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Characterization of the Dynamic Germination of Individual *Clostridium difficile* Spores Using Raman Spectroscopy and Differential Interference Contrast Microscopy

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ABSTRACT

The Gram-positive spore-forming anaerobe *Clostridium difficile* is a leading cause of nosocomial diarrhea. Spores of *C. difficile* initiate infection when triggered to germinate by bile salts in the gastrointestinal tract. We analyzed germination kinetics of individual *C. difficile* spores using Raman spectroscopy and differential interference contrast (DIC) microscopy. Similar to *Bacillus* spores, individual *C. difficile* spores germinating with taurocholate plus glycine began slow leakage of a ~15% concentration of a chelate of Ca^{2+} and dipicolinic acid (CaDPA) at a heterogeneous time T_1, rapidly released CaDPA at T_{lag}, completed CaDPA release at $T_{release}$, and finished peptidoglycan cortex hydrolysis at T_{lysis}. T_1 and T_{lag} values for individual spores were heterogeneous, but $\Delta T_{release}$ periods ($T_{release} - T_{lag}$) were relatively constant. In contrast to *Bacillus* spores, heat treatment did not stimulate spore germination in the two *C. difficile* strains tested. *C. difficile* spores did not germinate with taurocholate or glycine alone, and different bile salts differentially promoted spore germination, with taurocholate and taurodeoxycholate being best. Transient exposure of spores to taurocholate plus glycine was sufficient to commit individual spores to germinate. *C. difficile* spores did not germinate with CaDPA, in contrast to *B. subtilis* and *C. perfringens* spores. However, the detergent dodecylamine induced *C. difficile* spore germination, and rates were increased by spore coat removal although cortex hydrolysis did not follow $T_{release}$, in contrast with *B. subtilis*. *C. difficile* spores lacking the cortex-lytic enzyme, SleC, germinated extremely poorly, and cortex hydrolysis was not observed in the few *sleC* spores that partially germinated. Overall, these findings indicate that *C. difficile* and *B. subtilis* spore germination exhibit key differences.

IMPORTANCE

Spores of the Gram-positive anaerobe *Clostridium difficile* are responsible for initiating infection by this important nosocomial pathogen. When exposed to germinants such as bile salts, *C. difficile* spores return to life through germination in the gastrointestinal tract and cause disease, but their germination has been studied only with population-wide measurements. In this work we used Raman spectroscopy and DIC microscopy to monitor the kinetics of germination of individual *C. difficile* spores, the commitment of spores to germination, and the effect of germinant type and concentration, sublethal heat shock, and spore decoating on germination. Our data suggest that the order of germination events in *C. difficile* spores differs from that in *Bacillus* spores and provide new insights into *C. difficile* spore germination.

Clostridium difficile is a Gram-positive, spore-forming, strictly anaerobic bacterium that has become a leading cause of antibiotic-associated diarrhea worldwide (1). Antibiotic treatment disrupts the normal colonic flora that typically suppresses *C. difficile* growth and allows ingested *C. difficile* spores to germinate, outgrow, and proliferate rapidly in the gastrointestinal tract (2–5). Once established in the gastrointestinal tract, *C. difficile* secretes two toxins, the enterotoxin TcdA and the cytotoxin TcdB, that cause massive damage to the intestinal epithelium and induce strong inflammatory responses (6). During growth in the gastrointestinal tract, *C. difficile* strongly induces a transcriptional program that leads to spore formation (7). Spore formation is essential for *C. difficile* to survive exit from the host and transmit disease because its vegetative cells are exquisitely sensitive to oxygen (8). Furthermore, *C. difficile* spores are resistant to antibiotics (9), attacks from the host's immune system (10), and disinfectants commonly used in hospital settings due to their metabolic dormancy and intrinsic resistance properties (11–14). Thus, understanding the mechanisms controlling *C. difficile* spore germination may have practical applications in the management of *C. difficile* infections as this knowledge may lead to new methods for preventing spore germination or efficiently promoting it to facilitate killing of the less resistant germinated spores.

Although spore germination is essential for *C. difficile* to initiate disease (15), our knowledge of *C. difficile* spore biology lags behind that of other well-studied spores, such as those of *Bacillus*

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subtilis (16). *B. subtilis* spore germination can be triggered by a variety of factors, including nutrients, enzymes, hydrostatic pressure, cationic surfactants such as dodecylamine, and a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA) (CaDPA) (17, 18). Nutrient germinants for spores of *Bacillus* species include L-amino acids, purine nucleosides, and D-sugars as well as some mixtures of such compounds. These compounds trigger germination by binding to and interacting with germination receptors (GRs) present in the spore's inner membrane (IM), leading to a series of events taking place in a defined order (19, 20). First, exposure of spores to nutrient germinants causes a reaction that commits spores to germinate even if the germinant is removed or displaced from its cognate GRs (20–22). This commitment step is followed by release of monovalent cations, as well as the spore core's large pool of CaDPA. Ultimately, the spore's peptidoglycan cortex is degraded by either of two redundant cortex-lytic enzymes (CLEs), CwlJ and SleB, and subsequently the spore core swells and takes up water, leading to initiation of metabolism (17).

The process of an individual spore's germination has been divided into four phases according to a spore's optical intensity in differential interference contrast (DIC) or phase-contrast microscopy, with the different phases ending at times T_1, T_{lag}, $T_{release}$, and T_{lysis} (23, 24). T_1 is the time when slow CaDPA leakage begins after germinant addition and is probably coincident with the time of commitment, T_{lag} is the time after the start of slow CaDPA leakage when the initiation of very rapid CaDPA release begins, and $T_{release}$ is the time for completion of rapid CaDPA release; following $T_{release}$, there is a further small decline in spore refractility due to the hydrolysis of the spore cortex and spore core swelling, and the time when spore refractility becomes constant is termed T_{lysis}.

While there are some similarities between *B. subtilis* and *C. difficile* spore germination, there are a number of notable differences. A major difference is that the *C. difficile* genome lacks homologs of the GRs that exist in *Bacillus* species and in many *Clostridium* species (16, 25). Instead, *C. difficile* uses the CspC pseudoprotease as a noncanonical germination receptor to directly sense bile salts and activate germination (15). Whereas *B. subtilis* spores germinate in response to amino acids, nucleosides, and small cations, *C. difficile* spores germinate in response to specific bile salts, in particular, cholate and its derivatives (taurocholate, glycocholate, cholate, and deoxycholate), with glycine acting as a cogerminant; glycine on its own is insufficient to trigger *C. difficile* spore germination (26, 27). It should be noted that spores of some *C. difficile* strains can germinate in rich medium without taurocholate (28), suggesting that the triggers for *C. difficile* spore germination may be complex. In addition to these differences in germinant sensing, the regulation of cortex hydrolysis differs between *C. difficile* and *B. subtilis* (15, 16, 29). *C. difficile* spores require only a single CLE, SleC (30), which is made as a zymogen that is proteolytically activated by the serine protease CspB, similar to the situation in *Clostridium perfringens* (31–33). Thus, the environmental signals that trigger germination, the germinant receptors, and the regulation of cortex hydrolysis exhibit notable differences between *C. difficile* and *B. subtilis*.

To date the germination of *C. difficile* spores has been studied only with spore populations although it is known that the generally considerable heterogeneity in germination rates between individuals in spore populations can obscure individual germination events (34). In the current work we have used laser tweezers, Raman spectroscopy, and DIC microscopy to analyze the kinetics of the germination of multiple individual *C. difficile* spores. The roles of a variety of factors important in spore germination were also investigated, including variations in germination behavior between spores of different strains, responses to different bile salts, the dependence on bile salt and glycine concentrations, effects of sublethal heat shock or decoating prior to germination, and germination by dodecylamine and CaDPA. The results of the study provide new insight into the germination of *C. difficile* spores.

MATERIALS AND METHODS

Bacterial strains and spore preparation. The *C. difficile* strains used in this study were ATCC 43593 and ATCC 43601 (35). The PCR ribotype for each *C. difficile* strain used was ribotype 060 for ATCC 43593 and ribotype 031 for ATCC 43601. Strain JIR8094 and its isogenic *sleC* mutant were also used to look at the effect of SleC on cortex hydrolysis and spore germination (29). The *B. subtilis* strain used was strain PS533, an isogenic derivative of a laboratory 168 strain and carrying plasmid pUB110 providing resistance to 10 μg/ml kanamycin (36). Spores of *C. difficile* were prepared on 70:30 sporulation medium agar (37) and were harvested, purified, and stored as described previously (29). Briefly, *C. difficile* spores were washed extensively with water and then purified on a Histodenz gradient, followed by subsequent water washes. Spores of *B. subtilis* were prepared at 37°C on 2× Schaeffer's-glucose medium agar plates and were purified as described previously (38). All spores used in this study were >98% free of sporulating cells, germinated spores, and debris, as observed by phase-contrast microscopy. Where indicated below, *C. difficile* spores were chemically decoated by incubation of spores at an optical density at 600 nm (OD_{600}) of ~10 at 37°C in 1% SDS, 2% β-mercaptoethanol, and 0.1 M borate buffer (pH 10.0) (33). After 15 min of incubation, decoated spores were washed twice with 0.1 M borate buffer (pH 10.0) and five times with distilled water, and the washed spores were stored in water.

Spore germination. In order to examine the effects of prior sublethal heat shock on spore germination, *C. difficile* spores were incubated in water at 65°C for 30 min and then cooled on ice before germination. However, for most experiments, spores were not heat shocked since this treatment did not stimulate spore germination (see Results). Germination was carried out with different kinds and concentrations of bile salts and glycine (obtained from Sigma-Aldrich, Inc., St. Louis, MO) in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl. To check the effects of glycine on *C. difficile* spore germination, 0.25% taurocholate and variable glycine concentrations were used, as were constant glycine concentrations and variable taurocholate concentrations. The additional bile salts used in this work include deoxycholate, cholate, glycocholate, taurodeoxycholate, chenodeoxycholate, and taurolithocholate. These bile salts were used at 0.5% (~10 mM) with 19.6 mM glycine. Spore germination was also carried out at 37°C with 60 mM CaDPA made to pH 7.4 with Tris base and with 1.2 mM dodecylamine at 42°C in 25 mM K-HEPES buffer (pH 7.4) as described previously (24).

In experiments measuring spores' commitment to germinate, *C. difficile* spores on a coverslip (see below) were exposed to 0.25% taurocholate and 15.5 mM glycine in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl at 37°C for 1 to 2 min, followed by germinant removal, five rinses with 10 mM Tris-HCl (pH 7.4)–150 mM NaCl by vacuum pump suction, and further incubation at 37°C in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl until 30 min.

Measurement of CaDPA level and Raman spectra of individual spores by laser tweezers Raman spectroscopy. The CaDPA levels of individual spores of various strains were determined by laser tweezers Raman spectroscopy at 25°C (23, 39). Briefly, an individual spore was captured with laser tweezers, and its Raman spectrum was acquired with an integration of 20 s and a laser power of 20 mW at 780 nm. Spectra of 30 individual spores were measured and averaged. The CaDPA level in an individual spore was determined from the peak intensity at 1,017 cm^{-1} in its Raman spectrum relative to the peak intensity of the same Raman band

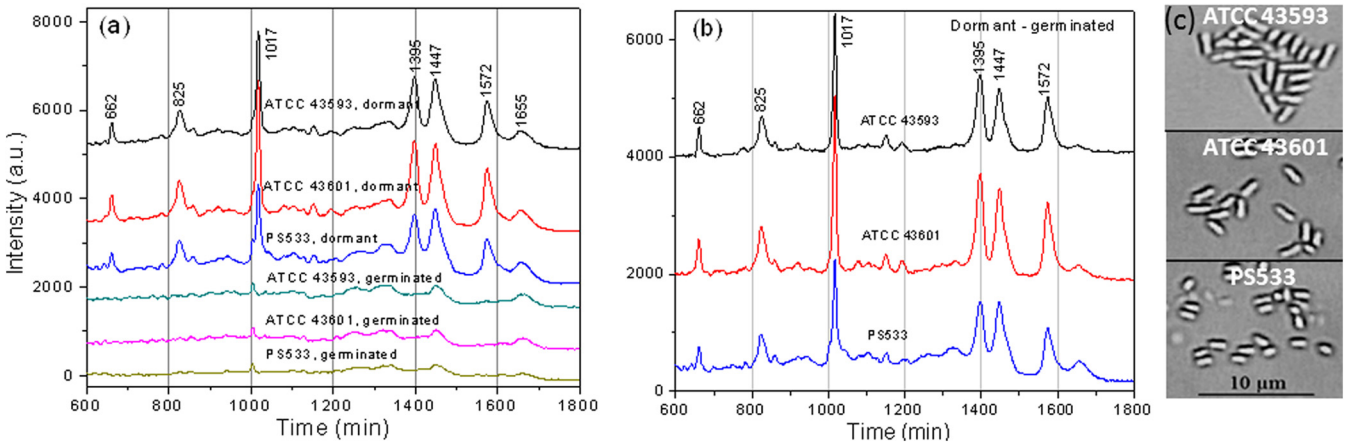

FIG 1 Average Raman spectra of 30 individual dormant and germinated spores of *C. difficile* and *B. subtilis* PS533 (a), difference spectra between dormant and germinated spores (b), and bright-field images of dormant spores (c) are shown. *C. difficile* spores were germinated at 37°C with 1.2% taurocholate and 15.5 mM glycine in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl for 60 min. *B. subtilis* spores were heat shocked for 30 min at 70°C, cooled on ice, and then germinated at 37°C with 10 mM L-valine in 25 mM K-HEPES buffer (pH 7.4) for 60 min. The bright-field images of *C. difficile* and *B. subtilis* spores were recorded with a video camera. au, arbitrary units.

from a CaDPA solution of known concentration (60 mM) and by multiplying this concentration value by the excitation volume of 1 fl to obtain attomoles of CaDPA/spore (23).

Monitoring germination of multiple individual spores. The kinetics of CaDPA release during germination of individual *C. difficile* spores optically trapped by laser tweezers was measured simultaneously by Raman spectroscopy and DIC microscopy as described previously (23, 40). The CaDPA level in an individual spore during germination was determined from the intensities of the CaDPA-specific Raman band at 1,017 cm^{-1}, while the intensity of the DIC image was recorded. As found previously (23, 40), the end of the rapid fall in DIC image intensity during spore germination corresponded to the point at which release of CaDPA was complete, and this time point was defined as $T_{release}$. At this time, the DIC image intensity ($I_{release}$) was 25 to 30% of that at T_0, when image intensity at T_0 was set at 1 and the intensity at the end of measurements was set at zero. Consequently, the CaDPA content of wild-type spores at any time relative to T_0 could be estimated from the DIC image intensity, I_t, as $100\% \times (I_t - I_{release})/(1 - I_{release})$ since the DIC intensity was found to be nearly coincident with the CaDPA level prior to $T_{release}$ (see Fig. 2a). In addition to $T_{release}$, a number of other spore germination parameters have been previously described (23, 24, 40), including the following: T_1, the time when CaDPA leakage starts; CaDPA leakage, the percent CaDPA released slowly between T_1 and T_{lag}; T_{lag}, the time after T_1 when very rapid CaDPA begins; $T_{release}$, the time when CaDPA release is complete; T_{lysis}, the time when spore refractility becomes constant; and $\Delta T_{release}$ and ΔT_{lysis}, which are calculated as $T_{release} - T_{lag}$ and $T_{lysis} - T_{release}$, respectively.

The germination of multiple individual spores was monitored by DIC microscopy as described previously (34, 40). In brief, spores were spread on the surface of a coverslip that was dried in a vacuum desiccator for ~10 min, and the coverslips were mounted on and sealed to a microscope sample holder kept at a constant temperature. The DIC images of multiple spores adhered on coverslips were recorded at a rate of 1 frame per 15 s for 30 to 120 min by a digital charge-coupled-device camera (16 bits; 1,600 by 1,200 pixels) following the addition of preheated germinant solution to the spores on the coverslips. The averaged pixel intensity of an area of 40 by 40 pixels that covered each individual spore's DIC image was calculated, the DIC image intensity of each individual spore was plotted as a function of the incubation time with a resolution of 15 s and with initial image intensity at the first time of measurement, T_0, normalized to 1, and the intensity at the end of the measurement period was normally set at zero. Invariably, the DIC image intensity had been constant for ≥10 min at the end of measurements. The degree of germination of spore populations was measured by simultaneously monitoring the germination of >200 individual spores by DIC microscopy, and at various times the percentage of these spores that had released their CaDPA was determined as described above.

RESULTS

Raman spectra and average CaDPA levels of individual *C. difficile* spores. Previous work has found that bands from CaDPA dominate the Raman spectra of individual spores of *Bacillus* species and *C. perfringens* (23, 41), and this was also the case for spores of two *C. difficile* strains (Fig. 1a). The average intensity of the CaDPA-specific 1,017 cm^{-1} Raman band from ~30 individual spores indicated that the CaDPA levels in spores of *C. difficile* ATCC 43593 and ATCC 43601 were 498.1 ± 156.5 and 664.2 ± 162.0 amol/spore, respectively. Both of these values are larger than the value for *B. subtilis* PS533 spores (432.6 ± 48.6 amol/spore), suggesting that there is more CaDPA in spores of *C. difficile* species. However, this may be due to the larger size of *C. difficile* spores than *B. subtilis* spores, as seen in bright-field images (Fig. 1c). Average Raman spectra of ~30 individual dormant spores with average germinated spores' Raman spectra subtracted also showed an obvious difference in the ratio between the 1,395 cm^{-1} and 1,447 cm^{-1} Raman spectral bands due to CaDPA (Fig. 1b), suggesting that the CaDPA environments in *C. difficile* and *B. subtilis* spores are somewhat different.

Kinetics of germination of individual *C. difficile* spores with taurocholate and glycine. Given the dominant CaDPA-specific band in the Raman spectrum of *C. difficile* spores and previous methods developed to follow the germination of individual spores of *Bacillus* species (23, 40), we used laser tweezers Raman spectroscopy and DIC microscopy to analyze the dynamic germination of individual *C. difficile* spores. The results with a single optically trapped *C. difficile* spore germinating with taurocholate and glycine showed that the fall in the spore's DIC image intensity corresponded to the release of CaDPA (Fig. 2a). In addition, this *C. difficile* spore gave a kinetic germination curve with the same four phases seen with spores of *Bacillus* species, including T_1, T_{lag}, $T_{release}$, and T_{lysis} (Fig. 2). The same results were also obtained

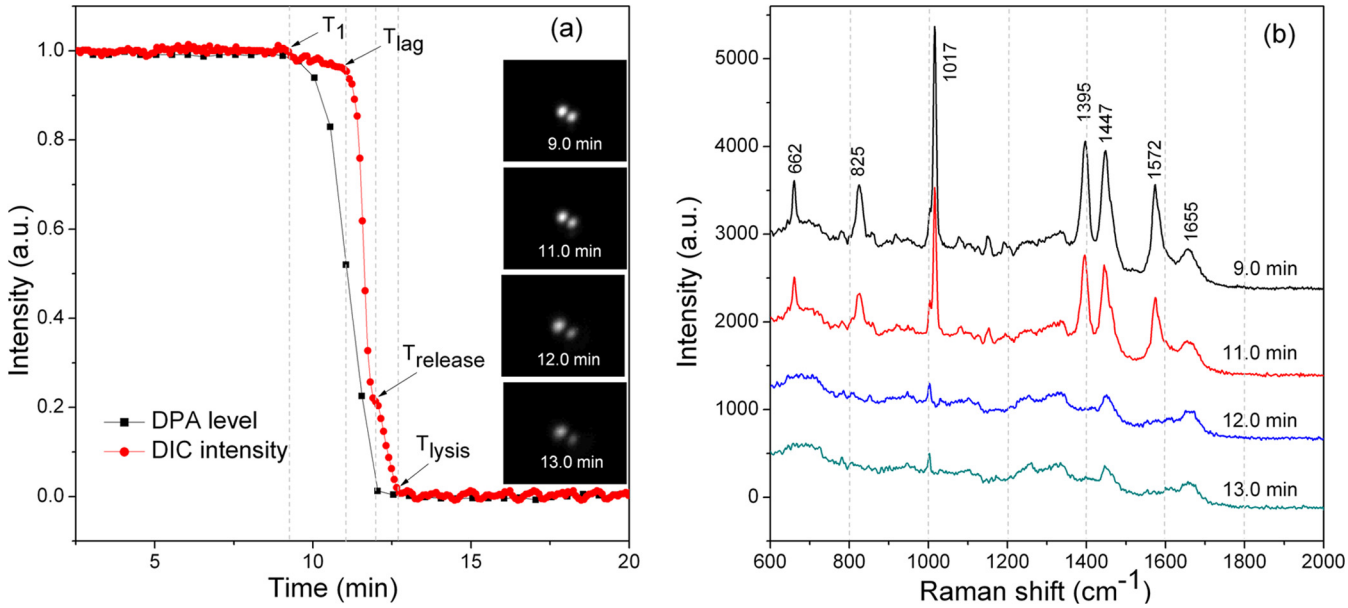

FIG 2 Germination of a single optically trapped *C. difficile* spore monitored by Raman spectroscopy and DIC microscopy. An optically trapped *C. difficile* ATCC 43593 spore was germinated with 1.2% taurocholate and 15.5 mM glycine, and DIC images and Raman spectra were recorded, all as described in Materials and Methods. The trapped spore appears as two bright spots in the DIC image due to the differential interference of the illumination polarized light (40). (a) The germination curves were determined from either the loss in spore DIC image intensity or the intensities of the CaDPA-specific Raman band at 1,017 cm^{-1}. The DIC image and Raman band intensities in arbitrary units (a.u.) were normalized to 1 based on the respective values at the first time of measurement, and DIC image intensities at the end of the experiment were set at 0. T_1, T_{lag}, $T_{release}$, and T_{lysis} for this spore are indicated. (b) Sequential Raman spectra during germination of the single trapped spore were obtained as described in Materials and Methods.

when the germination of multiple individual optically trapped wild-type *C. difficile* spores was followed by monitoring CaDPA release and loss of DIC image intensity (Fig. 3 and Table 1). Notably, 13 to 16% of total spore CaDPA was released between T_1 and T_{lag}, similar to results with nutrient germination of spores of *Bacillus* species (24). Given the good correspondence between CaDPA release and loss of DIC image intensity in spore germination, in further experiments we used DIC microscopy to monitor the germination of multiple individual *C. difficile* spores.

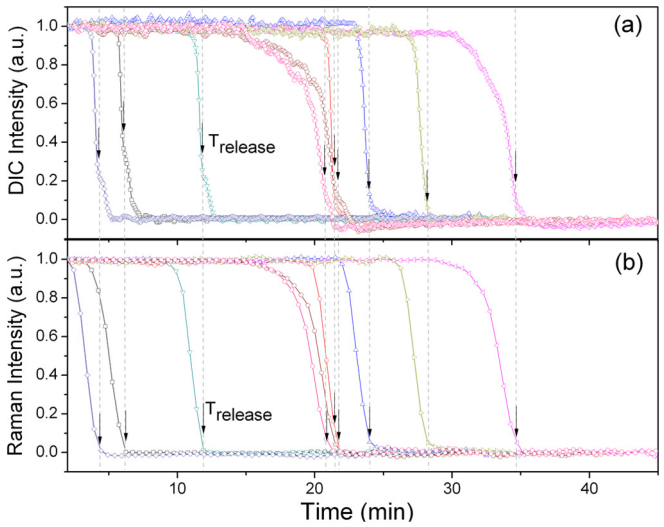

FIG 3 Germination of multiple individual optically trapped *C. difficile* spores as monitored simultaneously by DIC microscopy and Raman spectroscopy. *C. difficile* ATCC 43593 spores were germinated with 1.2% taurocholate and 15.5 mM glycine, and spore germination was monitored, all as described in Materials and Methods. Germination curves were determined from the loss in spore DIC image intensity (a) or the intensities of the CaDPA-specific Raman bands at 1,017 cm^{-1} (b). The DIC image and Raman band intensities in arbitrary units (a.u.) were normalized to 1 based on the respective values at the first time of measurement, and DIC image intensities at the end of the experiment were set at 0. $T_{release}$ (arrow) is indicated for each individual spore.

The germination of spores of the *C. difficile* wild-type strain JIR8094 and its isogenic *sleC* derivative were also examined to assess the effects of SleC on the overall germination process of *C. difficile* spores (see Fig. S4 in the supplemental material). Strikingly, while ~90% of the wild-type spores germinated in ~20 min with taurocholate plus glycine, at most 1% of *sleC* spores germinated in this time and even with higher germinant concentrations; even after 600 min, only ~5% of the *sleC* spores had germinated (see Fig. S4a and b). It was also striking that the individual *sleC* spores that germinated did not show any cortex hydrolysis period (ΔT_{lysis}) in the curves of DIC image intensity and displayed much higher absolute DIC image intensity at the end of the experiment than the wild-type spores (see Fig. S4c and d). This suggests that *sleC* spores do not undergo any cortex hydrolysis process, similar to *C. perfringens sleC* mutant spores (41). It was also notable that the $\Delta T_{release}$ period for CaDPA in the wild-type spores that germinated was ~2.5 min, while for the *sleC* spores that germinated this value was much larger at ~40 min. The accelerated CaDPA $\Delta T_{release}$ times by cortex hydrolysis have also been seen for germinating *B. subtilis* and *Bacillus megaterium* spores (42, 43).

Effect of heat shock on *C. difficile* spore germination. A short treatment at a high but generally sublethal temperature termed a heat shock can generally potentiate and synchronize germination of spores of *Bacillus* and at least some *Clostridium* species (44). Although the mechanism of this effect is unknown, it is generally seen only for GR-dependent spore germination (45). Examination of the effects of such a heat shock on *C. difficile* spores showed

TABLE 1 Kinetic parameters of *C. difficile* spores with or without heat shock and germinating with taurocholate and glycine[a]

C. difficile strain	Heat shock	T_1 (min)	CaDPA leakage (%)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lysis} (min)	% germination in 60 min (no. of germinated spores)
ATCC 43593	No	3.8 ± 2.1	15.5 ± 7.6	5.5 ± 2.7	6.9 ± 2.9	1.4 ± 0.4	1.1 ± 1.2	92.5 (271)
	Yes	3.4 ± 2.1	15.5 ± 9.2	5.7 ± 2.7	7.2 ± 2.9	1.5 ± 0.5	1.6 ± 1.3	77.3 (250)
ATCC 43601	No	3.3 ± 3.0	13.4 ± 8.3	5.0 ± 3.6	6.5 ± 3.8	1.7 ± 1.6	1.0 ± 1.2	89.5 (282)
	Yes	11.9 ± 12.2	13.1 ± 6.2	13.9 ± 12.6	15.5 ± 12.5	1.6 ± 0.7	1.2 ± 1.0	45.1 (100)

[a] Spores were germinated as described in Materials and Methods, and germination parameters were determined, all as indicated in the legend to Fig. 1 and in Materials and Methods. Values are means ± standard deviations.

that heat shock actually decreased the extent of spore germination with taurocholate plus glycine (Fig. 4; Table 1). In addition, while the kinetic parameters of ATCC 43593 spore germination were similar for spores with and without heat shock, the heat-shocked ATCC 43601 spores had much larger values of T_1, T_{lag}, and $T_{release}$ (Fig. 4 and 5; Table 1). However, $\Delta T_{release}$ times (corresponding to rapid CaDPA release) of ATCC 43601 spores were relatively unaffected by heat shock, as were the amounts of CaDPA leakage between T_1 and T_{lag} (Table 1). The lack of stimulation of *C. difficile* spore germination by a heat shock is consistent with several previous reports examining germination of spore populations (15, 46), and together these results suggest that heat activation is not important in *C. difficile* spore germination, at least for the spores of strains that have been studied to date.

Effects of taurocholate and glycine concentrations and various bile salts on *C. difficile* spore germination. Previous work found that taurocholate and glycine act as cogerminants for *C. difficile* spore germination (26). Initially, we examined the germination of multiple individual spores of the two *C. difficile* strains with different concentrations of taurocholate and glycine (Fig. 6). In the presence of 0.25% taurocholate, the extent of germination of the *C. difficile* spores increased with increasing glycine concentrations up to 15.5 and 19.6 mM glycine for ATCC strains 43593 and 43601, respectively (Fig. 6a and c). When the glycine concentration was held constant at 15.5 and 19.6 mM glycine for ATCC 43593 and 43601, respectively, the extent of spore germination increased until the taurocholate concentration reached 0.25% (Fig. 6b and d). In the presence of 15.5 mM glycine, T_1, T_{lag}, and $T_{release}$ times and the CaDPA leakage between T_1 and T_{lag} of ATCC 43593 spore germination were all reduced when taurocholate concentrations were increased from 0.05% to 0.25% although $\Delta T_{release}$ and ΔT_{lysis} (corresponding to presumed cortex hydrolysis) times were relatively unchanged (Table 2; also data not shown). In the presence of 0.25% taurocholate, T_1, T_{lag}, and $T_{release}$ times of ATCC 43593 spore germination decreased markedly when glycine concentrations increased from 9.1 to 15.5 mM although $\Delta T_{release}$ and ΔT_{lysis} times and the amounts of CaDPA leakage between T_1 and T_{lag} were relatively unchanged (Table 3). Notably, low concentrations of glycine (1.3 mM) were insufficient to stimulate germination in the presence of 0.1% taurocholate for both *C. difficile* strains even after incubation for 120 min (data not shown).

In addition to effects of concentrations of bile salts and glycine on spore germination rates, different bile salts also have various effects on *C. difficile* spore germination (26, 28). Analysis of the effects of different bile salts on *C. difficile* spore germination showed that, among the bile salts tested, only taurodeoxycholate resulted in germination at an extent similar to that of taurocholate

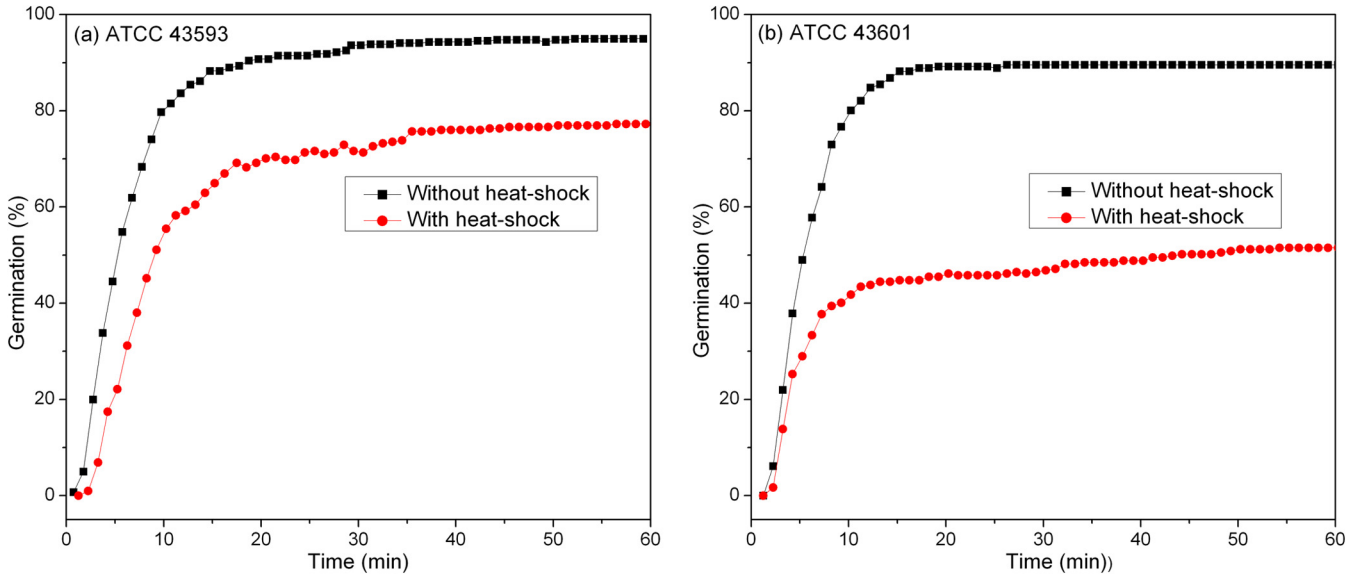

FIG 4 Effect of heat shock on germination of spores of two *C. difficile* strains. ATCC 43593 and ATCC 43601 spores (>200 each) with or without heat shock were germinated at 37°C with 0.25% taurocholate and either 15.5 mM glycine (a) or 19.6 mM glycine (b), and spore germination percentages were determined by DIC microscopy, all as described in Materials and Methods.

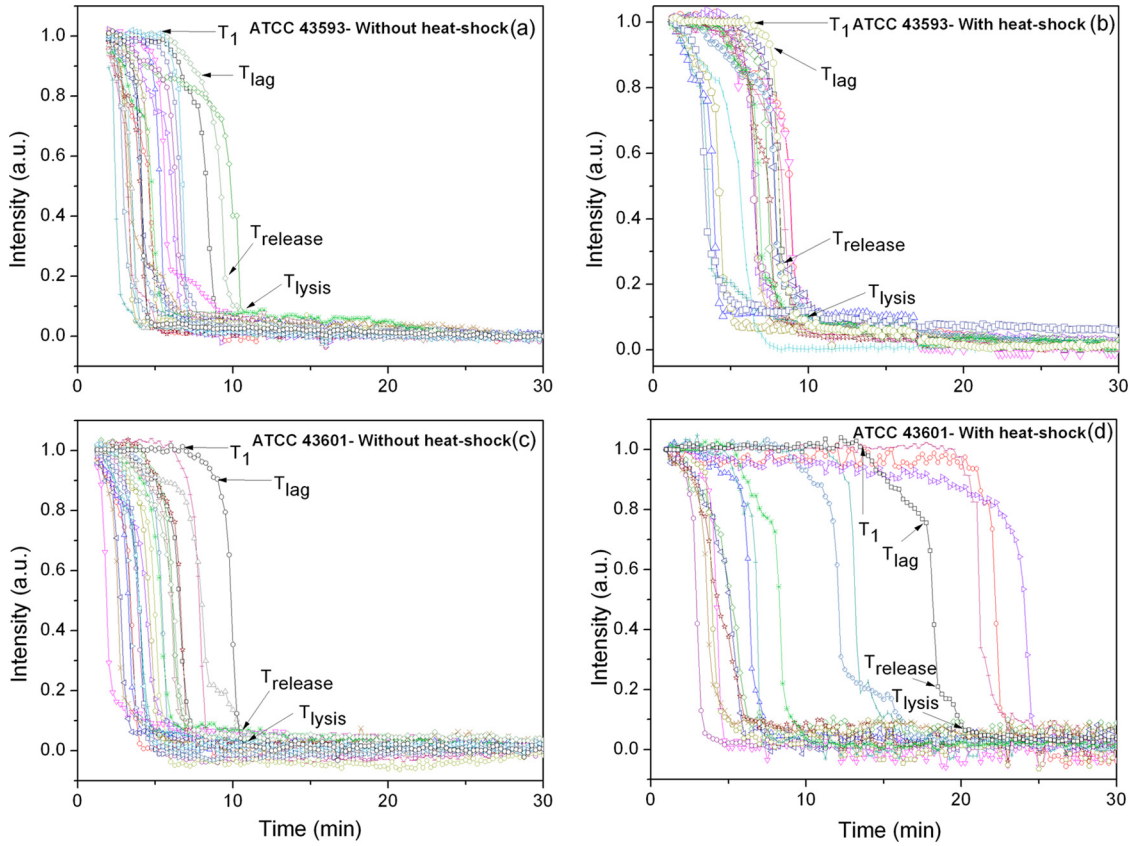

FIG 5 Germination curves of multiple individual *C. difficile* spores with or without a prior heat shock. ATCC 43593 and ATCC 43601 spores were germinated with 0.25% taurocholate and either 15.5 mM (a and b) or 19.6 mM (c and d) glycine for 30 min, and DIC image intensities of multiple individual spores were determined, all as described in Materials and Methods. The image intensities (a.u.) were normalized to 1 based on the respective values at the first time of measurement, and image intensities at the end of the experiment were set at 0. T_1, T_{lag}, $T_{release}$, and T_{lysis} are indicated for a single spore.

(Fig. 7). This finding contrasted with the prior observation that taurodeoxycholate is ~70% less effective at activating *C. difficile* strain 630 spores (27) and may reflect strain-specific differences, as has been observed by previous groups (28). Deoxycholate, cholate, or glycocholate partially activated germination although the magnitude of this germination differed somewhat between the spores of the two *C. difficile* strains and was always significantly less than that with taurocholate (Fig. 7; Tables 4 and 5). The major differences in the kinetic parameters of the germination of the *C. difficile* spores with the different bile salts were in the T_1, T_{lag}, and $T_{release}$ times and the amounts of CaDPA leakage between T_1 and T_{lag}, while $\Delta T_{release}$ and ΔT_{lysis} times were essentially identical for the five bile salts that induced at least some spore germination (Tables 4 and 5 and data not shown). As found in previous work, chenodeoxycholate (47) and taurolithocholate (27) did not trigger germination of *C. difficile* spores (Fig. 7). Consistent with the prior observation indicating that chenodeoxycholate is a competitive inhibitor of taurocholate for *C. difficile* UK1 spores, 0.25% chenodeoxycholate significantly inhibited germination stimulated by treatment with 0.25% taurocholate plus glycine of spores of both the *C. difficile* ATCC 43593 and 43601 strains. In contrast, 0.25% taurolithocholate failed to inhibit germination of spores of both strains under the same conditions (compare Fig. S1 in the supplemental material with Fig. 4), consistent with prior observations with *C. difficile* 630 spores (27).

***C. difficile* spore germination with CaDPA and dodecylamine.** In addition to GR-dependent germinants, spores of *Bacillus* and some *Clostridium* species can also germinate with the GR-independent germinants CaDPA and dodecylamine (18–20, 48, 49). To test whether *C. difficile* spores were similarly responsive to these nonnutritional GR-independent germinants, the germination of *C. difficile* spores in the presence of CaDPA or dodecylamine was also tested. This analysis showed that spores of both *C. difficile* strains exhibited no (<0.3% with 300 spores examined) germination with CaDPA in 60 min, under conditions where *B. subtilis* spores routinely germinated >90% (data not shown). In contrast, intact spores of both *C. difficile* strains germinated with dodecylamine. ATCC 43593 spores had much lower average T_1 times than ATCC 43601 spores (Fig. 8; Table 6). Notably, dodecylamine germination failed to decrease DIC image intensity after $T_{release}$ in either intact or decoated spores (Fig. 8c to f; see also Fig. S2 in the supplemental material), suggesting that cortex lysis either does not take place or takes place in parallel with CaDPA release during dodecylamine germination. However, the fact that the absolute DIC image intensity fell much less during dodecylamine germination than during germination with taurocholate plus glycine (see Fig. S3a and b in the supplemental material) strongly suggests that there is minimal cortex lysis during dodecylamine germination. There was also more CaDPA leakage between T_1 and T_{lag} during dodecylamine germination than during

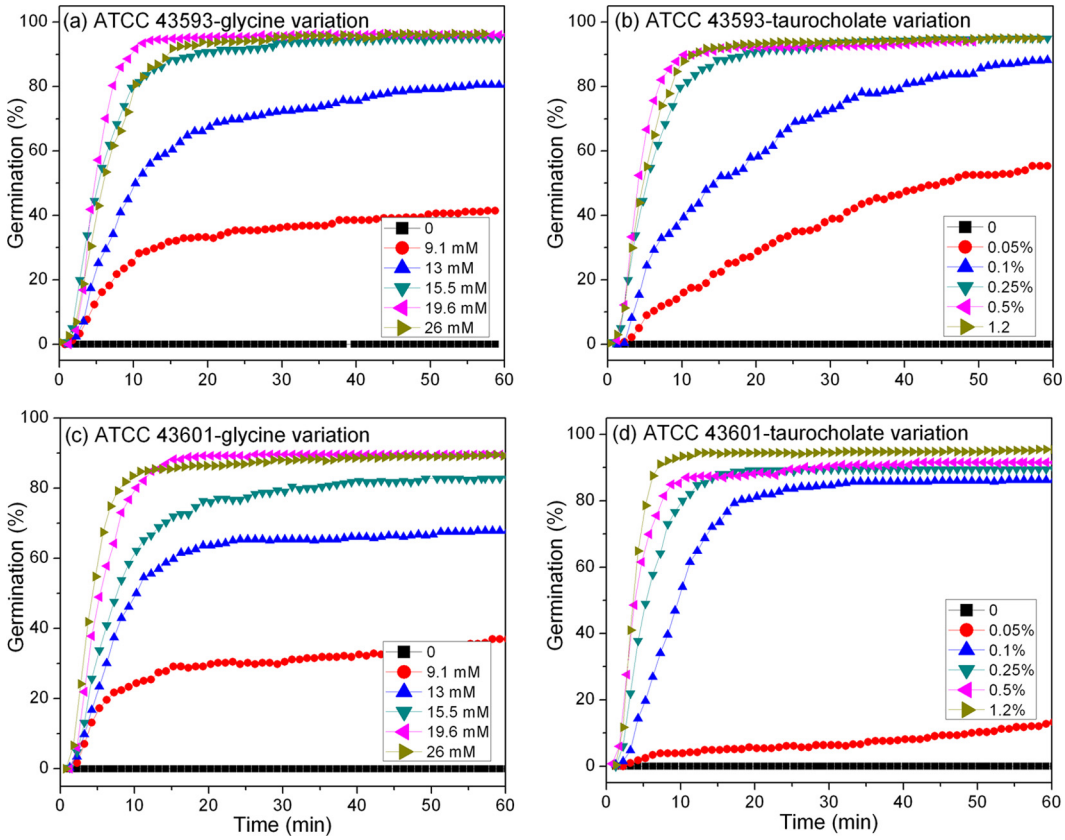

FIG 6 Effects of glycine and taurocholate concentrations on *C. difficile* spore germination. More than 200 individual ATCC 43593 or ATCC 43601 *C. difficile* spores were germinated at with 0.25% taurocholate and various glycine concentrations (a and c) or with 15.5 mM (b) or 19.6 mM (d) glycine and various taurocholate concentrations. Spore germination was monitored by DIC microscopy, all as described in Materials and Methods.

germination by taurocholate plus glycine (Tables 1 and 6). Notably, the absolute DIC image intensity of *C. difficile* spores germinating with taurocholate plus glycine and chenodeoxycholate fell by an amount similar to that of spores germinating with taurocholate plus glycine (see Fig. S3a and c), indicating that chenodeoxycholate inhibits not only cortex hydrolysis but also CaDPA release. This observation is consistent with the observation that a *cspC* mutant fails to release DPA (15) as well as activate SleC cleavage (data not shown).

The reasons for differences in rates of dodecylamine germination of spores of different strains are not known. Since chemical decoating can greatly increase rates of *Bacillus* spore germination with dodecylamine (48), we tested the effects of chemical decoating on *C. difficile* spore germination. While decoating had no major effect on the overall rate of the dodecylamine germination of the ATCC 43593 spore population (Fig. 8a), chemical decoating greatly increased ATCC 43601 spore germination with dodecylamine (Fig. 8b). In addition, decoating significantly decreased the average times between T_1 and T_{lag} for the dodecylamine germination of spores of both *C. difficile* strains (Table 6). As seen with intact spores germinating with dodecylamine, there was also no obvious cortex lysis period during dodecylamine germination of decoated *C. difficile* spores (Fig. 8e and f), consistent with the observation that decoating removes *C. difficile* spores' only cortex-lytic enzyme, SleC (data not shown). Indeed, as expected, the decoated *C. difficile* spores exhibited <0.3% germination with 0.5% taurocholate or taurodeoxycholate plus 19.6 mM glycine when 300 spores were examined for 60 min (data not shown).

TABLE 2 Kinetic parameters of *C. difficile* spores germinating with constant glycine and variable taurocholate concentrations[a]

Taurocholate concn (%)	T_1 (min)	CaDPA leakage (%)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lysis} (min)	% germination in 60 min (no. of germinated spores)
0.05	15.7 ± 13.4	21.4 ± 11.3	21.2 ± 14.8	23.3 ± 14.7	1.6 ± 0.7	2.2 ± 2.7	55.4 (168)
0.1	11.3 ± 13.3	19.6 ± 8.1	14.8 ± 13.5	16.7 ± 13.4	1.8 ± 0.5	1.5 ± 1.7	88.3 (283)
0.25	3.8 ± 2.1	15.5 ± 9.2	5.5 ± 2.7	6.9 ± 2.9	1.4 ± 0.4	1.1 ± 1.2	92.5 (271)
0.5	2.1 ± 1.2	14.3 ± 7.1	3.3 ± 1.8	5.0 ± 1.9	1.6 ± 0.4	1.1 ± 1.2	93.9 (252)
1.2	2.6 ± 1.4	14.9 ± 9.3	4.6 ± 2.2	6.4 ± 2.4	1.7 ± 0.4	1.2 ± 1.1	95.0 (440)

[a] ATCC 43593 spores were germinated as described in Materials and Methods with 15.5 mM glycine and various taurocholate concentrations. The germination of >250 individual spores was followed by DIC microscopy, and kinetic parameters of spore germination were calculated as indicated in the legend to Fig. 1 and in Materials and Methods. Values are means ± standard deviations.

TABLE 3 Kinetic parameters of *C. difficile* spore germination with constant taurocholate and various glycine concentrations[a]

Glycine concn (mM)	T_1 (min)	CaDPA leakage (%)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lysis} (min)	% germination in 60 min (no. of germinated spores)
9.1	9.5 ± 12.1	15.4 ± 11.0	11.7 ± 12.6	13.6 ± 12.6	1.8 ± 0.4	1.0 ± 1.3	41.5 (163)
13.0	9.2 ± 11.5	13.5 ± 8.0	10.9 ± 11.8	13.1 ± 11.9	1.8 ± 0.5	0.9 ± 1.4	81.9 (266)
15.5	3.8 ± 2.1	15.5 ± 9.2	5.5 ± 2.7	6.9 ± 2.9	1.4 ± 0.4	1.1 ± 1.2	92.5 (271)
19.6	2.1 ± 1.2	15.2 ± 7.2	3.8 ± 1.7	6.2 ± 1.9	2.0 ± 0.6	1.6 ± 1.7	96.3 (354)
26.0	3.6 ± 2.5	14.8 ± 6.5	5.7 ± 3.1	7.7 ± 3.2	1.8 ± 0.5	0.9 ± 1.1	96.3 (333)

[a] ATCC 43593 spores were germinated as described in Materials and Methods with 0.25% taurocholate and various glycine concentrations. The germination of >250 individual spores was followed by DIC microscopy, and kinetic parameters of spore germination were calculated as indicated in the legend to Fig. 1 and in Materials and Methods. Values are means ± standard deviations.

Measurement of commitment of *C. difficile* spores to germinate. A notable feature of *B. subtilis* spores is their irreversible commitment to germination, followed by some slow CaDPA leakage after a short exposure to germinant, with commitment taking place at approximately T_1 (22, 24, 50). Since *C. difficile* spores also showed a T_1 time followed by some slow CaDPA leakage, we examined if *C. difficile* spores also showed commitment after exposure to taurocholate and glycine for various periods, followed by germinant removal, extensive rinsing, and further incubation in buffer (Fig. 9). Like *Bacillus* spores, *C. difficile* spores given a short germinant exposure continued to release CaDPA after germinant removal. Indeed, spores given a 2-min exposure to germinants that gave only ~10% germination at the time of germinant removal ultimately reached ~76% germination (Fig. 9). The commitment phenomenon in germination with taurocholate plus glycine was not eliminated if 0.25% chenodeoxycholate was added after committed spores were rinsed and prior to subsequent incubation (data not shown). Taken together, these results suggest that *C. difficile* spores, similar to *B. subtilis* spores, also commit to germination shortly after exposure to germinant.

DISCUSSION

Analysis of the germination of individual *C. difficile* spores indicated that both the kinetics and the various stages of this process were essentially the same as with individual spores of a number of *Bacillus* species, *Geobacillus stearothermophilus*, and *Clostridium perfringens* (Fig. 1 and 2) (24, 41, 51). This result was somewhat surprising because the signal transduction pathway in germination of at least *Bacillus* spores is quite different from that in *C. difficile* spore germination (17, 18, 52). In *Bacillus* sp. spore germination, binding of nutrient germinants to their germinant receptors triggers CaDPA release, and this in turn triggers cortex lysis. However, in *C. difficile* spores, binding of bile salt germinants to CspC triggers cortex lysis and CaDPA release (15), with the proviso that the precise mechanism of action of the cogerminant glycine is not known.

As a result, while the overall kinetics of *C. difficile* spore germination resembled that of *B. subtilis*, the order of CaDPA release appeared to differ between these two organisms. During *C. difficile* spore germination, CaDPA release appears to precede cortex lysis, or at least the great majority of cortex lysis (Fig. 8; see also Fig. S3 in the supplemental material). How can CaDPA release precede cortex hydrolysis during *C. difficile* spore germination? One simple explanation is that only a small amount of cortex hydrolysis is needed to trigger CaDPA release, and this cortex hydrolysis is not reflected in changes in the DIC image intensity of the spores, perhaps because there are no such changes until later. Indeed, while GR-dependent CaDPA release can take place in *Bacillus* spore germination even in the absence of both redundant CLEs, values of

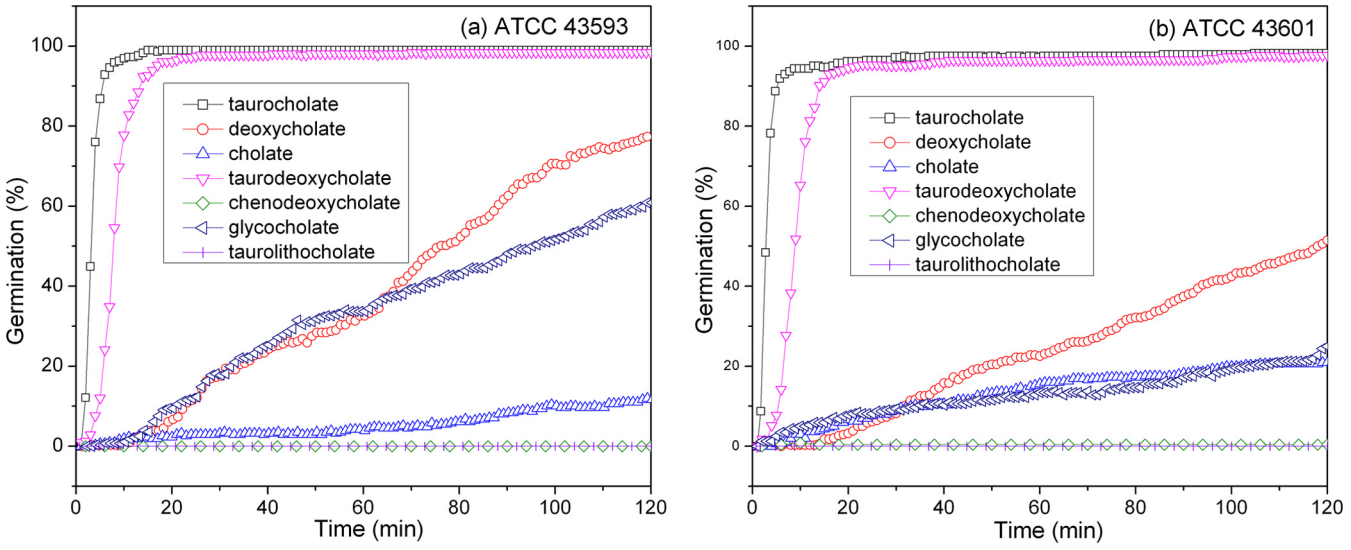

FIG 7 Germination of *C. difficile* spores with different bile salts. Spores of *C. difficile* strains ATCC 43593 and ATCC 43601 were germinated with different bile salts at 0.5% plus 19.6 mM glycine, and germination of >200 spores was monitored by DIC image intensity changes, all as described in Materials and Methods.

TABLE 4 Kinetic parameters of *C. difficile* ATCC 43593 spore germination with different bile salts[a]

Bile salt	T_1 (min)	CaDPA leakage (%)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lysis} (min)	% germination in 120 min (no. of germinated spores)
Taurocholate	1.0 ± 0.5	17.4 ± 8.1	2.5 ± 1.1	3.7 ± 1.2	1.2 ± 0.3	1.0 ± 0.9	99.0 (293)
Taurodeoxycholate	5.0 ± 2.1	11.7 ± 7.7	7.0 ± 3.2	9.0 ± 3.3	2.0 ± 0.6	2.0 ± 1.8	98.4 (310)
Glycocholate	50.6 ± 33.6	12.0 ± 7.4	52.8 ± 33.4	54.6 ± 33.4	1.8 ± 0.6	2.1 ± 1.8	60.9 (168)
Deoxycholate	60.0 ± 29.7	8.8 ± 10.0	61.8 ± 29.3	63.8 ± 29.4	1.9 ± 0.6	1.5 ± 1.4	77.3 (242)
Cholate	67.4 ± 38.4	15.4 ± 7.6	70.3 ± 39.1	72.2 ± 38.9	1.9 ± 1.0	2.2 ± 2.5	11.7 (36)

[a] ATCC 43593 spores were germinated as described in Materials and Methods with 0.5% different bile salts plus 19.6 mM glycine. The germination of >276 individual spores was followed by DIC microscopy, and kinetic parameters of spore germination were calculated as indicated in the legend to Fig. 1 and in Materials and Methods. Values are means ± standard deviations.

$\Delta T_{release}$ are increased 5- to 10-fold simply by the loss of one specific CLE, CwlJ (42, 43), and this was also seen in this work with *sleC* mutant *C. difficile* spores. Thus, it seems likely that there is some cortex hydrolysis during taurocholate-induced *C. difficile* spore germination, beginning perhaps at or even before T_1, that causes changes in spore IM permeability or leakiness of the CaDPA channel, and then CaDPA leakage begins. This CaDPA leakage, combined with further cortex hydrolysis, could trigger faster CaDPA release. Notably, the same kinetics of GR-dependent germination are also seen in the germination of *C. perfringens* spores, in which GR-dependent germinants can trigger some CaDPA release even in the absence of SleC although loss of both SleC and the auxiliary CLE SleM greatly reduced CaDPA release in GR-dependent germination (31).

It was also notable that while germination of *C. difficile* spores was slowed tremendously in the absence of SleC, a small percentage of the *sleC* mutant spores released CaDPA but did not exhibit cortex hydrolysis. The latter observation is consistent with the low viability of *sleC* mutant *C. difficile* spores and the restoration of significant viability by inclusion of lysozyme in plating medium (30). An important question then concerns the mechanism of the germination of the small percentage of *sleC* mutant spores that germinated in 10 h, as measured by their release of CaDPA, as this is perhaps analogous in some ways to the spontaneous germination of small percentages of *B. subtilis* spores that lack all GRs (53).

Another surprising result was that there was no evidence of cortex hydrolysis during dodecylamine germination of *C. difficile* spores, in contrast to clear cortex hydrolysis during dodecylamine germination of *Bacillus* spores (42, 48). Notably, there also does not appear to be any cortex hydrolysis during *C. perfringens* spore germination with dodecylamine (41). In *Bacillus* spore germination with dodecylamine, this agent is thought to bind to and activate the spore's IM channel for CaDPA composed of SpoVA proteins (17, 18). Many of these SpoVA proteins are highly conserved in spore-forming members of *Bacillales* and *Clostridiales* including *C. difficile* (16). Dodecylamine may bind specifically to the conserved SpoVAC protein, which was recently shown to have mechanosensitive properties (54). As noted above, in *Bacillus* spores CaDPA release activates CLEs, at least CLE probably directly, such that cortex hydrolysis is triggered in the process of dodecylamine germination. However, during germination of *Clostridiales* spores, neither CaDPA release nor its absence from spores appears to trigger CLE action (16) (Fig. 8; see also Fig. S3 in the supplemental material). Whereas DPA-less *B. subtilis* spores that lack the SpoVA proteins essential for CaDPA uptake in sporulation spontaneously germinate very rapidly during sporulation (55), DPA-less *spoVA C. perfringens* spores are completely stable (56). Presumably this will also be the case for *spoVA C. difficile* spores. Consequently, *C. difficile* spores triggered directly to release CaDPA may have no way to efficiently activate the hydrolysis of their cortex peptidoglycan and thus cannot activate events downstream of CaDPA release. In further support of this simple model for *Clostridium* spore germination, the rapid GR-dependent CaDPA release from *C. perfringens* spores as measured by $\Delta T_{release}$ values is not altered notably by loss of SleC (41), in contrast to the large increases in $\Delta T_{release}$ values due to loss of the CLE CwlJ (42, 43).

The sensitive Raman spectroscopy and DIC microscopy techniques used in this study allowed us not only to measure DPA release and cortex hydrolysis in real time but also to analyze for the first time the responsiveness of individual *C. difficile* spores to different germinants. Until now, germinant responsiveness of *C. difficile* spores was generally restricted to population-wide measurements using OD_{600} or bulk DPA release assays. Using assays of individual spores, we observed that spores of different *C. difficile* strains exhibited marked differences in their responsiveness to heat treatment (Fig. 4; Table 1) and bile salts plus glycine (Fig. 6 and 7; Tables 4 and 5), similar to results with spores of other *C. difficile* strains (28). The most notable difference in the germination of the spores of the two strains examined in this work was in

TABLE 5 Kinetic parameters of *C. difficile* ATCC 43601 spore germination with different bile salts

Bile salt	T_1 (min)	CaDPA leakage (%)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lysis} (min)	% germination in 120 min (no. of germinated spores)
Taurocholate	3.4 ± 2.1	14.1 ± 6.3	5.0 ± 2.8	6.5 ± 3.0	1.4 ± 0.4	0.8 ± 0.9	91.6 (262)
Taurodeoxycholate	5.1 ± 3.9	10.2 ± 7.7	7.3 ± 3.9	9.6 ± 4.1	2.3 ± 0.9	1.5 ± 2.2	97.7 (377)
Glycocholate	72.1 ± 36.1	14.8 ± 13.3	73.8 ± 35.5	75.5 ± 35.4	1.7 ± 0.6	1.3 ± 1.6	25.0 (80)
Deoxycholate	64.3 ± 32.5	7.0 ± 11.3	66.2 ± 32.4	68.3 ± 32.3	2.1 ± 0.9	0.7 ± 0.9	51.5 (176)
Cholate	41.4 ± 32.1	11.2 ± 9.7	43.0 ± 32.1	44.6 ± 32.2	1.7 ± 0.5	0.9 ± 1.5	20.9 (63)

[a] ATCC 43601 spores were germinated as described in Materials and Methods with 0.5% different bile salts plus 19.6 mM glycine. The germination of >286 individual spores was followed by DIC microscopy, and kinetic parameters of spore germination were calculated as indicated in the legend to Fig. 1 and in Materials and Methods. Values are means ± standard deviations.

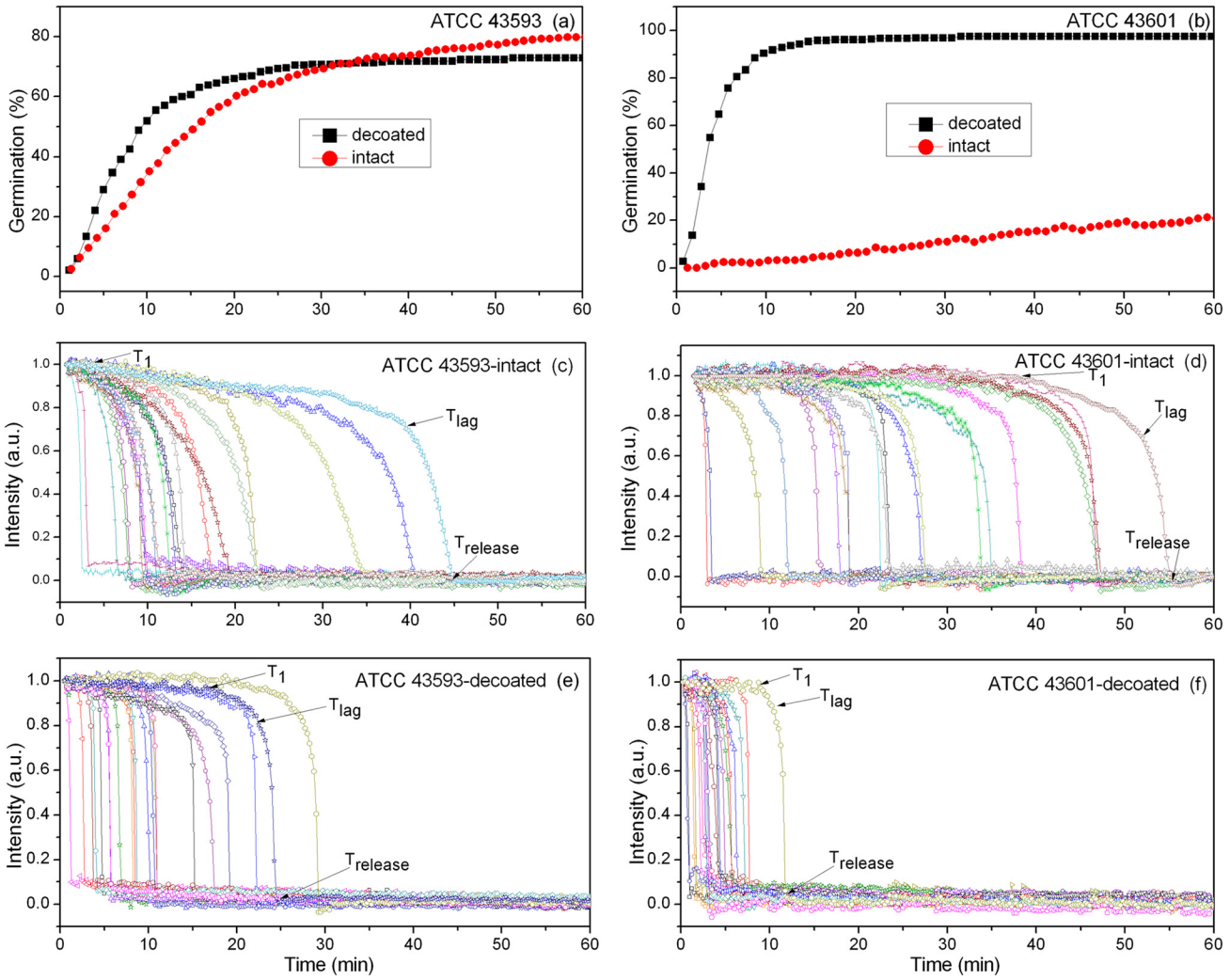

FIG 8 Germination of *C. difficile* spores with dodecylamine. Spores of *C. difficile* strains (ATCC 43593 and ATCC 43601), either intact or decoated, were germinated with dodecylamine as described in Materials and Methods. (a and b) Germination of >200 intact and decoated spores as a function of time. (c to f) DIC intensities of 20 individual intact spores as a function of time were measured as described in Materials and Methods. In panels c to f, T_1, T_{lag}, and $T_{release}$ for one spore are indicated.

their germination with dodecylamine as ATCC 43601 spores were much slower to respond to this germinant than ATCC 43593 spores (Fig. 8; Table 6). Since this difference was not seen with decoated spores, this suggests that precise structures of spores' outer layers, the coats and/or outer membranes, are major factors in spores' rates of germination with dodecylamine. Differences in the permeability of the outer layers of *C. difficile* spores between strains may lead to differential responsiveness to bile salt germi-

TABLE 6 Kinetic parameters of intact or decoated *C. difficile* spores germinating with 1.2 mM dodecylamine[a]

Strain and spore coat status	T_1 (min)	CaDPA leakage (%)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	$I_{release}$[b]	% germination in 60 min (no. of germinated spores)
ATCC 43593							
Intact	4.8 ± 5.3	26.5 ± 11.0	14.3 ± 10.2	17.0 ± 10.8	3.0 ± 0.9	0.07 ± 0.08	81.6 (425)
Decoated	5.5 ± 5.0	7.5 ± 6.4	7.9 ± 6.5	9.0 ± 6.7	1.2 ± 0.5	0.07 ± 0.06	73.0 (267)
ATCC 43601							
Intact	22.9 ± 14.6	18.3 ± 6.8	27.2 ± 16.0	28.9 ± 16.3	1.6 ± 0.6	0.01 ± 0.08	21.3 (102)
Decoated	2.6 ± 2.4	9.4 ± 4.8	3.7 ± 2.7	4.7 ± 2.8	1.0 ± 0.5	0.07 ± 0.07	97.5 (357)

[a] Intact or decoated spores of *C. difficile* strains were germinated with dodecylamine as described in Materials and Methods. The germination of >366 individual spores was followed by DIC microscopy, and kinetic parameters of spore germination were calculated as indicated in the legend to Fig. 1 and in Materials and Methods. Values are means ± standard deviations.

[b] $I_{release}$, DIC image intensity at $T_{release}$.

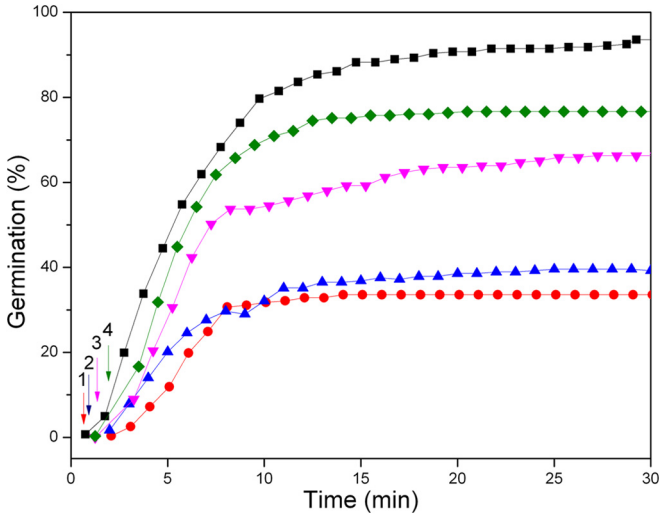

FIG 9 Commitment in *C. difficile* spore germination. ATCC 43593 spores were exposed to 0.25% taurocholate and 15.5 mM glycine in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl at 37°C for short periods (arrows denote ends of germinant exposures), followed by germinant removal, rinsing with vacuum suction, and further incubation, as described in Materials and Methods. The exposure periods were as follows: 1 to 0.83 min (red), 2 to 1 min (blue), 3 to 1.5 min (pink), and 4 to 2 min (green). ATCC 43593 spores were also germinated continuously (black) with 0.25% taurocholate and 15.5 mM glycine. In all cases, the germination of >200 spores was monitored by DIC microscopy as described in Materials and Methods.

nants (52, 57), especially since the differences in dodecylamine sensitivity of the ATCC 43593 and 43601 strains largely disappeared following decoating. However, the exact mechanism restricting dodecylamine access to the spore's inner membrane in intact spores is not yet clear. In spores of *Bacillus* species, the GerP proteins, a group of small proteins likely present in the spore coat, are thought to be crucial to ensure rapid access of some germinants to spores' IMs (58, 59). However, proteins analogous to GerP proteins have not been identified in *Clostridium* spores.

A second notable finding in this work was that *C. difficile* spores exhibited no detectable germination with exogenous CaDPA (data not shown). This is in contrast to spores of *Bacillus* species in which exogenous CaDPA or CaDPA released from the spore core activates the CLE CwlJ, perhaps directly. In contrast, CaDPA appears to activate a GR in *C. perfringens* spores (49). Given this finding, the absence of IM GRs in *C. difficile* spores (16) would then be consistent with these spores' lack of germination with CaDPA. This would further suggest that CaDPA plays no direct role in the signaling pathways in *C. difficile* spore germination, as suggested above based on the results with dodecylamine germination.

The final notable observation in this work is that *C. difficile* spores showed the phenomenon of commitment in spore germination, whereby brief treatment with bile salts plus glycine resulted in germination events much later than and well after germinant removal. Prior work also found that a short exposure to taurocholate or deoxycholate enhanced colony formation by *C. difficile* spores, and it was suggested that this indicated that *C. difficile* spores also exhibit commitment to germinate (26). *Bacillus* spores also exhibit commitment in GR-dependent germination (18, 22, 24, 32, 50) although the mechanism of this commitment is not known. However, in *Bacillus* spores, commitment appears to parallel a large change in the permeability of either the spore IM or the CaDPA channel, such that CaDPA leakage begins at about the time of commitment. Since *C. difficile* spores also exhibited slow CaDPA leakage early in germination, it is tempting to speculate that this is also associated with commitment in *C. difficile* spore germination. The mechanism of establishing commitment is, however, unclear, although one obvious possible mechanism is that commitment reflects the activation of significant CspB protease activity by bile salts plus glycine. In this model, activated CspB protease persists after germinant removal and catalyzes pro-SleC activation, which leads to cortex hydrolysis followed by slow leakage of CaDPA release that transitions to fast CaDPA release. Since the precise signal transduction pathway in *C. difficile* spore germination is not yet clear, testing this model will yield important insights into the mechanisms regulating *C. difficile* spore germination.

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