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_i) with a pH optimum of 6. The *in vitro* germination of spores of CDAD strains with KCl and P_i, two molecules present at significant levels in the GI tract, suggests that *C. difficile* spores germinate in the human body by sensing P_i in the early segments of the duodenum and KCl in the colon.

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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.*, 1990; 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 pyridine-2,6-dicarboxylic 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⁺), divalent cations (Ca²⁺, Mg²⁺ and Mn²⁺), and the spore large depot (~20% of core dry weight) of DPA (Setlow, 2003). DPA is

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Abbreviations: CDAD, *Clostridium difficile*-associated disease; CLE, cortex-lytic enzyme; DPA, dipicolinic acid; GI, gastrointestinal; PG, peptidoglycan.

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 (P_i); 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 sporulation 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 ($\sim 10^7$ 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 $\text{OD}_{600} \sim 6$ 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 $[\text{c.f.u.} (\text{OD}_{600} \text{ unit})^{-1} \text{ ml}^{-1}] / [\text{spore count} (\text{OD}_{600} \text{ unit})^{-1} \text{ ml}^{-1}] \times 100\%$.

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_2HPO_4 buffer (pH 7.5) to reduce the background germination caused by P_i (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 $\sim 65\%$ corresponds to $\geq 99\%$ 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} of 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 $\leq 15\%$ from the average.

For germination with dodecylamine, spores at $\text{OD}_{600} \sim 1$ 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 OD₂₇₀ 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 OD₆₀₀ ~ 1.5 and incubated at 40 °C with Ca-DPA (50 mM CaCl₂, 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 OD₂₇₀ of the supernatant fluid as described previously (Cabrera-Martinez *et al.*, 2003; Setlow *et al.*, 2003). The change in the OD₆₀₀ 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 OD₆₀₀ 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				Average percentage germination* within 60 min in:		
	Spores (OD ₆₀₀ unit) ⁻¹ ml ⁻¹	c.f.u. (OD ₆₀₀ unit) ⁻¹ ml ⁻¹ †		Efficiency (%)‡	BHI	BSC§	BST§
		No HA	HA				
JIR8094	4.8 × 10 ⁷	3.4 × 10 ⁶	1.3 × 10 ⁷	26	47	12	15
Pitt33	7.3 × 10 ⁷	2.9 × 10 ⁶	3.9 × 10 ⁷	53	79	11	12
Pitt40	5.1 × 10 ⁷	2.2 × 10 ⁶	7.8 × 10 ⁶	14	32	15	17
Pitt221	1.2 × 10 ⁸	2.0 × 10 ⁶	4.7 × 10 ⁶	4	12	9	9
Pitt251	4.7 × 10 ⁷	2.6 × 10 ⁶	2.3 × 10 ⁷	47	63	9	12
Pitt301	8.7 × 10 ⁷	3.4 × 10 ⁶	1.2 × 10 ⁷	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 OD₆₀₀ 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. (OD₆₀₀ unit)⁻¹ ml⁻¹]/[spore count (OD₆₀₀ unit)⁻¹ ml⁻¹] × 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).

Fig. 1. Effect of activation temperature and bile salts on *C. difficile* spore germination with nutrients. (a) Spores of strains JIR8094 (circles), Pitt40 (triangles) and Pitt 301 (squares) were heat activated at 80 °C for 10 min (filled symbols) or at 60 °C for 20 min (open symbols), and germinated in BHI broth at 40 °C. (b) Spores of strains JIR8094 (open symbols) and Pitt301 (filled symbols) were activated at 80 °C for 10 min and germinated in: BHI broth (squares); BHI broth plus 1 % sodium cholate (circles); and BHI broth plus 1 % sodium taurocholate (triangles). (c) Spores of strains JIR8094 (open symbols) and Pitt301 (filled symbols) were heat activated at 80 °C for 10 min and germinated in: 1.3 mM glycine in 10 mM sodium phosphate buffer (pH 7.5) (squares); 1.3 mM glycine in 10 mM sodium phosphate buffer (pH 7.5) plus 1 % sodium cholate (circles); and 1.3 mM glycine in 10 mM sodium phosphate buffer (pH 7.5) plus 1 % sodium taurocholate (triangles). Spore germination was measured by monitoring the OD₆₀₀ of germinating cultures as described in Methods. In all cases the approximate degree of spore germination was confirmed by phase-contrast microscopy.

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 $\leq 10\%$ 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.

Strain	Mean percentage germination in 60 min with:										
	H ₂ O	Phosphate*	Ala	Ser	Asn	AK†	KCl	NaCl	KPO ₄ ‡	KI	KBr
JIR8094	0	22	12	17	17	63	82	25	77	43	45
Pitt33	5	9	12	14	12	80	92	20	60	29	75
Pitt40	0	18	22	22	22	37	54	17	35	26	29
Pitt221	3	9	8	9	8	18	31	18	14	9	11
Pitt251	0	14	11	11	12	100	82	20	15	42	52
Pitt301	2	28	31	37	34	71	80	26	57	40	65

*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 OD₆₀₀ of germinating cultures or by phase-contrast microscopy (Table 2). Interestingly, phosphate buffer also triggered the germination of a small but significant percentage of *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 *C. perfringens* (Paredes-Sabja *et al.*, 2008c), *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 *C. difficile* isolates, while asparagine alone was ineffective (Table 2). In contrast to the stimulation of *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 $\approx$ KH₂PO₄

> KI (Table 2). Previous work has shown that spores of *C. perfringens* (Paredes-Sabja *et al.*, 2008c) and *Bacillus megaterium* QM B1551 (Christie & Lowe, 2007; Rode & Foster, 1962) are also germinated by salts, in particular K⁺ salts, alone.

The pH-dependence of KCl-induced germination of spores of two representative *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 $\sim$ 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_i on the rate of germination of *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 OD₆₀₀ 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 P_i 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 P_i 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 P_i 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 P_i 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 P_i-induced germination of these spores was maximal at ~200 mM P_i (Fig. 2e). Pitt301 spores incubated with 200 mM P_i 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 P_i 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 (OD₆₀₀ 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* (Sebahia *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 OD₆₀₀ 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 OD₆₀₀ 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.*

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 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 means of duplicate experiments, and the variance in all cases was less than 10 % of the mean.

Strain	Mean percentage germination in 60 min in:							
	Sodium phosphate at pH:				Sodium citrate at pH:		MOPS at pH:	
	3	4	6	7	6	7	6	7
JIR8094	15	17	40	31	20	26	18	17
Pitt33	22	25	80	17	31	17	9	8
Pitt40	14	17	25	20	17	20	6	15
Pitt221	12	20	15	11	11	14	3	9
Pitt251	28	26	40	17	15	15	14	14
Pitt301	22	26	60	26	34	23	23	28

Fig. 3. Germination of spores of *C. difficile* strains with dodecylamine. Spores of strains JIR8094 (■) and Pitt301 (▲) were incubated with dodecylamine and their germination was assessed by measuring DPA release, as described in Methods.

perfringens also germinate with Ca-DPA (Paredes-Sabja *et al.*, 2008c).

Bioinformatic 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*-acetylmuramyl-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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