ± 13·6	2·1 ± 1·0	1·8 ± 1·4	70·0 ± 9·9	59·9 (232)
	0·1 mmol l^{-1} KCl, 30°C	41·3 ± 18·1	42·9 ± 18·0	44·9 ± 18·0	1·6 ± 0·7	1·9 ± 1·5	70·6 ± 7·6	20·2 (367)
	0 mmol l^{-1} KCl, 30°C	49·1 ± 26·1	50·9 ± 26·0	54·1 ± 26·6	1·8 ± 1·0	3·2 ± 3·4	66·9 ± 11·1	9·4 (341)
	60 mmol l^{-1} CaDPA, 40°C	36·5 ± 25·8	38·6 ± 25·9	42·2 ± 26·9	2·1 ± 0·6	3·6 ± 5·1	72·1 ± 7·8	61·6 (349)
	0·8 mmol l^{-1} dodecy-lamine, 45°C	13·0 ± 10·0	15·5 ± 12·1	nc	2·6 ± 1·1	nc	61·4 ± 14·9	86·6 (347)
DPS121	100 mmol l^{-1} KCl, 40°C	37·6 ± 31·9	40·2 ± 32·3	nc	2·6 ± 0·9	nc	65·1 ± 15·2	10·0 (346)
	100 mmol l^{-1} KCl, 30°C	29·9 ± 16·4	32·9 ± 16·8	nc	2·9 ± 1·3	nc	61·0 ± 13·3	12·7 (438)
	0·8 mmol l^{-1} dodecy-lamine, 45°C	10·5 ± 11·0	13·3 ± 12·3	nc	2·8 ± 1·7	nc	72·4 ± 19·4	85·8 (305)

nc – Values of T_{lys} and ΔT_{lys} for dodecylamine germination of MRS101 spores and all germinations of DPS121 spores were not calculated because of the absence of periods of obvious cortex hydrolysis in these germinating spores.

**Clostridium perfringens* spores were germinated with Ca-dipicolinic acid (CaDPA), dodecylamine or various concentrations of KCl as described in Materials and methods. The measurement time was 60 min for the germination of MRS101 spores with 100 mmol l^{-1} and 10 mmol l^{-1} KCl, and 90 min for all other germinations. Values for germination parameters are averages that were calculated from individual values obtained from 30 individual spores that germinated as shown in Figs 3, 4 and 6. The percentages of spore germination at the ends of the incubations under the various conditions were determined as described in Materials and methods. The number of spores tested is given in the brackets following the percentage of spore germination.

their CaDPA during a 90-min incubation in the germination buffer alone (Fig. 2).

More detailed analysis of the germination of multiple individual MRS101 spores by Raman spectroscopy found that following addition of KCl, for most spores, there was little to no change in the CaDPA level during a variable T_{lag} period, followed by rapid and essentially complete CaDPA release in *c.* 2 min (the $\Delta T_{release}$ period) (Fig. 3a; Table 1). A few spores did exhibit significant albeit slow CaDPA release during T_{lag}, as seen previously with some spores of *Bacillus* species as well (Chen *et al.* 2006; Zhang *et al.* 2010b; Kong *et al.* 2011; Wang *et al.* 2011). The DIC image intensities of the same individual spores examined by Raman spectroscopy were also monitored, and some but not all spores also exhibited slow falls in DIC image intensity during T_{lag} (Fig. 3b; Table 1). However, all spores exhibited rapid falls in DIC image intensity during $\Delta T_{release}$. In addition, following $T_{release}$, all MRS101 spores exhibited a further slow fall in DIC image intensity (Fig. 3a). Previous work with germinating spores of *Bacillus* species has shown that this latter slow fall in DIC image intensity is because of hydrolysis of the spores' PG cortex and water uptake by the spore core as it expands once CaDPA has been released and the cortex is hydrolysed (Zhang *et al.* 2010b; Kong *et al.* 2011). This also appears to be the case with *Cl. perfringens* MRS101 spores, as the slow fall in DIC image intensity following CaDPA release was not seen during the KCl germination of DPS121 spores that cannot degrade their cortex because of the absence of the *Cl. perfringens* spores' only CLE SleC (Paredes-Sabja *et al.* 2009b) (Fig. 3c). Consequently, while the fall in spore DIC image intensity to a constant value was *c.* 74% for germinating MRS101 (wild-type) spores, this value became constant after only an *c.* 38% fall in DIC image intensity for germinating DPS121 (*sleC*) spores.

Effect of KCl concentration on *Clostridium perfringens* spore germination heterogeneity

It was clear from the results described above that there is significant heterogeneity between individual *Cl. perfringens* spores in their germination with KCl, because even when germinated under identical conditions, individual spores exhibited quite variable T_{lag} times, although had very similar $\Delta T_{release}$ values (Fig. 3; Table 1). An obvious question is what causes the variability in T_{lag} times during KCl germination. One of the factors affecting the germination heterogeneity of spores of *Bacillus* species with GR-dependent germinants is the germinant concentration, with lower germinant concentrations giving larger and more variable T_{lag} times (Zhang *et al.* 2010a). Consequently, the effects of various KCl concentrations on the heterogeneity of MRS101 spore germination as well as the overall germination kinetics were examined (Fig. 4; Table 1). Notable results from these experiments were as follows. (i) Higher KCl concentrations increased the amount of MRS101 spore germination. With

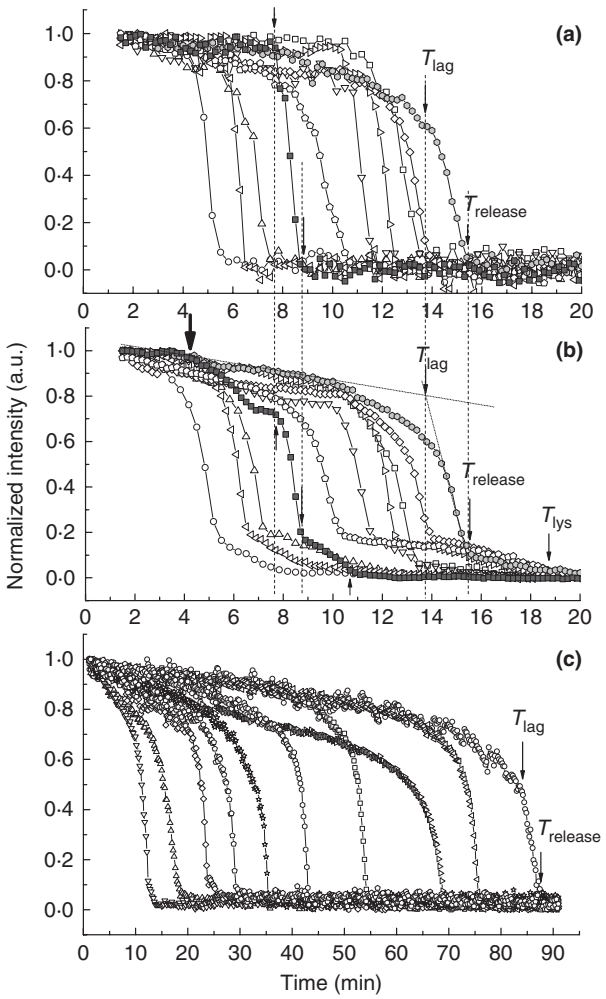

Figure 3 Kinetics of germination of individual *Clostridium perfringens* MRS101 (a,b) and DPS121 (c) spores as monitored by (a) Raman spectroscopy and (b,c) differential interference contrast (DIC) microscopy. The germination of 10 individual spores with 100 mmol l^{-1} KCl at 30°C was followed by Method A, and the intervals between each Raman spectrum or DIC image were 20 s. In (a) and (b) the same MRS101 spores were monitored simultaneously by Raman spectroscopy and DIC microscopy, and the time points of T_{lag}, $T_{release}$ and T_{lys} (thin arrows in a,b) are illustrated by data for the two spores denoted with grey or black symbols. T_{lag} was defined as the point of the intersection of the slow Ca-dipicolinic acid (CaDPA) release slope with the rapid CaDPA release slope. $T_{release}$ is the time that CaDPA release from the spore core was complete as determined by Raman spectroscopy, and T_{lys} is the time when spore cortex hydrolysis was complete. The bold arrow in (b) indicates the initiation of a slow fall in DIC image intensity prior to fast CaDPA release at T_{lag} for the spore denoted by the black symbols.

≥2 mmol l^{-1} KCl, >80% of spores germinated in 90 min, although the average T_{lag} values and their variation increased as the KCl concentration decreased. KCl concentrations <2 mmol l^{-1} gave less spore germination, and even higher average T_{lag} values that also exhibited even more individual variability. (ii) Like DPS121 spores, a small amount of MRS101 spores germinated in germination buffer alone, and with even longer and more variable average T_{lag} times. (iii) Despite the effects of KCl concentrations on germination heterogeneity, MRS101 spores germinated with various KCl concentrations all exhibited similar values of $\Delta T_{release}$ and ΔT_{lys}. (iv) Some individual MRS101 spores exhibited a slow fall in DIC image intensity prior to T_{lag}, and this was more noticeable at lower KCl concentrations (Fig. 4c,d; bold arrows). (v) Average I_{lag} values also decreased somewhat as average T_{lag} values increased, perhaps because there was more slow CaDPA release from spores with long T_{lag} periods.

Similar results were obtained when multiple spores' germination at various KCl concentrations were monitored by Method C (Fig. 4e). Again there was a small amount (*c.* 10%) of germination of the MRS101 spores in 2 h in Na-Hepes buffer alone, but the time of spores' germination in the buffer alone was spread out over the 2 h measurement period and was actually greatest in the last 20 min. In contrast, with 100 mmol l^{-1} KCl, *c.* 93% of the spores germinated in 2 h, and almost all within 20 min. With lower KCl concentrations, germination was less complete in 2 h and the germination times of individual spores in the population were shifted to slightly longer times, consistent with the longer T_{lag} times of MRS101 spores germinating at lower KCl concentrations as measured by Method B.

Effect of heat activation on *Clostridium perfringens* spore germination heterogeneity

Another parameter that alters the germination heterogeneity of spores of *Bacillus* species is the degree of spore heat activation, as optimal heat activation decreases T_{lag} values and their variability as well as increasing the percentage of spores that germinate (Zhang *et al.* 2010a). Therefore, we examined the effects of heat activation conditions on *Cl. perfringens* MRS101 spore germination and its heterogeneity (Fig. 5; Table 2). This work led to a number of notable conclusions as follows. (i) With no heat activation, only *c.* 2% of these spores germinated with 100 mmol l^{-1} KCl in 90 min (Fig 5a). (ii) Heat activation at higher temperatures allowed more spores to germinate with KCl, but heat activation at 80°C for 10 or 30 min had similar effects, suggesting that 10 min at 80°C gives optimal heat activation for KCl germination. (iii) ≥80% of the spores heat activated at 80°C for 10 or 30 min that germinated in 90 min germinated in the first *c.* 15 min, in the first *c.* 30 min after heat activation at 80°C for 5 min or 70°C 10 min, and in the first

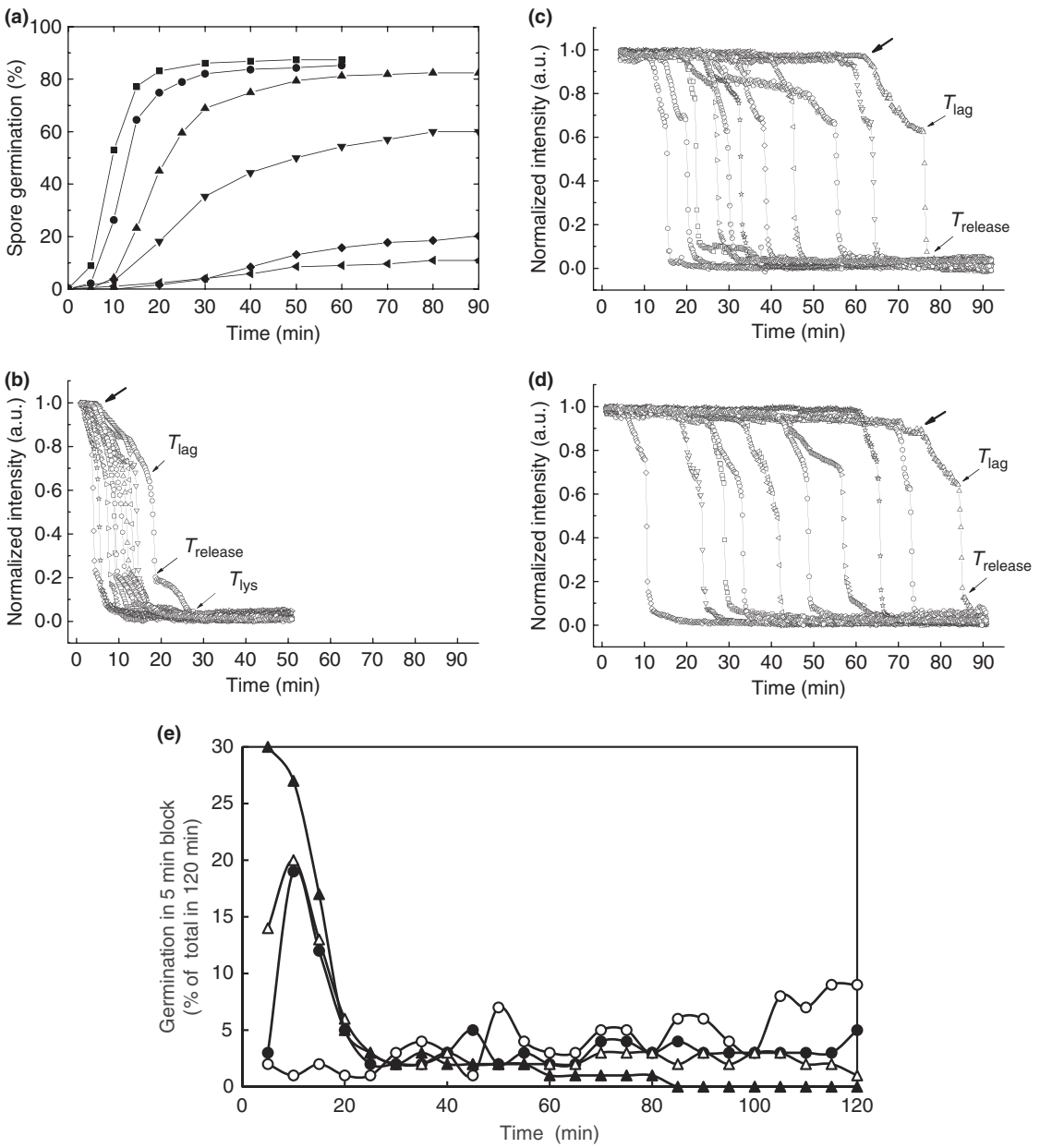

Figure 4 Germination of *Clostridium perfringens* MRS101 spores with various KCl concentrations. Spores were germinated in 25 mmol l^{-1} Na-Hepes buffer (pH 7·4) at 30°C with various KCl concentrations, and spore germination was followed by differential interference contrast (DIC) microscopy using Methods B (a–d) or C (e) as described in Materials and methods. (a) The concentrations of KCl used were: ■, 100 mmol l^{-1}; ●, 10 mmol l^{-1}; ▲, 2 mmol l^{-1}; ▼, 0·5 mmol l^{-1}; ♦, 0·1 mmol l^{-1}; ◀, 0 mmol l^{-1}, and each curve represents the cumulative behaviour of *c.* 300 starting spores. (b–d) The germination of ten individual spores with (b) 100 mmol l^{-1} KCl, (c) 0·5 mmol l^{-1} KCl and (d) 0·1 mmol l^{-1} KCl was followed by Method B. For one spore each in panels b–d, the time of initiation of the slow fall in DIC image intensity prior to fast Ca-dipicolinic acid release is indicated with a bold arrow, and the T_{lag}, $T_{release}$ and T_{lys} points are indicated with thin arrows. (e) The germination of *c.* 1000 individual spores incubated with different KCl concentrations was followed by Method C. The KCl concentrations used (and the % of spores that germinated in 2 h) were: ○ – no KCl (11%); ● – 3 mmol l^{-1} KCl (43%); △ – 5 mmol l^{-1} KCl (68%); and ▲ – 100 mmol l^{-1} KCl (89%). Data are shown as the spores that germinated in each 5-min block divided by the total number of spores that germinated in the 2 h measurement period.

c. 40 min after heat activation at 60°C for 10 min (Fig. 5a). These latter results were consistent with the *c.* 2·5-fold increased average T_{lag} values as heat activation temperatures decreased (Table 2); note that this effect was probably much >2·5-fold, because most spores heat activated at ≤60°C did not germinate in 90 min and were

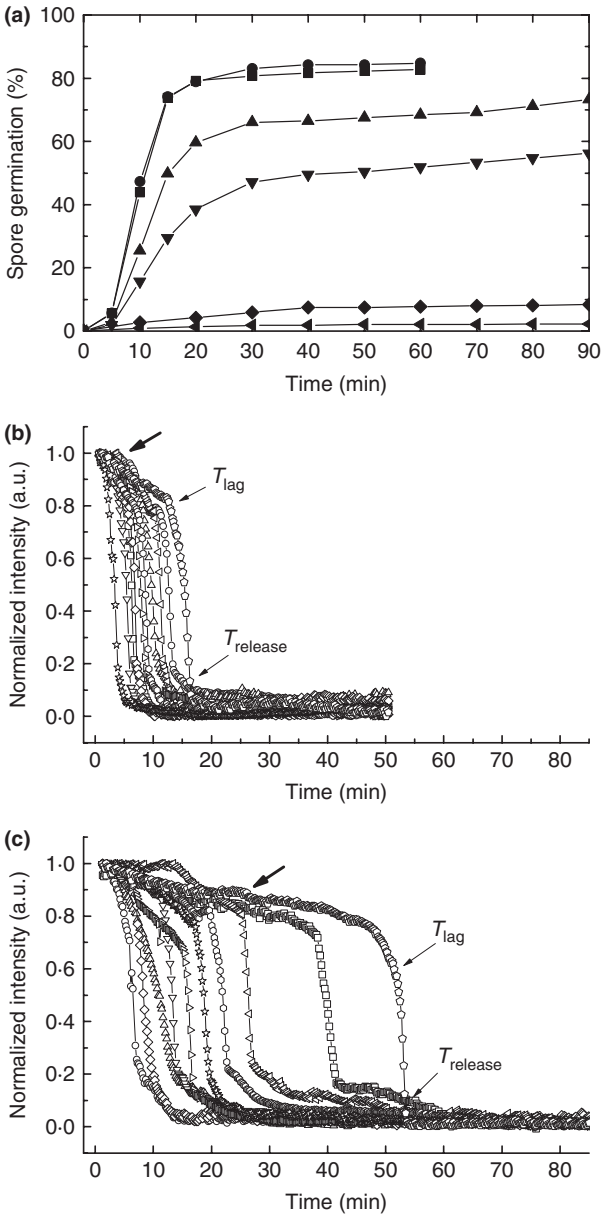

Figure 5 KCl germination of MRS101 spores with various heat activation treatments. In (a), the spores were given various heat activation treatments and then cooled at 25°C for 5 min prior to germination with 100 mmol l^{-1} KCl at 30°C. Spore germination was followed by Method B, and each curve represents the cumulative behaviour of *c.* 300 starting spores. The heat activation treatments were: ■, 80°C for 30 min; ●, 80°C for 10 min; ▲, 80°C for 5 min; ▼, 70°C for 10 min; ♦, 60°C for 10 min; and ◄, no heat activation. In (b and c), the germination of ten individual spores was followed by differential interference contrast (DIC) microscopy by Method B. Spores were germinated with 100 mmol l^{-1} KCl at 30°C after heat activation at (b) 80°C for 30 min or at (c) 70°C for 10 min. For one spore in panels b,c, the time of initiation of the slow fall in DIC image intensity prior to fast Ca-dipicolinic acid release is indicated with a bold arrow, and the T_{lag} and $T_{release}$ points are indicated with thin arrows.

not included in calculations of T_{lag}. (iv) Despite differences in average T_{lag} values, spores heat activated at different temperatures exhibited almost identical $\Delta T_{release}$ values, except perhaps for spores heat activated at 60°C, and values of I_{lag} were also almost identical (Table 2). (v) At low heat activation temperatures, it appeared that the time period for the slow fall in DIC intensity prior to T_{lag} was prolonged (Fig. 5b,c; bold arrows indicate the initiation of the slow fall in DIC image intensity). The average time of the slow fall in DIC image intensity prior to T_{lag} was *c.* 6 min for spores heat activated at <80°C, but *c.* 3 min for those heat activated at 80°C for 10 or 30 min (Fig. 5b,c; and data not shown). (vi) At low heat activation temperatures, the time for cortex hydrolysis (ΔT_{lys}) was also slightly increased (Table 2).

Germination heterogeneity with dodecylamine and exogenous CaDPA

In addition to germinants that trigger germination via GRs, the cationic surfactant dodecylamine triggers spore germination without GR participation (Setlow 2003; Setlow and Johnson 2007). Germination of spores of *Bacillus* species by CaDPA is also not dependent on GRs, although it is reported to be GerK dependent in spores of *Cl. perfringens* (Setlow 2003; Paredes-Sabja *et al.* 2009b). About 60% of MRS101 *Cl. perfringens* spores germinated in 90 min with 60 mmol l^{-1} CaDPA, and the kinetics of this germination for individual spores were similar to those seen for *B. subtilis* spore germination with CaDPA (Fig. 6a,b) (Peng *et al.* 2009). However, most DPS121 spores did not germinate with exogenous CaDPA, presumably because of the absence of SleC, although a small percentage of these spores may germinate in the absence of any germinant (Fig. 2).

In contrast to KCl and CaDPA germination that were quite different for MRS101 and DPS121 *Cl. perfringens* spores, germination of these spores with dodecylamine was almost identical (Fig. 6). In particular, during dodecylamine germination: (i) *c.* 85% of the MRS101 and DPS121 spores germinated in 90 min and most in 40 min (Fig. 6a); (ii) the spores of these two strains exhibited similar average T_{lag}, $T_{release}$ and $\Delta T_{release}$ values (Table 1); and (iii) the kinetics of change in DIC image intensity of individual spores were extremely similar (Fig. 6c,d).

Discussion

While there were many similarities in the germination of individual spores of *Cl. perfringens* and *Bacillus* species seen in the current work as described below, two differ-

Table 2 Mean values and standard deviations of KCl germination parameters of *Clostridium perfringens* MRS101 spores given various heat activation treatments*

Activation treatment	T_{lag} (min)	$T_{release}$ (min)	T_{lys} (min)	$\Delta T_{release}$ (min)	ΔT_{lys} (min)	I_{lag} (%)	Germination (%)
80°C, 30 min	7·4 ± 3·4	9·4 ± 3·4	12·5 ± 3·7	2·0 ± 0·8	3·1 ± 1·7	78·6 ± 9·6	82·7 (313)
80°C, 10 min	7·9 ± 3·0	10·0 ± 3·1	14·3 ± 5·3	2·1 ± 0·6	4·9 ± 3·3	80·6 ± 10·5	84·0 (261)
80°C, 5 min	11·8 ± 11·4	13·9 ± 11·3	18·0 ± 11·2	2·1 ± 0·7	4·1 ± 2·6	74·6 ± 11·3	73·2 (295)
70°C, 10 min	15·0 ± 10·5	17·2 ± 10·9	24·4 ± 14·0	2·2 ± 0·9	7·2 ± 5·7	77·7 ± 10·6	56·2 (315)
60°C, 10 min	17·4 ± 16·0	20·4 ± 16·0	27·0 ± 16·9	3·0 ± 1·1	6·1 ± 5·7	75·8 ± 10·6	8·4 (403)

**Clostridium perfringens* MRS101 spores were given various heat activation treatments, cooled and germinated with 100 mmol l^{-1} KCl in 25 mmol l^{-1} Na-Hepes buffer (pH 7·4) at 30°C as described in Materials and methods. Germination was monitored for 60 min for spores activated at 80°C for 10 and 30 min, and 90 min for all other activation treatments. Values for various germination parameters are averages that were calculated from individual values obtained from 30 individual spores that germinated as shown in Figs 3 and 5. The per cent germination of the various spore preparations at the end of measurement periods was determined as described in Materials and methods. The number of spores tested is given in the brackets following the percentage of spore germination.

ences were also noted in the germination of the CLE-deficient spores of the two species. One was that for individual *Bacillus megaterium* and *B. subtilis* spores lacking their two redundant CLEs, CwlJ and SleB, $\Delta T_{release}$ times for GR-dependent germination average 3·5- and 15-fold slower, respectively, than for the corresponding wild-type spores (Sarker *et al.* 1999; Peng *et al.* 2009). This finding indicates that CLE action greatly increases the rates of rapid CaDPA release during GR-dependent germination of these spores. However, the average $\Delta T_{release}$ times in GR-dependent germination of wild-type and *sleC Cl. perfringens* spores were only *c.* 1·5-fold slower for the *sleC* spores. While this difference may be significant, it was certainly a smaller difference than seen with spores of *Bacillus* species. The mechanism of stimulation of CaDPA release by cortex hydrolysis in *Bacillus* spore germination is not known, but it is perhaps notable that the T_{lag} values for *sleC Cl. perfringens* spores germinating with 100 mmol l^{-1} KCl were much longer than for wild-type *Cl. perfringens* spores, while this is not true for wild-type and CLE-deficient *Bacillus* spores germinating via GRs (Sarker *et al.* 1999; Peng *et al.* 2009). This suggests that in *Cl. perfringens* spores, SleC action somehow decreases T_{lag} values for GR-dependent germination, a different situation than in *Bacillus* spore germination. However, SleC action did not noticeably affect T_{lag} values in dodecylamine germination of *Cl. perfringens* spores.

The second difference in the germination of *Cl. perfringens* and *Bacillus* spores that lack CLEs was that the *sleC Cl. perfringens* spores germinated very poorly with the GR-dependent germinant KCl as measured by CaDPA release and reported previously (Paredes-Sabja *et al.* 2009b). In contrast, *cwlJ sleB B. megaterium* and *B. subtilis* spore populations release their CaDPA much more efficiently (>90%) with GR-dependent germinants (Sarker *et al.* 1999; Peng *et al.* 2009), although the reason for this difference between the germination of the spores of these two orders is not clear. Note also that invariably CaDPA release from both individual wild-type and *sleC Cl. perfringens* spores was an all or none phenomenon, as no individual spores incubated with germinants were ever observed that released CaDPA only partially. This is also the case for individual spores of *Bacillus* species incubated with germinants (Chen *et al.* 2006; Peng *et al.* 2009; Kong *et al.* 2011; Wang *et al.* 2011). However, while *c.* 10% of the *sleC Cl. perfringens* spores germinated in 90 min with 100 mmol l^{-1} KCl, it seems likely that there was minimal if any cortex PG hydrolysis in these germinating spores, because the colony formation efficiency of *sleC* spores is ≤0·1% of that of wild-type spores (Paredes-Sabja *et al.* 2009b). The *sleC Cl. perfringens* spores also germinated poorly with CaDPA as reported previously (Paredes-Sabja *et al.* 2009b), and the amount of these spores that did germinate was similar to the amount that germinated with germination buffer alone. The reason for this apparent germinant-independent germination of these spores, as well as possible differences between those spores that exhibited this type of germination and the great majority of spores that did not germinate with CaDPA or a GR-dependent germinant is not clear. It is perhaps notable that wild-type *Cl. perfringens* spores also exhibited significant apparently GR-independent germination as well, and this behaviour has also been noted in spores of *Bacillus* species that lack functional GRs (Paidhungat and Setlow 2000). However, the mechanism of this apparently spontaneous germination is not clear.

Despite the differences in some aspects of the germination of individual wild-type and *sleC Cl. perfringens* spores and spores of *Bacillus* species noted above, it was probably most notable that the germination of individual *Cl. perfringens* spores with KCl was generally quite similar to the GR-dependent germination of spores of *Bacillus* species. Thus, wild-type spores of both genera exhibit long and variable T_{lag} periods following GR-dependent

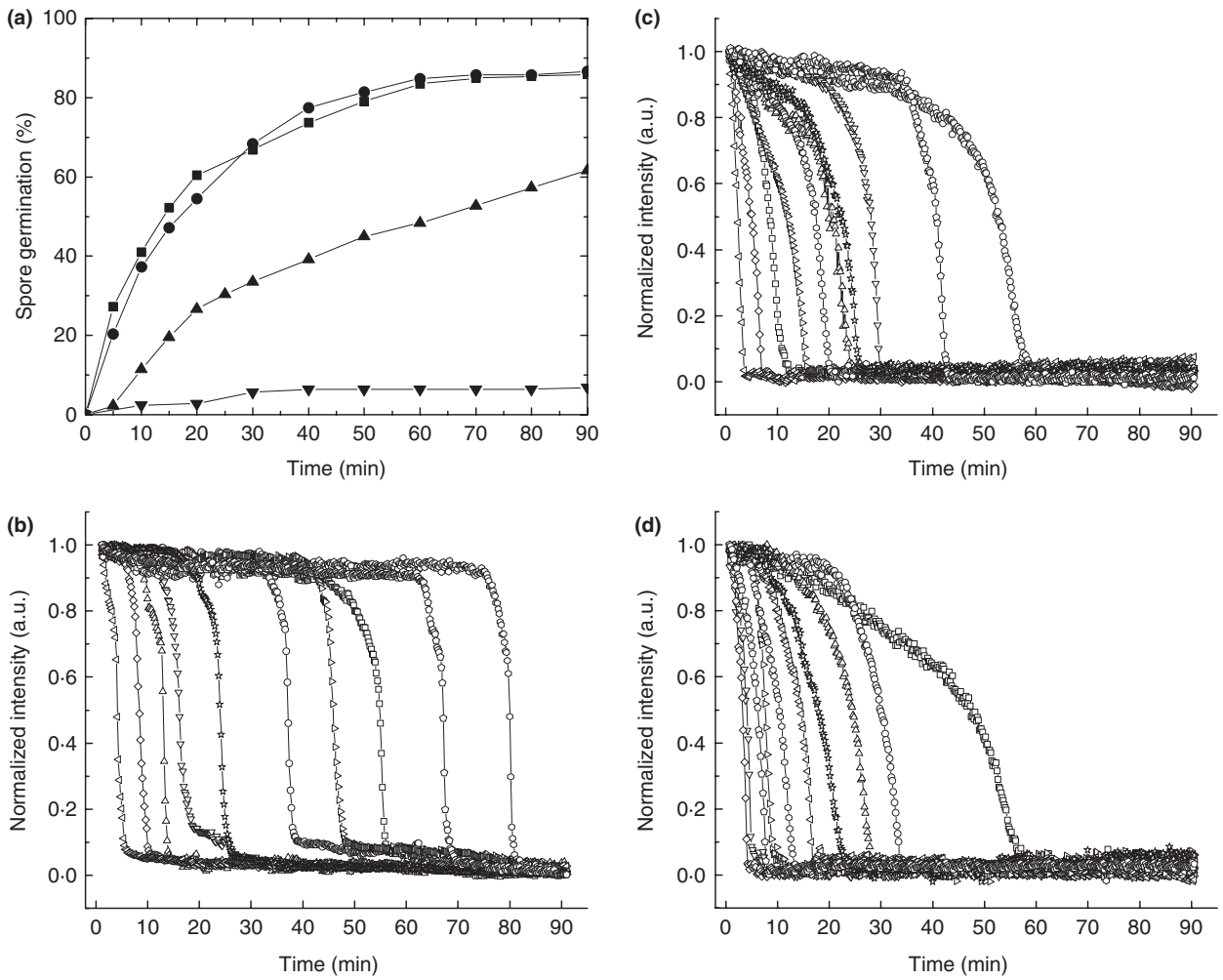

Figure 6 (a) Germination of *Clostridium perfringens* spores with Ca-dipicolinic acid (CaDPA) at 40°C or dodecylamine at 45°C. Spores were germinated with CaDPA or dodecylamine and germination of *c.* 300 individual spores was assessed by differential interference contrast (DIC) microscopy using Method B as described in Materials and methods. The spore germination conditions were: ■, MRS101 spores with 0·8 mmol l^{-1} dodecylamine; ●, DPS121 spores with 0·8 mmol l^{-1} dodecylamine; ▲, MRS101 spores with 60 mmol l^{-1} CaDPA; and ▼, DPS121 spores with 60 mmol l^{-1} CaDPA. (b–d) Kinetics of germination of ten individual spores of: (b) strain MRS101 with CaDPA; (c) strain MRS101 with dodecylamine; and (d) strain DPS121 with dodecylamine were monitored by DIC microscopy using Method B as described in Materials and methods.

germinant addition during which there is often some slow CaDPA release paralleled by decreases in spores' DIC image intensity (Chen *et al.* 2006; Peng *et al.* 2009; Kong *et al.* 2011; Wang *et al.* 2011). With wild-type spores of both genera the length of T_{lag} is also decreased by both increased GR-dependent germinant concentrations and more optimal heat activation treatments. The variable T_{lag} period was followed by rapid release of all remaining CaDPA, and this was paralleled again by a rapid fall in the spore's DIC image intensity, with both of these events ending at $T_{release}$. The time for this rapid CaDPA release $\Delta T_{release}$ is constant for a given germination temperature, and essentially independent of T_{lag} values. Finally, following $T_{release}$ there is a further fall of *c.* 25% in DIC image intensity and in spores of *Bacillus* species this is because of spore cortex hydrolysis and attendant spore core swelling and water uptake (Sarker *et al.* 1999; Peng *et al.* 2009). This also appears to be the case with *Cl. perfringens* spores, because *sleC* spores germinating with KCl had $\Delta T_{release}$ values similar to those for the wild-type spores, but exhibited no fall in DIC image intensity following $T_{release}$ and these spores are known not to degrade their PG cortex during KCl germination (Paredes-Sabja *et al.* 2009b). In addition to these similarities in GR-dependent germination, the kinetic patterns of the CaDPA and dodecylamine germination of wild-type spores of *Cl. perfringens* and *Bacillus* species were also similar, as was the fact that lack of the essential

CLE SleC in *Cl. perfringens* or the two redundant CLEs, CwlJ and SleB, in *Bacillus* species has no effect on the kinetics of spore germination with dodecylamine (Peng *et al.* 2009; Setlow *et al.* 2009; Wang *et al.* 2011). As a consequence of the similarities noted above, certainly, the major conclusion from the current work is that the germination of spores of *Cl. perfringens* and *Bacillus* species with a number of different germinants are remarkably similar.

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