Germination of spores of *Clostridium difficile* strains, including isolates from a hospital outbreak of *Clostridium difficile*-associated disease (CDAD)

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*Clostridium difficile* is an emerging nosocomial pathogen and one of the major causes of antibiotic-associated diarrhoea. Cases of *Clostridium difficile*-associated disease (CDAD) are likely initiated by the ingestion of dormant *C. difficile* spores, which then germinate, outgrow and rapidly proliferate to cause gastrointestinal (GI) infections. To understand the initial stages of CDAD pathogenesis, we have characterized the germination of spores from a collection of *C. difficile* strains, including some clinical isolates obtained from a CDAD outbreak (CDAD isolates). Spores of one laboratory strain and five CDAD isolates did not germinate with amino acids, but did germinate on a nutrient-rich medium. However, bile salts had little effect on spore germination, either alone or in a nutrient-rich medium. These spores also germinated with KCl, as well as the non-nutrient germinants dodecylamine and a 1:1 chelate of Ca2+ and dipicolinic acid. An unexpected finding was that spores of most of the *C. difficile* strains also germinated with inorganic phosphate (P) with a pH optimum of 6. The *in vitro* germination of spores of CDAD strains with KCl and P, two molecules present at significant levels in the GI tract, suggests that *C. difficile* spores germinate in the human body by sensing P in the early segments of the duodenum and KCl in the colon.

**INTRODUCTION**

*Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacterium, and is the causative agent of *Clostridium difficile*-associated disease (CDAD). Human CDAD is responsible for 25% of all cases of antibiotic-associated diarrhoea (Bartlett, 1992), with approximately three million cases annually (Bean et al., 1990; Johnson et al., 1989; McFarland et al., 1989) and an estimated cost of $1.1 billion in the USA (Kyne et al., 2002). CDAD generally occurs subsequent to a course of antibiotic treatment, which disrupts the normal colonic flora and allows the ingested *C. difficile* spores to germinate, outgrow and proliferate rapidly, filling empty niches (Kelly et al., 1994; Kelly & LaMontt, 1998). During vegetative growth, *C. difficile* cells produce two major toxins, TcdA and TcdB (Borriello et al., 1990; Voth & Ballard, 2005), which cause a wide spectrum of nosocomial diseases, ranging from mild, self-limiting diarrhoea to serious and life-threatening pseudomembranous colitis and toxic megacolon (Borriello, 1998; Hurley & Nguyen, 2002).

Spore germination has been well studied in *Bacillus subtilis*, and can be initiated by a variety of factors (termed germinants), including nutrients, cationic surfactants such as dodecylamine, enzymes, hydrostatic pressure and a 1:1 chelate of Ca2+ and dipicolinic acid (dipicolinic acid; DPA) (Paidhungat & Setlow, 2000; Paidhungat et al., 2002). Nutrient germinants for spores of *Bacillus* species include L-alanine, D-glucose, inosine and a mixture of L-asparagine, D-glucose, D-fructose and K+ ions (AGFK) (Clements & Moir, 1998; Moir et al., 2002; Setlow, 2003). The receptors that sense nutrient germinants are located in the spore inner membrane, and nutrient germinant receptor–ligand binding stimulates the release of monovalent cations (H+, Na+ and K+) (Clements & Moir, 1998; Moir et al., 2002; Setlow, 2003). DPA is a potent endospore germinant, and its role in CDAD pathogenesis is unclear. The goal of this study was to characterize the germination of spores from a collection of *C. difficile* strains, including some clinical isolates obtained from a CDAD outbreak (CDAD isolates). Spores of one laboratory strain and five CDAD isolates did not germinate with amino acids, but did germinate on a nutrient-rich medium. However, bile salts had little effect on spore germination, either alone or in a nutrient-rich medium. These spores also germinated with KCl, as well as the non-nutrient germinants dodecylamine and a 1:1 chelate of Ca2+ and dipicolinic acid. An unexpected finding was that spores of most of the *C. difficile* strains also germinated with inorganic phosphate (P) with a pH optimum of 6. The *in vitro* germination of spores of CDAD strains with KCl and P, two molecules present at significant levels in the GI tract, suggests that *C. difficile* spores germinate in the human body by sensing P in the early segments of the duodenum and KCl in the colon.
released in exchange for some water as a 1:1 chelate with divalent cations, predominantly Ca$^{2+}$ (Ca–DPA), and Ca–DPA release triggers downstream events in spore germination. Most important among the latter is the hydrolysis of the spore peptidoglycan (PG) cortex by one or more cortex-lytic enzymes (CLEs), which allows the core to expand and take up even more water, thus reaching the hydration levels found in growing cells. The latter event restores protein movement and enzyme action in the spore core, and leads to the resumption of energy metabolism and macromolecular synthesis (Cowan et al., 2003; Setlow, 2006). Spore germination in Clostridium species is less well studied than in B. subtilis. Limited studies have shown that spores of Clostridium botulinum, Clostridium sporogenes and Clostridium perfringens germinate in response to certain amino acids and salts (Ando, 1974; Broussolle et al., 2002; Paredes-Sabja et al., 2008c). Spores of C. difficile strains also germinate in nutrient media with bile salts, and glycine and bile salts have recently been reported to be co-germinants (Sorg & Sonenshein, 2008; Wilson et al., 1982; Wilson, 1983). However, the precise mechanism of action of germinants in spores of this species is unclear, since genes for nutrient germinant receptors appear to be absent from the C. difficile genome (Sebaihia et al., 2006).

In this study, we have investigated the germination of spores of C. difficile, including some clinical isolates obtained from a CDAD outbreak (CDAD isolates) (McEllistrem et al., 2005). Major findings on these C. difficile spores were that: (1) they germinated well with a complex nutrient-rich medium, but not with individual nutrients; (2) their germination was not accelerated significantly by bile salts; (3) they germinated well with KCl or inorganic phosphate (Pi); and (4) they germinated well with both Ca–DPA and the cationic surfactant dodecylamine.

**METHODS**

**Bacterial strains.** The laboratory C. difficile strain JIR8094, an erythromycin-sensitive derivative of strain 630, the genome of which has been sequenced (O’Connor et al., 2006), and five CDAD isolates (Pitt33, Pitt40, Pitt221, Pitt251 and Pitt301; McEllistrem et al., 2005) were used in this work.

**Spore preparation.** Starter C. difficile cultures (10 ml) were prepared by overnight growth at 37 °C in TGY-vegetative medium (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine). To prepare C. difficile spores, we first compared sporeulation of C. difficile strain JIR8094 in a sporulation medium (SM) used earlier for C. difficile sporulation (Wilson et al., 1982) versus Duncan–Strong (DS) medium, a sporulation medium used routinely for C. perfringens (Duncan & Strong, 1968; Kokai-Kun et al., 1994). Sporulating cultures were prepared by inoculating 0.6 ml TGY starter culture into 10 ml of each medium, followed by incubation for 24 h at 37 °C. Since similar levels of sporulation of C. difficile were observed (~10$^7$ spores ml$^{-1}$) in both SM and DS media (data not shown), C. difficile spores were routinely prepared using DS medium.

For spore purification, spore suspensions were prepared in 600 ml DS medium. Spores were cleaned of debris by repeated centrifugation and washing with sterile distilled water, and were resuspended in distilled water at OD$_{600}$~6 and stored at −20 °C until use (Paredes-Sabja et al., 2008c). All spore preparations used in this work were >99% free of sporulating cells, cell debris and germinated spores, as determined by phase-contrast microscopy.

**Assessment of colony-forming efficiency of spores.** To assess the colony-forming efficiency of C. difficile spores, spore suspensions at an OD$_{600}$ of 1 were heat activated at 80 °C for 10 min, aliquots of dilutions were plated on Brain Heart Infusion (BHI) agar, the plates were incubated at 37 °C anaerobically for 24 h, and colonies were counted to determine c.f.u. ml$^{-1}$. The number of spores per ml was counted directly by phase-contrast microscopy using a counting chamber (Model Z30000, Weber Scientific), and the percentage of spores that gave rise to colonies on BHI agar was calculated as [c.f.u. (OD$_{600}$ unit)$^{-1}$ ml$^{-1}$]/[spore count (OD$_{600}$ unit)$^{-1}$ ml$^{-1}$] × 100%.

**Spore germination.** Preliminary experiments indicated that C. difficile spore germination was more efficient when spores were heat activated at 80 °C for 10 min rather than at 60, 70 or 90 °C (data not shown), as is also the case for C. perfringens spores (Paredes-Sabja et al., 2008c). Consequently, all germination experiments used heat-activated spores unless noted otherwise. After heat activation, spores were cooled to room temperature, sonicated briefly to break up any clumps and incubated at 40 °C for 10 min before addition of germinants, and the OD$_{600}$ of the spore suspensions was measured to assess spore germination (SmartSpec 3000 Spectrophotometer, Bio-Rad Laboratories); levels of spore germination were also confirmed by phase-contrast microscopy.

Germination in nutrient medium was performed in BHI broth. Germination with bile salts and/or glycine was carried out in 10 mM Na$_2$HPO$_4$ buffer (pH 7.5) to reduce the background germination caused by Pi (see below). Germination was routinely carried out aerobically, since no difference in germination kinetics was detected under anaerobic conditions (data not shown), and was in 25 mM sodium phosphate buffer (pH 7.5) unless noted otherwise. Spore germination kinetics exhibited no significant difference when assessed at 37 or 40 °C (data not shown). Thus, to allow direct comparison with previous studies of germination of C. perfringens spores (Paredes-Sabja et al., 2008b,c), spore germination of C. difficile was carried at 40 °C. No noticeable spore clumping was observed during germination assays. The extent of spore germination was determined by measuring the decrease in OD$_{600}$ of germinating spore suspensions, and was expressed as a percentage of the initial OD$_{600}$. Since a decrease in OD$_{600}$ of ~65% corresponds to ~99% spore germination as assessed by phase-contrast microscopy, the percentage decrease in the OD$_{600}$ was converted to the percentage germination by taking an OD$_{600}$ decrease of 65% as 100% germination. The rate of germination was expressed as the maximum rate of loss of OD$_{600}$ spore suspensions relative to initial values. To evaluate effects of pH on spore germination rates, germination was performed in 25 mM sodium citrate buffer (pH 2 and 4), 25 mM sodium phosphate buffer (pH 2, 5 and 7.5) or 25 mM Tris/HCl buffer (pH 8.5) at 40 °C. All values reported are averages of two experiments performed with two independent spore preparations, and individual values varied by ≤15% from the average.

For germination with dodecylamine, spores at OD$_{600}$ ~1 were used without heat activation, since this had no effect on germination with this agent, as has been found for spores of other species (Paredes-Sabja et al., 2008c). Spores were incubated at 60 °C with 1 mM dodecylamine in 25 mM Tris/HCl buffer (pH 7.4). Aliquots (1 ml) of germinating cultures were centrifuged at 16110 g for 2 min in a microcentrifuge, and DPA in the supernatant fluid was measured by monitoring OD$_{270}$ The total OD$_{270}$ that could be released from these spores was determined by boiling a sample of dormant spores at an OD$_{600}$ of 1 for 60 min, followed by cooling on ice, centrifugation and
measurement of the OD$_{270}$ of the supernatant fluid as described previously (Cabrera-Martinez et al., 2003; Setlow et al., 2003). All experiments with dodecylamine were repeated at least twice, and results for different experiments differed by $\leq 5\%$.

For germination with Ca–DPA, spores were germinated with or without prior heat activation, cooled to room temperature, diluted to OD$_{600}$ $\sim 1.5$ and incubated at 40°C with Ca–DPA (50 mM CaCl$_2$, 50 mM DPA adjusted to pH 8.0 with Tris/HCl), as described previously (Paredes-Sabja et al., 2008c). At various times, 1 ml aliquots were centrifuged for 2 min in a microcentrifuge, and the spore pellet was washed four times with sterile distilled water and suspended in 1 ml sterile water. Residual spore core DPA content was determined by boiling samples for 60 min, centrifuging them for 5 min, and measuring the OD$_{270}$ of the supernatant fluid as described previously (Cabrera-Martinez et al., 2003; Setlow et al., 2003). The change in the OD$_{600}$ of spore cultures during germination with Ca–DPA was also measured as described above. All experiments with Ca–DPA were repeated at least twice, and results for different experiments differed by $\leq 5\%$.

**Measurement of spore core DPA content.** Spore DPA content was measured by boiling 1 ml spores at OD$_{600}$ 6 for 60 min, followed by cooling on ice, centrifuging at 16 110 $g$ for 5 min, and assaying DPA in the supernatant fluid chemically as described elsewhere (Rotman & Fields, 1968).

### RESULTS

**Nutrient germination of C. difficile spores**

Spore germination is essential for C. difficile spores to cause disease, but the specific requirements for C. difficile spore germination are relatively poorly defined. As expected (Wilson et al., 1982; Wilson, 1983), spores of the six C. difficile strains gave colonies on a complex nutrient medium, BHI (Table 1). Although there was significant variability in the colony-forming efficiency among spores of the different isolates, heat activation increased spore titres significantly (Table 1) and the titres were generally similar to those observed previously with C. perfringens spores (Paredes-Sabja et al., 2008c), except for spores of C. difficile Pitt221 (Table 1). A relatively high level of germination was also observed for the spores of all six C. difficile strains after 60 min incubation in BHI broth (Table 1, Fig. 1a), although which of the individual components of BHI broth triggers C. difficile spore germination is not clear. These results are different from those obtained in a recent study, in which spores of two other C. difficile strains, CD196 and UK14, germinated poorly in BHI broth (Sorg & Sonenshein, 2008). However, a significant difference between the current work and the study of Sorg & Sonenshein (2008) is that the heat-activation temperature was 80°C for 10 min in the current work versus 60°C for 20 min in the other study. Indeed, the C. difficile spores germinated to a significantly lesser extent when heat activated at 60°C for 20 min compared with spores that were heat activated at 80°C for 10 min (Fig. 1a; data not shown).

The study of Sorg & Sonenshein (2008) also reported that bile salts and glycine act as co-germinants to give a significant increase in the germination of C. difficile spores. To test if this is also the case for spores of the six isolates used in the current work, purified spores were incubated in BHI broth with bile salts. However, the bile salts actually reduced the germination of spores of the six C. difficile isolates in BHI broth, irrespective of heat-activation conditions (Table 1, Fig. 1b). In addition, no significant differences were seen in the germination of the spores of the six C. difficile strains that were heat activated at 60°C.

**Table 1.** Colony-formation efficiency and germination of C. difficile spores in rich medium

| Strain | Colony-forming efficiency |  |  |  |
|------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
|      | Spores (OD$_{600}$ unit)$^{-1}$ ml$^{-1}$ | c.f.u. (OD$_{600}$ unit)$^{-1}$ ml$^{-1}$ | Efficiency (%) | Average percentage germination* within 60 min in: |
|      | NO HA | HA | NO HA | HA | BHI | BSC$\S$ | BST$\S$ |
| JIR8094 | $4.8 \times 10^7$ | $3.4 \times 10^6$ | $1.3 \times 10^2$ | 26 | 47 | 12 | 15 |
| Pitt33 | $7.3 \times 10^7$ | $2.9 \times 10^6$ | $3.9 \times 10^2$ | 53 | 79 | 11 | 12 |
| Pitt40 | $5.1 \times 10^7$ | $2.2 \times 10^6$ | $7.8 \times 10^2$ | 14 | 32 | 15 | 17 |
| Pitt221 | $1.2 \times 10^8$ | $2.0 \times 10^6$ | $4.7 \times 10^2$ | 4 | 12 | 9 | 9 |
| Pitt251 | $4.7 \times 10^7$ | $2.6 \times 10^6$ | $2.3 \times 10^2$ | 47 | 63 | 9 | 12 |
| Pitt301 | $8.7 \times 10^7$ | $3.4 \times 10^6$ | $1.2 \times 10^2$ | 14 | 49 | 16 | 19 |

*Spores were heat activated (80°C, 10 min) and incubated at 40°C for 60 min in BHI broth with or without bile salts. The OD$_{600}$ of germinating cultures was measured, expressed as the percentage germination as described in Methods and the approximate degree of spore germination was confirmed by phase-contrast microscopy. Results are averages of duplicate experiments, and the variance in all cases was less than 8%.

†Spores were counted microscopically, plated on BHI agar before (NO HA) and after (HA) heat activation at 80°C for 10 min and incubated at 37°C overnight, and colonies were counted as described in Methods.

‡Efficiency was determined using heat-activated spore suspensions and calculated as: [c.f.u. (OD$_{600}$ unit)$^{-1}$][spore count (OD$_{600}$ unit)$^{-1}$] $\times 100\%$; experiments were repeated at least twice and the variation was $\leq 30\%$.

§BHI broth supplemented with 1% sodium cholate (BSC) or sodium taurocholate (BST).
for 20 min and incubated with glycine or bile salts alone or with glycine plus bile salts (data not shown). Indeed, the germination of these spores was similar to that of the spores in buffer (10 mM sodium phosphate buffer, pH 7.5) alone, as ≤10% of spores became phase dark after 60 min incubation at 40 °C. Heat activation at 80 °C for 10 min enhanced germination of the spores of these strains with glycine plus bile salts, but only to a small extent, and the germination of JIR8094 and Pitt301 spores with glycine with or without bile salts was significantly less than that obtained in BHI broth (Table 1, Fig. 1a, c).

While combinations of bile salts and glycine seemed to have no major effect on germination of C. difficile spores, individual amino acids and some salts can induce germination of spores of many Bacillus and Clostridium species (Broussolle et al., 2002; Clements & Moir, 1998; Paredes-Sabja et al., 2008c; Rode & Foster, 1962). Consequently, a number of individual components of BHI broth, in particular free amino acids, were tested to identify the specific germinant(s) that trigger C. difficile spore germination. However, none of the 20 tested amino acids induced germination of spores of the six C. difficile

### Table 2. Germination of C. difficile spores in the presence of amino acids and salts

All experiments were performed in 25 mM sodium phosphate buffer (pH 7.5), with or without 100 mM germinant. Spores were heat activated (80 °C, 10 min) and incubated at 40 °C for 60 min; the OD₆₀₀ of the germinating cultures was measured and expressed as the percentage germination as described in Methods, and the approximate degree of spore germination was confirmed by phase-contrast microscopy. Results are the means of duplicate experiments, and the variance in all cases was less than 10% of the mean.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean percentage germination in 60 min with:</th>
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<tbody>
<tr>
<td></td>
<td>H₂O</td>
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<tr>
<td>JIR8094</td>
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</tr>
<tr>
<td>Pitt33</td>
<td>5</td>
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<tr>
<td>Pitt40</td>
<td>0</td>
</tr>
<tr>
<td>Pitt221</td>
<td>3</td>
</tr>
<tr>
<td>Pitt251</td>
<td>0</td>
</tr>
<tr>
<td>Pitt301</td>
<td>2</td>
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</table>

*Phosphate: 25 mM sodium phosphate buffer, pH 7.5, only.
†AK is a mixture of 100 mM L-asparagine and 100 mM KCl.
‡KPO₄ was at 100 mM (pH 7.5) and without the sodium phosphate.
strains (Table 2; data not shown). Indeed, germination with these amino acids was similar to that with phosphate buffer alone when germination was assessed either by the decrease in OD_{600} of germinating cultures or by phase-contrast microscopy (Table 2). Interestingly, phosphate buffer also triggered the germination of a small but significant percentage of \textit{C. difficile} spores after 60 min of incubation, with 5–20\% of the spores becoming phase dark (Table 2; data not shown). As found with spores of \textit{C. perfringens} (Paredes-Sabja et al., 2008c), \textit{C. difficile} spores did germinate in AK (a mixture of 100 mM asparagine plus 100 mM KCl) (Table 2). However, much of the effect of AK appeared to be due to the KCl, as KCl alone gave significant germination of spores of all \textit{C. difficile} isolates, while asparagine alone was ineffective (Table 2). In contrast to the stimulation of \textit{C. difficile} spore germination by KCl, NaCl was ineffective (Table 2). Other potassium salts stimulated spore germination, but not as well as KCl; the general order of effectiveness was KCl > KBr > KH_{2}PO_{4} > KI (Table 2). Previous work has shown that spores of \textit{C. perfringens} (Paredes-Sabja et al., 2008c) and \textit{Bacillus megaterium} QM B1551 (Christie & Lowe, 2007; Rode & Foster, 1962) are also germinated by salts, in particular K\textsuperscript{+} salts, alone.

The pH-dependence of KCl-induced germination of spores of two representative \textit{C. difficile} strains (JIR8094 and Pitt301) was similar, with a pH optimum of 6.0–7.5 (Fig. 2a). The rate of KCl-induced germination of JIR8094 and Pitt301 spores was dependent on the KCl concentration, with a maximum germination response at 100–200 mM (Fig. 2b). While JIR8094 spores incubated with 100 mM KCl exhibited a constant rate of germination for ~60 min (Fig. 2c), Pitt301 spores incubated similarly germinated much more rapidly, and almost completely within the first 10 min of incubation (Fig. 2c), with \(\geq 99\%\) of the spores from both isolates becoming phase dark after 60 min incubation (data not shown).

**Fig. 2.** Effect of pH, KCl and P\textsubscript{i} on the rate of germination of \textit{C. difficile} spores. Spores of strains JIR8094 (▲) and Pitt301 (■) were heat activated at 80 °C for 10 min and germinated at 40 °C in: (a) 100 mM KCl in 25 mM sodium phosphate buffer at various pH values; (b) various concentrations of KCl in 25 mM sodium phosphate buffer, pH 7.0; (c) 100 mM KCl in 25 mM sodium phosphate buffer, pH 7.0. Heat-activated spores of strain Pitt301 were incubated at 40 °C in: (d) 25 mM sodium phosphate buffer at various pH values; (e) various concentrations of sodium phosphate buffer, pH 6.0; and (f) 200 mM sodium phosphate buffer, pH 6.0. Spore germination was measured by monitoring the OD_{600} of germinating cultures and either the maximum rate (Max.) was determined over 60 min (a, b, d, e) or germination was monitored continuously (c, f), as described in Methods. In all cases the approximate degree of spore germination was confirmed by phase-contrast microscopy.
Effect of Pi on C. difficile spore germination

Interestingly, during the study of the pH optimum of KCl-induced germination (Fig. 2a), we noticed that Pitt301 and JIR8094 spores germinated with 25 mM sodium phosphate at pH 6.0 alone. This led us to hypothesize that Pi might also induce germination of spores of C. difficile isolates. To test this, we examined the germination of spores of the six C. difficile strains in 25 mM sodium phosphate at pH values from 3 to 7.5. The highest extent of germination of spores of the surveyed isolates was obtained with sodium phosphate at pH 6.0, although this was lower than that obtained with KCl plus sodium phosphate (Tables 2 and 3). To determine whether it was the Pi or the pH of 6.0 that was inducing C. difficile spore germination, we also carried out germination using citrate and MOPS buffers. Germination in these buffers at pH 6.0 or 7.0 was greater than in water alone but lower than in sodium phosphate at pH 6.0, and these results were confirmed by phase-contrast microscopy (Table 3). The latter results strongly suggest that Pi is a significant factor in inducing C. difficile spore germination. The optimum pH for germination with 25 mM sodium phosphate of spores of a fast-germinating isolate (Pitt301) was 6.0 (Fig. 2d), and the rate of Pi-induced germination of these spores was maximal at ~200 mM Pi (Fig. 2e). Pitt301 spores incubated with 200 mM Pi at pH 6.0 exhibited a high level of germination after 10 min of incubation, as was observed with KCl (Fig. 2c, f). These results further indicate that Pi at pH 6.0 is capable of inducing germination of C. difficile spores relatively efficiently.

Dodecylamine germination of C. difficile spores

Dodecylamine, a cationic surfactant, can also germinate spores of many Bacillus and Clostridium species (Setlow et al., 2003). In B. subtilis spores, dodecylamine may act by opening a channel in the spore inner membrane allowing DPA release (Setlow et al., 2003; Vepachedu & Setlow, 2005, 2007). Levels of DPA in spores of C. difficile JIR8094 and Pitt301 were 17.3 ± 1.6 and 18 ± 0.6 μg DPA (OD$_{600}$ unit)$^{-1}$ ml$^{-1}$, respectively, values similar to those for B. subtilis (Paidhungat et al., 2000) and C. perfringens (Paredes-Sabja et al., 2008a, b) spores. Incubation of JIR8094 and Pitt301 spores with dodecylamine at 60 °C caused the release of ~80% of the spore DPA after 80 min (Fig. 3), although no DPA was released upon incubation at 60 °C in buffer alone (data not shown). However, Pitt301 spores incubated with dodecylamine released DPA approximately fourfold faster than did JIR8094 spores (Fig. 3), similar to that which was seen with KCl-induced germination (Fig. 2c).

Germination by exogenous Ca–DPA

Previous work has shown that B. subtilis spores can also germinate in the presence of exogenous Ca–DPA, which acts to promote cortex hydrolysis by activation of the CLE CwlJ (Paidhungat et al., 2001). Similar, albeit not identical, CLEs have been identified in other endospore-forming species (Foster & Johnstone, 1987; Ishikawa et al., 1998; Makino et al., 1994; Miyata et al., 1995, 1997; Shimamoto et al., 2001), and bioinformatic analyses reveal that similar CLEs are also present in C. difficile (Sebaihia et al., 2006). When spores of strains JIR8094 and Pitt301 without prior heat activation were incubated with Ca–DPA and germination was measured, there were no significant changes in OD$_{600}$ or spore refractility, and no release of DPA (data not shown). This is in agreement with the lack of effect of Ca–DPA on C. perfringens spores that have not been heat activated (Paredes-Sabja et al., 2008c). However, as described in Methods, within 60 min heat-activated JIR8094 and Pitt301 spores germinated ≥80% with Ca–DPA, as measured by both OD$_{600}$ decrease and loss of DPA (data not shown). These results were confirmed by phase-contrast microscopy, as ~80% of JIR8094 and Pitt301 spores became phase dark after 60 min incubation with Ca–DPA (data not shown). Heat-activated spores of C. difficile, 2005, pH 6.0, C.

Table 3. Germination of C. difficile spores in different buffers

Heat-activated (80 °C, 10 min) spores were incubated in 25 mM buffer at 40 °C for 60 min; the OD$_{600}$ of the germinating cultures was measured and expressed as the percentage germination as described in Methods, and the approximate degree of spore germination was confirmed by phase-contrast microscopy. Results are means of duplicate experiments, and the variance in all cases was less than 10% of the mean.

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<th>Strain</th>
<th>Mean percentage germination in 60 min in:</th>
<th>Sodium phosphate at pH</th>
<th>Sodium citrate at pH</th>
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perfringens also germinate with Ca–DPA (Paredes-Sabja et al., 2008c).

Biinformatic analyses of *C. difficile* germination apparatus

Although no homologue of the *gerA* family of operons that encode nutrient germinant receptors is present in the genome of *C. difficile* 630 (Sebaihia et al., 2006), *C. difficile* spores must have some way to sense when conditions are appropriate for the spores to germinate. Indeed, current and previous studies (Sorg & Sonenshein, 2008; Wilson et al., 1982; Wilson, 1983) have shown that spores of various *C. difficile* strains are able to initiate germination in response to individual compounds. However, how these compounds are sensed is not clear; perhaps they directly activate CLEs or the release of Ca–DPA.

While genes encoding nutrient germinant receptors appear to be absent from the *C. difficile* 630 genome, genes encoding CLEs and SpoVA orthologues are present (Sebaihia et al., 2006), with the SpoVA proteins possibly involved in DPA release during spore germination and in DPA uptake during sporulation (Vepachedu & Setlow 2007). The *spoVA* locus in *C. difficile* consists of three ORFs, CD0773, CD0774 and CD0775, and the organization of the *C. difficile* *spoVA* operon resembles that of *C. perfringens* with the order *spoVAC*, *spoVAD* and *spoVAE* (Myers et al., 2006; Sebaihia et al., 2006). Three CLE orthologues are encoded in the *C. difficile* genome. *C. difficile* ORF CD0551 encodes a 423-residue protein with high similarity (67 %) to a *C. perfringens* CLE, SleC, that is suggested to cause local changes in the structure of cortical PG through its lytic transglycosylase and N-acetylmur-amy1-l-alanine amidase activity (Kumazawa et al., 2007). CD0552 is annotated as *sleB* and encodes a 238-residue protein, but shares no similarity with CLEs from *C. perfringens* and *B. subtilis*. The third potential CLE in *C. difficile* is ORF CD3563, which encodes a 168-residue protein with 70 % similarity to *B. subtilis sleB*.

**DISCUSSION**

CDAD is thought to be initiated by the ingestion of *C. difficile* spores, which then germinate, grow and produce virulence factors that cause severe damage to the intestinal epithelium, leading to an acute inflammatory response (Borriello, 1998; Hurley & Nguyen, 2002). To understand the initial stages of pathogenesis of CDAD, we have characterized the germination of five CDAD isolates (McEllistrem et al., 2005) and one laboratory strain of *C. difficile*. Probably the most notable finding in this work is the identification of compounds that can induce the germination of *C. difficile* spores, since the identity of such compounds has long been controversial. The germination of spores of *C. difficile* by potassium salts alone was somewhat unexpected, but by no means unique, since spores of some *C. perfringens* and *B. megaterium* strains (Ando, 1974; Christie & Lowe, 2007; Paredes-Sabja et al., 2008c; Rode & Foster, 1962) germinate well with KI, KBr and KCl. K+ ions are also essential for the AGFK-initiated germination of *B. subtilis* spores (Setlow, 2003). The precise mechanism of spore germination by K+ ions alone is not known, but in *B. megaterium* spores this appears likely to be via the activation of one or more of the spore nutrient germinant receptors (Christie & Lowe, 2007; Cortezzo et al., 2004; Rode & Foster, 1962), and this also appears to be the case, at least in part, for *C. perfringens* spores (Paredes-Sabja et al., 2008c). This cannot be the case for *C. difficile* spores, since at least strain JIR8094, a derivative of the sequenced strain 630 (Sebaihia et al., 2006), also appears to lack the nutrient germinant receptors that are orthologues of the GerA family of proteins, and thus K+ ions may trigger germination of these spores by either activating a CLE or triggering Ca–DPA release directly. In addition, based on their germination phenotype, the spores from CDAD outbreak isolates also appear to lack the GerA family of proteins, and the variability observed might be due to differences in the number of downstream effectors of spore germination, such as SpoVA proteins or CLEs. These findings differ from those of earlier studies (Nakamura et al., 1985; Wilcox et al., 2000) that used lysozyme to enhance *C. difficile* spore germination and recovery. This enzyme bypasses the whole germination apparatus and degrades the PG cortex directly, allowing spore outgrowth, and is able to recover spores with an intact or even a damaged germination apparatus, as well as spores that have not been subjected to proper heat activation. However, it is possible that the triggering of *C. difficile* spore germination by K+ ions is relevant to the ability of these spores to cause CDAD. The concentration of K+ ions in the human GI tract increases from ~10 mM in the ileum to ~75 mM in the colon (Johnson, 2000). Thus, while the colon is low in nutrients, the concentration of K+ ions is certainly more than high enough to induce
germination of C. difficile spores, although the activation factors necessary for the germination of C. difficile spores in the gut are not known. The germination of C. difficile spores seen with BHI broth may also be due, at least in part, to P_i (≥18 mM in BHI broth) and to the K+ ions present in this medium, since the levels of K+ ions are likely significant. Levels of K+ ions and P_i are also significant in meat products (50–80 and 42–60 mM, respectively) (USDA; http://www.nal.usda.gov/fnic/food-comp/search/), so there could be significant spore germination in foods that are held for significant periods at temperatures that allow germination. However, we have not studied the K+ - and P_i-dependent germination of C. difficile spores at different temperatures, and the temperature at which foods are held could greatly influence K+ - and P_i-dependent C. difficile spore germination.

A novel and unexpected finding in this work was that C. difficile spores germinated well at pH 6 with P_i alone. To our knowledge, P_i has not been reported to induce germination of bacterial spores, although P_i at pH 5.5–6.7 induces sporulation of C. perfringens (Philippe et al., 2006). The precise mechanism of initiation of C. difficile spore germination by P_i is not known. However, again this might be advantageous for C. difficile spores in at least one of their natural habitats. The pH in the first centimetre of the duodenum is 2.0–3.5, and gradually increases due to secretion of bicarbonate, reaching pH 5.0–6.0 near the mid-duodenum (Rune, 1973), where P_i could trigger germination of C. difficile spores. P_i is ubiquitous in the diet of healthy Western adults, and is present in the human intestinal lumen at 15–30 mM (Lemann, 1993; Walton & Gray, 1979). As shown in this work, this P_i concentration will induce germination of C. difficile spores.

One major conclusion from the identification of K+ ions and P_i as effective germinants for C. difficile spores is that ingested spores that survive stomach acidity (JUMP et al., 2007) could germinate in any of three different segments of the GI tract: (1) the early duodenum, where the pH is ~6.0; (2) the small intestine, triggered by unidentified compounds, perhaps bile salts; and (3) the colon, where high levels of K+ would trigger germination of the remaining dormant spores. However, CDAD symptoms affect primarily the large intestine and rarely the small intestine (Hurley & Nguyen, 2002), perhaps because either most C. difficile spores germinate primarily in the large intestine due to the presence of K+ ions or spores do germinate in early segments of the GI tract but do not outgrow appreciably in the small intestine and only outgrow, colonize, release toxins (TcdA and TcdB) and cause CDAD symptoms in the large intestine.

Clearly, a significant unresolved paradox about the major findings in this work is how C. difficile spores respond to and initiate germination with K+ ions and P_i in the apparent absence of the receptors that recognize specific germinants. One possible explanation is that K+ ions and P_i directly activate a CLE and/or Ca–DPA release. Ongoing work is thus oriented towards investigating the roles played by a number of proteins, including CLEs that may be involved in cortex PG hydrolysis (Foster & Johnstone, 1987; Ishikawa et al., 1998; Makino et al., 1994; Miyata et al., 1995, 1997; Shimamoto et al., 2001) and SpoVA proteins implicated in Ca–DPA release (Paredes-Sabja et al., 2008b), in the germination of C. difficile spores with K+ and P_i. The resolution of the paradox noted above as well as a more thorough understanding of the germination of C. difficile spores may well have applications in the areas of public health and food safety.

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