



ORIGINAL ARTICLE

Characterization of a *Clostridioides difficile* ST-293 isolate from a recurrent infection in Argentina



P. Cecilia Soldavini Pelichotti^{a,b}, Daniela Cejas^{c,d}, Liliana Fernández-Caniggia^e, Fernando M. Trejo^a, Pablo F. Pérez^{a,b,*}

^a Cátedra de Microbiología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 115, La Plata, Argentina

^b Centro de Investigación y Desarrollo en Criotecnología de Alimentos, CCT La Plata, CONICET-UNLP, 47 y 116 (s/n), La Plata B1900AJI, Argentina

^c Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Instituto de Investigaciones en Bacteriología y Virología Molecular (IBaViM), Ciudad Autónoma de Buenos Aires, Argentina

^d Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

^e Laboratorio de Microbiología, Hospital Alemán, Av. Pueyrredón 1640, Ciudad Autónoma de Buenos Aires, Argentina

Received 8 April 2022; accepted 27 September 2022

Available online 2 January 2023

KEYWORDS

Clostridioides difficile;
Pathogenesis;
Sporulation;
Germination;
Recurrence;
MLST

Abstract *Clostridioides difficile* is an opportunistic spore-forming pathogen responsible for antibiotic-associated diarrhea in humans. *C. difficile* produces two main toxins: TcdA and TcdB as well as a third toxin named binary toxin (CDT) that is also involved in virulence. The present study aimed at characterizing the *C. difficile* isolate ALCD3 involved in a relapse episode of nosocomial infection. Molecular characterization showed that isolate ALCD3 belongs to toxinotype 0/v and the MLST analysis demonstrated allelic profile *adk:91*, *atpA:1*, *dxa:2*, *glyA:1*, *recA:27*, *sodA:1* and *tpi:1* which corresponds to ST293 (MLST clade: 1). During growth, isolate ALCD3 showed an early increase in the sporulation ratio as well as maximal values of heat resistant forms after 2 days of incubation. Both sporulation kinetics and production of heat resistant forms were faster for isolate ALCD3 than for the reference strain VPI 10463. Germination in the presence of the natural germinant taurocholate was faster for isolate ALCD3 than for strain VPI 10463, which indicates that isolate ALCD3 starts cortex hydrolysis earlier than strain VPI 10463. Furthermore, the co-germinant glycine, induces rapid release of dipicolinic acid (DPA) in isolate ALCD3. These findings indicate that isolate ALCD3 is particularly efficient in both sporulation and germination. The present work represents the first report of the circulation of *C. difficile* ST293 in Argentina. The ability of isolate ALCD3 to produce toxins and its high sporulation/germination capacity are key features compatible with a microorganism with high dissemination potential and the possibility of inducing recurrent infections.

© 2022 Asociación Argentina de Microbiología. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author.

E-mail address: pfp@biol.unlp.edu.ar (P.F. Pérez).

PALABRAS CLAVE

*Clostridioides
difficile*;
Patogénesis;
Esporulación;
Germinación;
Recurrencia;
MLST

Caracterización de un aislamiento de *Clostridioides difficile* ST-293 de una infección recurrente en Argentina

Resumen *Clostridioides difficile* es un patógeno esporulado oportunista responsable de diarrea asociada a antibióticos en humanos. *C. difficile* produce 2 toxinas principales: TcdA y TcdB, además de la toxina binaria (CDT), también asociada a la virulencia. Este estudio buscó caracterizar el aislamiento ALCD3, involucrado en un episodio de recurrencia de una infección nosocomial. La caracterización molecular mostró que dicho aislamiento pertenece al toxinotipo 0/v y el análisis por MLST demostró un perfil alélico *adk:91, atpA:1, dxr:2, glyA: 1, recA:27, sodA: 1* y *tpi:1*, lo cual corresponde al ST293 (MLST clado 1). Durante el crecimiento, el aislamiento ALCD3 mostró un incremento temprano de la tasa de esporulación y valores máximos de formas termorresistentes luego de 2 días de incubación. Tanto la cinética de esporulación como la producción de formas termorresistentes fueron más rápidas en el aislamiento ALCD3 que en la cepa de referencia VPI 10463. La germinación en presencia del germinante natural taurocolato fue más rápida en el aislamiento ALCD3 que en la cepa VPI 10463, lo que indica que aquel comienza la hidrólisis del córtex antes. También, el co-germinante glicina indujo una rápida liberación de ácido dipicolínico en ALCD3. Estos hallazgos indican que el aislamiento ALCD3 es particularmente eficiente en la esporulación y en la germinación. El presente trabajo representa el primer informe de la circulación de *C. difficile* ST293 en Argentina. La habilidad del aislamiento ALCD3 para producir toxinas y su alta capacidad de esporulación/germinación son características claves compatibles con un alto potencial de diseminación e inducción de infecciones recurrentes.

© 2022 Asociación Argentina de Microbiología. Publicado por Elsevier España, S.L.U. Este es un artículo Open Access bajo la licencia CC BY-NC-ND (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Clostridioides difficile is a spore-forming strict anaerobe that colonizes the intestinal tract of approximately 5% of adults and 15–70% of infants. These percentages could be higher in hospitalized patients and nursing home residents³⁵. This pathogen is the main etiological agent responsible for antibiotic-associated diarrhea²⁶, and was believed to be almost exclusively associated with nosocomial infections. However, there is increasing evidence of a shift in the epidemiology of this pathogen with the occurrence of young healthy carriers without a history of antibiotic use^{15,28} and the presence of alternative ways of spore transmission such as food, grass, compost, manure, animals, and other environmental sources^{9,14}. Clinical manifestations of *C. difficile* infection (CDI) include asymptomatic, mild, and self-limiting disease to severe, life-threatening pseudomembranous colitis leading to toxic megacolon, sepsis, and death^{3,20,39}. Virulence of *C. difficile* is mainly related to the production of two large protein toxins, i.e., toxin A (TcdA), toxin B (TcdB). These toxins glycosylate proteins of the Rho GTPase family thus leading to the disruption of the actin cytoskeleton, cell death and a strong inflammatory response²². A third toxin, the binary toxin (CDT), can be produced by *C. difficile* and is composed of two separate components: CDTa and CDTb⁴⁵. While CDTa presents ADP-ribosyltransferase activity that modifies actin, CDTb is responsible for the binding of the toxin complex to the host cell surface¹⁶. In a murine model, CDT expression along with TcdA and TcdB induce an exacerbated inflammatory response⁶. It has been suggested that the production of CDT is related to high fatality rates

in patients infected with CDT-producing strains compared to those infected with CDT-negative strains^{2,24,34}.

The infective cycle of *C. difficile* relies on the ability to sporulate/germinate. The spores are ingested and then they germinate giving rise to vegetative cells that in turn produce virulence factors in the host's intestine. Afterwards, sporulation favors the elimination of infective forms with feces and further spreading. Since the anaerobic nature of *C. difficile* makes it impossible for vegetative forms of bacteria to survive in aerobic environments²¹, the impaired ability to sporulate limits drastically its potential to persist in the host and further transmission⁸ and the correlation between increased sporulation ability and disease severity has been demonstrated⁵.

The ability of *C. difficile* spores to resist physical and chemical stress (e.g. heat, desiccation and disinfectants) lead to the possibility of survival in the environment thus favoring the transmission by the fecal–oral route^{8,31}. Spore germination allows for vegetative growth and toxin production²⁵. In this context, sporulation and germination are key events in the cell cycle of *C. difficile* and are crucial for virulence.

Antibiotic treatment favours *C. difficile* overgrowth through disruption of the intestinal microbiota and subsequent changes in bile salt metabolism thus leading to the increase of germinant concentrations (e.g. cholate, taurocholate) and further spore germination^{17,43}.

The present study aimed to characterize a *C. difficile* isolate involved in a relapse episode of nosocomial infection and to gain insight into its sporulation/germination ability.

Materials and methods

Bacterial strains and culture conditions

C. difficile ALCD3 was isolated from a recurrent episode of CDI. The patient, an 89-year-old woman received antibiotic therapy after a surgical resection of the right ureter due to a malignant tumor. In the post-surgery period, CDI was diagnosed by toxin detection in feces and treatment with vancomycin was prescribed (14 days, 4 doses of 125 mg per day). Ten days after recovery, she was re-admitted with symptoms compatible with CDI (confirmed by toxin detection in feces). She recovered after treatment with metronidazole–vancomycin and no further relapses were reported.

Isolation was done as follows: the fecal sample was treated with ethanol (1:1) for 30 min at room temperature. Then the material was homogenized with sterile phosphate buffered saline (PBS: 0.144 g/l KH₂PO₄, 9 g/l NaCl, 0.795 g/l Na₂HPO₄, pH 7.5). Afterwards, suspensions were streaked on Differential Clostridia Medium – (DCM) agar (Laboratorios Britania S.A., Argentina) supplemented with 0.1% w/v sodium taurocholate (Santa Cruz Biotechnology, Dallas, Texas, USA). Plates were incubated for 48 h at 37 °C in anaerobic conditions (AnaeroPak; Mitsubishi Gas Chemical Co, Inc.). Colonies were selected based on morphology and Gram staining and genetically characterized as indicated below.

The isolate and reference strain VPI 10463 were stored at –80 °C with 20% v/v of glycerol as cryoprotectant. Before the experiments, bacterial suspensions were thawed, inoculated (1%, v/v) in Brain Heart Infusion (BHI: Biokar Diagnostic, Beauvais, France) containing 0.05% w/v L-cysteine hydrochloride (BHIC) and incubated in anaerobic conditions at 37 °C for 22 h (AnaeroPack™ anaerobic system, Mitsubishi Gas Chemical America, Inc., New York, USA).

Toxinotyping

Spent culture supernatants (SCS) were obtained from a 72-h-old culture of ALCD3 in BHIC by centrifugation and further filter sterilization (0.45 µm). Presence of TcdA and TcdB in SCS was assessed by the dot blot assay by using mouse anti-TcdA (1/1000) or anti-TcdB (1/500) monoclonal antibodies (Meridian Life Science Inc., USA) respectively as previously described⁴⁴. Biological activity of SCS (mainly associated to TcdB) was determined in vitro by using cultured Vero cells⁴⁴. The coding genes for TcdA (*tcdA*), TcdB (*tcdB*) and components of the binary toxin (*cdtA* and *cdtB*) were detected according to Stubbs et al.⁴² and Rupnik et al.³⁷. To analyze the PaLoc region, the 3'-end of *tcdA* (A3) and 5'-end of *tcdB* (B1) were amplified by PCR. Next, A3 and B1 fragments were digested with EcoRI (Biolabs_{inc}, New England) or HincII/AclI (Biolabs_{inc}), respectively. An algorithm considering restriction fragment length polymorphism profile (PCR-RFLP), toxin production and presence of the CDT gen allowed to allocate the isolate to one of the 34 existing toxinotypes³⁸ (<http://www.mf.um.si/mf/tox/profile.html>). Details on primer sequences and PCR conditions are given as supplementary material (Tables S1 and S2).

Molecular typing

To perform the DNA extraction, *C. difficile* ALCD3 was grown in BHI broth supplemented with 0.05% w/v L-cysteine for 48 h at 37 °C in anaerobic conditions. After incubation, 1 ml of the culture was centrifuged (16 000 g, 3 min). Pellet was washed with 1 ml of 0.1 M NaCl, suspended in 300 µl of 6% w/v CHELEX (BIO-RAD, USA) and incubated at 60 °C for 20 min. After vortexing, the sample was heated at 100 °C for 8 min, centrifuged at 16 000 g for 3 min, aliquoted and stored at –20 °C until use.

Multilocus sequence typing (MLST) analysis was conducted by amplification and sequencing of the housekeeping genes: *adh*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi* as previously described by Griffiths et al.¹⁸ (details in supplementary material). Amplicons sequences were compared with the MLST database (<https://pubmlst.org/cdifficile/>) to identify the allelic profiles and the corresponding sequence type (ST).

Isolate ALCD3 was able to produce TcdA and TcdB, was positive for the *cdt* gene (binary toxin) and belonged to toxinotype 0/v. Strain VPI 10463 was used for comparison purposes.

Growth kinetics

A series of replicate cultures (one culture per planned timepoint) were done in BHIC at 37 °C under anaerobic conditions. At different timepoints, cell density was assessed by OD₆₀₀ readings from individual cultures (Thermo electron Co, HELIOSY spectrophotometer).

To evaluate viable counts at 24 h incubation, serial dilutions of samples in NaCl 0.9% w/v were plated onto DCM supplemented with 0.1% w/v of sodium taurocholate. Plates were incubated for 24 h at 37 °C under anaerobic conditions as indicated above.

Spore production and purification

Plates of solid DCM were inoculated with 100 µl of 22 h cultures in BHIC and incubated for 7 days at 37 °C under anaerobic conditions (see “Molecular typing” section). Next, spores were recovered and purified as described by Sorg and Sonenshein⁴¹. Briefly, cells were harvested from agar plates with ice-cold distilled water (1.5 ml, twice) and spore suspensions were stored for 72 h at 4 °C and washed 5 times with 1 ml sterile ice-cold water (7000 g for 5 min). Suspensions in distilled water (1 ml) were layered on top of 10 ml of 50% w/v sucrose in water and centrifuged in a swinging-bucket rotor at 3200 g for 20 min at 4 °C. After centrifugation, pellets containing mature spores were washed 5-times as described above, suspended in sterile distilled water and stored at –20 °C until use^{17,41}.

Sporulation kinetics

Microscopic evaluation of morphotypes

One-hundred microliters of an overnight culture, approximately 1.5×10^7 CFU/ml, (corresponding to OD_{600nm} = 1) of *C. difficile* were inoculated on DCM agar plates and

incubated for 1, 2, 5 or 7 days at 37 °C under anaerobic conditions. After incubation, cells were harvested with NaCl 0.9% w/v as described in "Bacterial strains and culture conditions" section and analyzed immediately by bright-field microscopy at 1000× magnification. Vegetative forms (Vg) and three spore morphotypes were detected, i.e., phase dark (D), phase bright (B) and free (F) spores corresponding to different sporulation stages¹⁰. At least 300 total forms (TF = Vg + D + B + F) were evaluated for each microorganism under study. Results were expressed as sporulation ratio (SR) calculated as the ratio between total sporulated cells (TSC = D + B + F) and total forms (TF). The ratio of each morphotype (MR) was calculated as the number of cells belonging to a determined morphotype divided by TSC.

Heat-resistance

One hundred and fifty microliters of spore suspensions harvested at different timepoints were heat-treated at 65 °C for 20 min. Ten-fold serial dilutions of both heat-treated and non-treated suspensions were plated on DCM supplemented with sodium taurocholate 0.1% w/v (DCM-TA). Plates were incubated at 37 °C for 24 h and colonies were counted. The heat resistance ratio (HRr) was calculated as the ratio between counts of heat-treated and non-treated samples at each time-point³².

Spore germination

Assessment of changes in optical density

Suspensions of purified spores ($OD_{620} = 0.8-1$) were incubated in flat-bottom 96 wells plates (Jet Biofil, DKSH Australia) in the presence of different concentrations (0, 50, 100 and 150 mM) of sodium taurocholate in PBS buffer (pH = 7.2–7.4). Decrease of $OD_{620\text{nm}}$, that evidences changes in spore refringence and correlate with early steps in germination, was monitored at 1 min intervals (TECAN microplate spectrophotometer Infinite F50)³³. Ratios between OD_{620} values at each timepoint and the initial value ($t = 0$) were defined as relative OD_{620} and were plotted against time. Germination rates ($V_{OD_{620\text{nm}}}$) were determined by calculating the slopes in the initial linear region of the relative $OD_{620\text{nm}}$ vs t plots.

Ca dipicolinate release (CaDPA)

CaDPA release is one of the early steps in germination of *C. difficile*. It was monitored in real time by measuring fluorescence in the presence of terbium (III) chloride⁴. An opaque flat-bottom 96-well plate (Greiner Bio-One) was prepared with 125 μl of 10 mM Tris (Sigma-Aldrich, St. Louis, USA) (pH 7.5), 150 mM NaCl (Cicarelli, Santa Fe, Argentina), 800 μM TbCl_3 (Sigma-Aldrich) and different sodium taurocholate concentrations (0, 0.1, 1.8, 12.5 and 100 mM). Two experimental conditions were tested. Condition 1: 1.5 μl of concentrated spore suspensions ($OD_{600\text{nm}} = 40$) were added per well; condition 2, glycine (Sigma-Aldrich) was used as co-germinant (final concentration 100 mM) and 5 μl of a heat-activated spore suspension (at 65 °C for 20 min) were added per well. Fluorescence was monitored for 80 min at 37 °C (Infinite 200 PRO TECAN fluorescence plate reader) using the following wavelengths: excitation = 270 nm; emission = 545 nm; cutoff = 420 nm. The ratio F_t/F_i was plotted

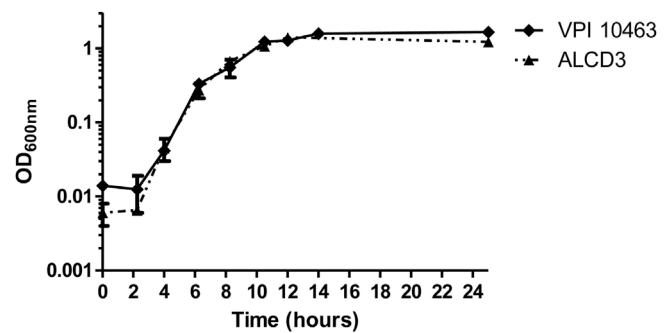


Figure 1 Growth kinetics of *C. difficile* strains in liquid BHIC assessed by optical density at 600 nm. Incubation was performed in an anaerobic atmosphere at 37 °C. Values represent averages of duplicate readings in a representative experiment.

versus time (min), where F_t is the fluorescence after t min and F_i is the initial fluorescence. To determine DPA release rate (V_{DPA}) the slopes of the linear region of F_t/F_i kinetics were calculated and plotted at each taurocholate concentration tested.

Statistical analysis

Growth kinetics and sporulation assays were performed in duplicate. Slopes were analyzed using the GraphPad slope comparison test that compares the simple models obtained of each dataset with a global model using an F-test. A one-way ANOVA with Tukey's multiple comparison test was used to analyze HR, $V_{OD_{620\text{nm}}}$ and V_{DPA} . Ratios between different morphotypes were compared by means of the exact Fisher's test. Statistical analysis was performed by using InfoStat software (InfoStat, version 2020 for Windows, FCA-UNC Córdoba, Argentina) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Characterization of isolate ALCD3

C. difficile ALCD3 was able to produce TcdA and TcdB. In addition, sequences of the genes associated to the binary toxin (*cdt*) and RFLP analysis, showed that this isolate belongs to toxinotype 0/v. MLST showed the allelic profile *adh*:91, *atpA*:1, *dxr*:2, *glyA*: 1, *recA*:27, *sodA*: 1 and *tpi*:1, which corresponds to ST293 (MLST clade: 1).

Growth kinetics

Growth kinetics in BHIC medium are shown in [Figure 1](#). Growth rates were 0.43 ± 0.1 (1/h) for ALCD3 and 0.66 ± 0.1 (1/h) for VPI 10463 and lag periods were around 2 h. After 12–14 h, cultures reached stationary phase with OD_{600} values ranging from 1.2 to 1.6 units. Viable counts in 24-h-old cultures were 5.6×10^6 (VPI 10463) and 1.4×10^7 (ALCD3) CFU/ml.

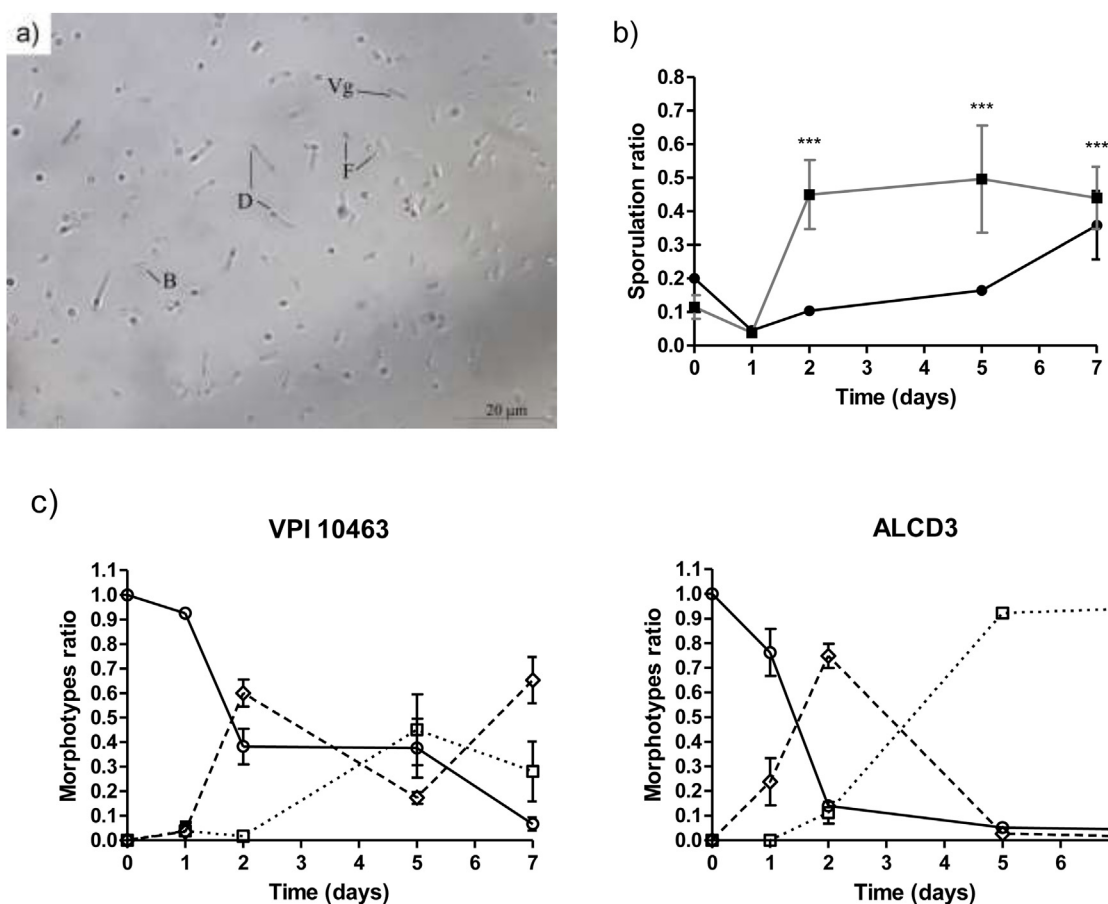


Figure 2 (a) Bright field micrography showing different morphotypes (1000 \times): Vg (vegetative cell), D (dark phase sporulated form), B (bright phase sporulated form) and F (free spore). (b) Kinetics of sporulation ratios for *C. difficile* strains in DCM medium at 37 $^{\circ}$ C. (■) ALCD3 and (●) VPI 10463. (c) Kinetics of morphotype ratios. (○) Dark phase, (◇) bright phase and (□) free spores. Ratios were compared with the Fisher exact test ($p < 0.05$).

Sporulation kinetics and morphotype evaluation

Morphological changes during sporogenesis can be assessed by microscopic examination. As shown in Figure 2a, different morphotypes were evidenced, i.e., vegetative forms (Vg) and three morphotypes: phase-dark (D), phase-bright (B) and free spores (F).

Kinetics of sporulation in DCM agar is shown in Figure 2b. At $t=0$ sporulation rates (ranging from 0.1 to 0.2) are due to the carry-over of spores from the initial inoculum. After 1-day incubation, sporulation ratios (SR) were low for strain VPI 10463 (0.045 ± 0.002) and isolate ALCD3 (0.040 ± 0.01). This represents, in absolute values, 4–13 sporulated forms respectively, per 300 total forms.

C. difficile ALCD3 showed an early increase in the sporulation ratio (reaching maximal values after 2 days of incubation (SR: 0.46 ± 0.03 ; Fig. 2b)). In contrast, sporulation ratios of *C. difficile* VPI 10463 increased until day 7 but were always below those of ALCD3 isolate. These results represent, in absolute values, numbers of total sporulated forms per 300 total forms of 107 ± 43 (VPI 10463) and 132 ± 40 (ALCD3).

Quantification of the different morphotypes (expressed as morphotype ratio, MR) revealed strain-dependent sporulation patterns (Fig. 2c). At time zero, the spores were in the

early stages of the cycle ($MR_D = 1$). These spores arise from the inoculum carryover. As expected, on day 1, high ratios of dark phase forms were observed for both microorganisms under study (ratios ranged from 0.75 to 0.95). On day 2, ratios of dark phase forms decreased, and bright phase forms were observed. Interestingly, free spores ($MR_F = 0.11$) were observed on day 2 for isolate ALCD3 that also showed high ratios of bright phase spores ($MR_B = 0.75$) at this time-point (Fig. 2c). These findings are in agreement with the sporulation kinetics for *C. difficile* ALCD3 strain shown in Figure 2b.

The maximal ratios of free spores (MR_F) were found on day 5: 0.92 for ALCD3 and 0.45 for VPI 10463. These values were significantly different ($p < 0.001$) and remained stable on day 7 (Fig. 2c).

Heat resistance

Progress of the sporulation process was monitored by assessing the ratio of thermo-resistant forms. On day 1, cultures were in stationary phase and total bacteria counts (before heating) were around 10^8 CFU/ml (Fig. 3A) with ratios of heat resistant forms ($HRr = \text{heat resistant}/\text{total counts}$) lower than 0.002 (Fig. 3B). Both microorganisms under study

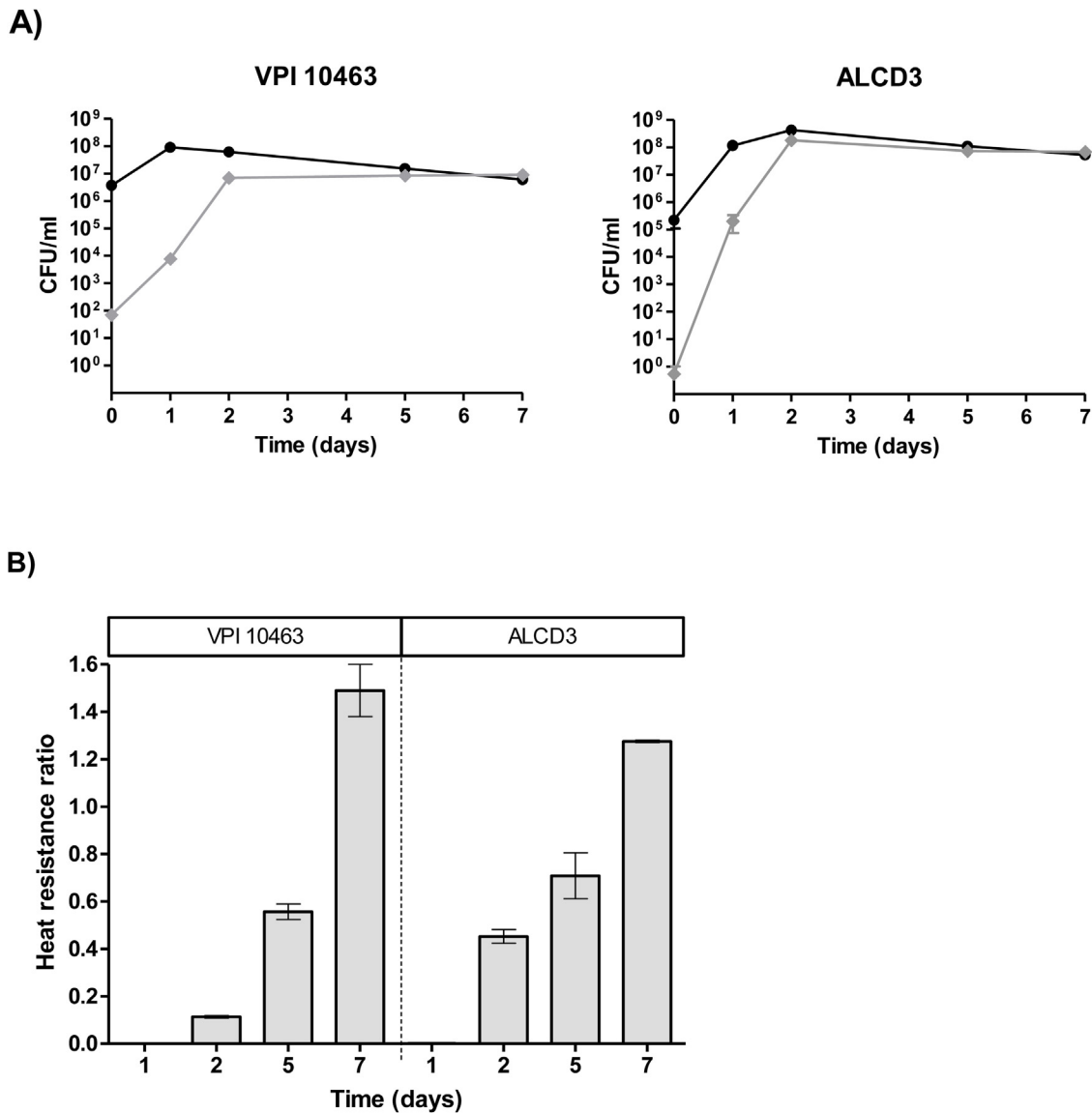


Figure 3 Kinetics (A) and ratios (B) of heat resistant forms for *C. difficile* strains. Viable counts were performed before (●) and after (◆) sample heating at 65 °C for 20 min. Results represent averages from two independent experiments and bars are the standard error of the means.

reached maximum values of heat resistant forms at 2 days of incubation. In addition, the ratio of heat-resistant forms steadily increased and HRr > 1 were found at 7 days of incubation for both strains (Fig. 3B).

Spore germination

As shown in Figure 4, isolate ALCD3 showed the most noticeable changes in the values of relative OD₆₂₀ when spores were exposed to 100 mM of taurocholate. As shown in Table 1, isolate ALCD3 showed the highest germination rates even at the lowest taurocholate concentration tested (50 mM). Furthermore, this microorganism responds to the increase of taurocholate from 50 to 100 mM by doubling the germination rate. No detectable germination was observed

at 50 mM taurocholate concentrations for isolate VPI 10463, but it increased 5 times the germination rate in the presence of 150 mM taurocholate.

Figure 5A shows a representative plot of DPA release from isolate ALCD3 in condition 2 (with glycine as co-germinant). As expected, the rate of DPA release depends on the germinant concentration. High F_t/F_i values were detected after 30 min incubation in the presence of 12.5 and 100 mM taurocholate.

Rates of DPA release (V_{DPA}) were calculated from the linear region of F_t/F_i vs t plots and were plotted for different taurocholate concentrations (Fig. 5B). As expected, the presence of the co-germinant significantly increased the rate of DPA release from heat-activated spores of the ALCD3 strain as compared with germination without glycine (Fig. 5B).

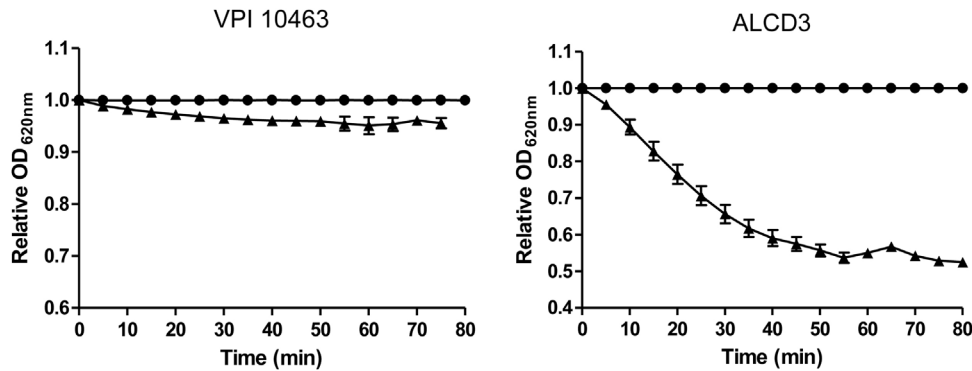


Figure 4 Kinetics of OD_{620} for spores of *C. difficile* incubated in the presence (▲) or not (●) of 100 mM sodium taurocholate at room temperature. Results show a representative experiment and bars indicate the standard error of the mean ($n=2$).

Table 1 Germination rates* for *C. difficile* strains in the presence of different taurocholate concentrations.

Taurocholate (mM)	VPI 10463	ALCD3
50	NG	74 ± 6^a
100	11 ± 1^a	140 ± 10^b
150	49 ± 1^b	ND

NG: no germination was detected; ND: not determined.

Different letters in the same column indicate significant differences ($p < 0.001$).

* Germination rates were defined as the slopes of the linear region of OD_t/OD_i plots (as per Fig. 4).

Discussion

This study found that *C. difficile* ALCD3 ST293 isolated from a recurrent infection presents high sporulation/germination efficiency. The deposit of ST293 in <https://pubmlst.org/cdifficile/> database corresponded to an isolate recovered in China in 2015; however, to the best of our knowledge there are no previous publications in the indexed literature reporting the relationship of this ST to CDI. Interestingly, ST293 is a single locus variant of ST3, which only differs in the allele of *recA*. ST3 was one of the prevalent ST in China associated with CDI and community-acquired infections. It is worth noting that ST3 is associated to PCR ribotypes 001, 009, 072 and 115 that are not related to typical hypervirulent strains^{18,27}.

It has been proposed that enhanced sporulation and toxin production correlate with an apparent increase in virulence in the isolates called "hypervirulent" associated with specific genotypes¹. High nosocomial spread of strains belonging to ribotype 027 was related to an increased resistance of its spores to environmental factors and disinfectants²⁰. It is evident that isolate ALCD3 was successful in spreading, infecting and producing toxins. However, although the ability to produce a high number of spores correlates with the dissemination/infectious potential, it is not directly associated to the ability to release toxins. In this context, strain VPI 10463 is able to release a higher concentration of toxins than other nosocomial strains but it is not considered a hypervirulent strain²⁹.

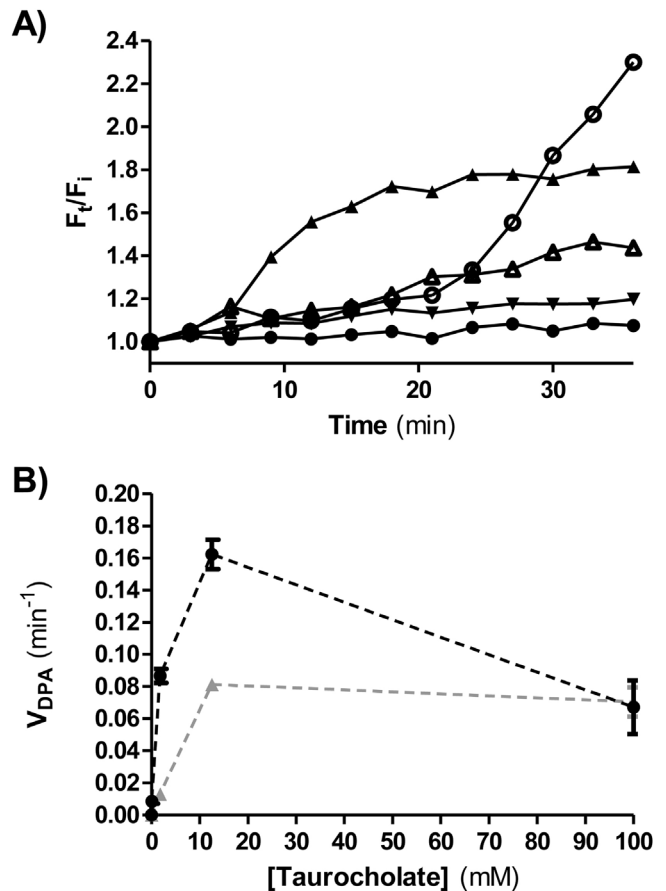


Figure 5 (A) Kinetics of CaDPA release from heat-activated spores of the ALCD3 strain in the presence of 100 mM glycine and different concentrations of sodium taurocholate. (▲) 100 mM, (○) 12.5 mM, (△) 1.8 mM, (▼) 0.1 mM and (●) 0 mM. (B) Initial rate of CaDPA release from spores of strain ALCD3 in the presence of different concentrations of sodium taurocholate with neither glycine nor heat-activation (▲) or with glycine 100 mM and heat-activation for 20 min at 65 °C (●). Results show a representative experiment and bars are the standard deviation of the linear regression.

Although the role of the binary toxin during infection is not well understood, the presence of the *cdt* gene could contribute to the virulence of *C. difficile* ALCD3. Indeed, a mouse model of infection with strain R20291 CDT⁺ produced higher mortality than the isogenic CDT⁻ strain^{7,27}.

Isolate ALCD3 showed high ratios of sporulated forms (Fig. 2b). The sporulation ratios and free spore ratios (Figs. 2b and c) evidence the efficiency in sporogenesis of the isolate.

Spore germination is a paramount step in the infective cycle. Dormant spores must sense environmental signals to germinate thus allowing bacterial growth. Germination of *C. difficile* spores is triggered by natural germinants and co-germinants such as taurocholate and glycine, respectively^{19,36}. Moreover, divalent ions such as calcium play a role as factors enhancing germination²³. As a particular mechanism to detect germinants, *C. difficile* uses the CspC pseudoprotease to sense cholate-derived bile acids such as taurocholate³⁶.

Isolate ALCD3 demonstrated high response to taurocholate (Table 1 and Fig. 4). Interestingly, the release of DPA was evident in the first 10 min of incubation with taurocholate. This indicates that the germinant rapidly diffuses in the spore coat thus reaching CspC. However, some spores required further activation since heat treatment and the presence of glycine as co-germinant in addition to taurocholate, significantly enhanced DPA release from the ALCD3 isolate.

In the present report, we demonstrate that isolate ALCD3 is efficient in germination (Figs. 4 and 5), which contrasts with the reference strain VPI10463 that was able to produce high amounts of spores but showed lower rate of cortex hydrolysis in the presence of 100 mM taurocholate. It must be pointed out that during germination of *C. difficile* spores, cortex hydrolysis precedes DPA release. This is opposite to *B. subtilis* germination¹³. Therefore, results shown in Figures 4 and 5 correspond to consecutive events. As expected, isolate ALCD3 responds to the co-germinant glycine but, noteworthy, it is also able to germinate when only taurocholate was used. These findings agree with the demonstrated effect of CspC conformation on germination. Indeed, a single point mutation in *cspC* dramatically modifies the mobility of relevant domains thus allowing germination with otherwise competitive inhibitors such as chenodeoxycholate¹². In addition, it has been proposed that there are mutants (*yabG* mutants) that fail to process CspBA to give rise to CspB, the cortex hydrolase^{11,30}. This condition leads to spores that are insensitive to the presence of co-germinants⁴⁰. Differences in response to germinants can be related to differences in the diffusion of germinants due to variations in the exosporium composition³¹.

It can be noted that isolate ALCD3 responds to increasing taurocholate concentrations by increasing the rate of cortex hydrolysis (Table 1). However, the results shown in Figure 5B demonstrated that DPA release peaked at 12.5 mM.

It is worth noting that isolate ALCD3 was obtained from a recurrent event, 10 days after the patient completed the standard treatment with oral vancomycin for 14 days.

Our study reports for the first-time the circulation of *C. difficile* ST293 outside China. We demonstrated that

C. difficile ALCD3 ST293 isolate presented high sporulation/germination efficiencies that could contribute to its high pathogenic and spread potential. The present report encourages further research to understand the real impact of this lineage in Argentina.

Conflict of interest

None declared.

Acknowledgements

Authors acknowledge financial support from Facultad de Ciencias Exactas (Universidad Nacional de La Plata, X816), CONICET (PIP 2018-511) and ANPCyT (PICT 2018-3512). Isolate ALCD3 was from Hospital Alemán, Ciudad Autónoma de Buenos Aires, Argentina. PCSP is fellow of the CONICET, Argentina. FMT, DC and PFP are members of the Carrera del Investigador Científico y Tecnológico, CONICET, Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ram.2022.09.004](https://doi.org/10.1016/j.ram.2022.09.004).

References

- Åkerlund T, Persson I, Unemo M, Norén T, Svenungsson B, Wullt M, Burman LG. Increased sporulation rate of epidemic *Clostridium difficile* type 027/NAP1. *J Clin Microbiol.* 2008;46:1530–3.
- Bacci S, Mølbak K, Kjeldsen MK, Olsen KEP. Binary toxin and death after *Clostridium difficile* infection. *Emerg Infect Dis.* 2011;17:976–82.
- Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. *Clin Infect Dis.* 2008;46 Suppl.:12–8.
- Bhattacharjee D, Francis MB, Ding X, McAllister KN, Shrestha R, Sorg JA. Reexamining the germination phenotypes of several *Clostridium difficile* strains suggests another role for the CspC germinant receptor. *J Bacteriol.* 2016;198:777–86.
- Carlson PEJ, Walk ST, Bourgis AET, Liu MW, Koplaku F, Lo E, Young VB, Aronoff DM, Hanna PC. The relationship between phenotype, ribotype, and clinical disease in human *Clostridium difficile* isolates. *Anaerobe.* 2013;24:109–16.
- Connor MC, McGrath JW, McMullan G, Marks N, Guelbenzu M, Fairley DJ. Emergence of a non-sporulating secondary phenotype in *Clostridium (Clostridioides) difficile* ribotype 078 isolated from humans and animals. *Sci Rep.* 2019;9:1–12.
- Cowardin CA, Buonomo EL, Saleh MM, Wilson MG, Burgess L, Kuehne SA, Schwan C, Eichhoff AM, Koch-Nolte F, Lyras D, Aktories K, Minton NP, Petri WA Jr. The binary toxin CDT enhances *Clostridium difficile* virulence by suppressing protective colonic eosinophilia. *Nat Microbiol.* 2017;1:1–21.
- Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, Fairweather NF, Dougan G, Lawley TD. The *Clostridium difficile* *spo0A* gene is a persistence and transmission factor. *Infect Immun.* 2012;80:2704–11.
- Dharmasena M, Jiang X. Isolation of toxigenic *Clostridium difficile* from animal manure and composts being used as biological soil amendments. *Appl Environ Microbiol.* 2018;84, <http://dx.doi.org/10.1128/AEM.00738-18>, e0078-18.

10. Edwards AN, McBride SM. Initiation of sporulation in *Clostridium difficile*: a twist on the classic model. *FEMS Microbiol Lett.* 2014;358:110–8.
11. Fimlaid KA, Jensen O, Donnelly ML, Francis MB, Sorg JA, Shen A. Identification of a novel lipoprotein regulator of *Clostridium difficile* spore germination. *PLoS Pathog.* 2015;11:e1005239, <http://dx.doi.org/10.1371/journal.ppat.1005239>.
12. Francis MB, Allen CA, Shrestha R, Sorg JA. Bile acid recognition by the *Clostridium difficile* germinant receptor, CspC, is important for establishing infection. *PLoS Pathog.* 2013;9:e1003356, <http://dx.doi.org/10.1371/journal.ppat.1003356>.
13. Francis MB, Sorg JA. Detecting cortex fragments during bacterial spore germination. *J Vis Exp.* 2016;25:e54146, <http://dx.doi.org/10.3791/54146>.
14. Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, Kuijper EJ, Wilcox MH. The changing epidemiology of *Clostridium difficile* infections. *Clin Microbiol Rev.* 2010;23:529–49.
15. Galdys AL, Nelson JS, Shutt KA, Schlackman JL, Pakstis DL, Pasculle AW, Marsh JW, Harrison LH, Curry SR. Prevalence and duration of asymptomatic *Clostridium difficile* carriage among healthy subjects in Pittsburgh, Pennsylvania. *J Clin Microbiol.* 2014;52:2406–9.
16. Gerding DN, Johnson S, Rupnik M, Aktories K. *Clostridium difficile* binary toxin CDT: mechanism, epidemiology, and potential clinical importance. *Gut Microbes.* 2014;5:15–27.
17. Giel JL, Sorg JA, Sonenshein AL, Zhu J. Metabolism of bile salts in mice influences spore germination in *Clostridium difficile*. *PLOS ONE.* 2010;5:e8740, <http://dx.doi.org/10.1371/journal.pone.0008740>.
18. Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, Golubchik T, Harding RM, Jeffery KJM, Jolley KA, Kirton R, Peto TE, Rees G, Stoesser N, Vaughan A, Walker AS, Young BC, Wilcox M, Dingle KE. Multilocus sequence typing of *Clostridium difficile*. *J Clin Microbiol.* 2010;48:770–8.
19. Haine ER, Rolff J, Siva-Jothy MT. Functional consequences of blood clotting in insects. *Dev Comp Immunol.* 2007;31:456–64.
20. Hensgens MPM, Keessen EC, Squire MM, Riley TV, Koene MGJ, de Boer E, Lipman LJA, Kuijper EJ. *Clostridium difficile* infection in the community: a zoonotic disease? *Clin Microbiol Infect.* 2012;18:635–45.
21. Jump RLP, Pultz MJ, Donskey CJ. Vegetative *Clostridium difficile* survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pump inhibitors and *C. difficile*-associated diarrhea? *Antimicrob Agents Chemother.* 2007;51:2883–7.
22. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature.* 1995;375:500–3.
23. Kochan TJ, Shoshiev MS, Hastie JL, Somers MJ, Plotnick YM, Gutierrez-Munoz DF, Foss ED, Schubert AM, Smith AD, Zimmerman SK, Carlson PE, Hanna PC. Germinant synergy facilitates *Clostridium difficile* spore germination under physiological conditions. *mSphere.* 2018;3:1–13.
24. Kuehne SA, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP. Importance of toxin A, toxin B, and CDT in virulence of an epidemic *Clostridium difficile* strain. *J Infect Dis.* 2014;209:83–6.
25. Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, Mastroeni P, Scott P, Raisen C, Mottram L, Fairweather NF, Wren BW, Parkhill J, Dougan G. Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun.* 2009;77:3661–9.
26. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JL, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC. Burden of *Clostridium difficile* infection in the United States. *N Engl J Med.* 2015;372:825–34.
27. Liu X-S, Li W-G, Zhang W-Z, Wu Y, Lu J-X. Molecular characterization of *Clostridium difficile* isolates in China from 2010 to 2015. *Front Microbiol.* 2018;9:845, <http://dx.doi.org/10.3389/fmicb.2018.00845>.
28. Manzoor SE, McNulty CAM, Nakiboneka-Ssenabulya D, Lecky DM, Hardy KJ, Hawkey PM. Investigation of community carriage rates of *Clostridium difficile* and *Hungatella hathewayi* in healthy volunteers from four regions of England. *J Hosp Infect.* 2017;97:153–5.
29. Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S, Gerding DN, Vedantam G. Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. *J Bacteriol.* 2010;192:4904–11.
30. Paredes-Sabja D, Setlow P, Sarker MR. SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. *J Bacteriol.* 2009;191:2711–20.
31. Paredes-Sabja D, Shen A, Sorg JA. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends Microbiol.* 2014;22:406–16.
32. Pishdadian K, Fimlaid KA, Shen A. SpoIIID-mediated regulation of σ K function during *Clostridium difficile* sporulation. *Mol Microbiol.* 2015;95:189–208.
33. Ramirez N, Liggins M, Abel-Santos E. Kinetic evidence for the presence of putative germination receptors in *Clostridium difficile* spores. *J Bacteriol.* 2010;192:4215–22.
34. Riley TV, Kimura T. The epidemiology of *Clostridium difficile* infection in Japan: a systematic review. *Infect Dis Ther.* 2018;7:39–70.
35. Rodriguez Diaz C, Seyboldt C, Rupnik M. Non-human *C. difficile* reservoirs and sources: animals, food, environment. *Adv Exp Med Biol.* 2018;1050:227–43.
36. Rohlffing AE, Eckenroth BE, Forster ER, Kevorkian Y, Donnelly ML, Benito de la Puebla H, Doublie S, Shen A. The CspC pseudoprotease regulates germination of *Clostridioides difficile* spores in response to multiple environmental signals. *PLoS Genet.* 2019;15:e1008224, <http://dx.doi.org/10.1371/journal.pgen.1008224>.
37. Rupnik M, Avesani V, Janc M, Von Eichel-Streiber C, Delmée M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol.* 1998;36:2240–7.
38. Rupnik M, Janezic S. An update on *Clostridium difficile* toxinotyping. *J Clin Microbiol.* 2016;54:13–8, <http://dx.doi.org/10.1128/JCM.02083-15>.
39. Rupnik M, Wilcox MH, Gerding DN. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol.* 2009;7:526–36.
40. Shrestha R, Sorg JA. Terbium chloride influences *Clostridium difficile* spore germination. *Anaerobe.* 2019;58:80–8.
41. Sorg JA, Sonenshein AL. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. *J Bacteriol.* 2010;192:4983–90.
42. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett.* 2000;186:307–12.
43. Theriot CM, Koenigsnecht MJ, Carlson PE, Hatton GE, Nelson AM, Li B, Huffnagle GB, Li JZ, Young VB. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase

- susceptibility to *Clostridium difficile* infection. Nat Commun. 2014;5:3114, <http://dx.doi.org/10.1038/ncomms4114>.
44. Trejo FM, Pérez PF, De Antoni GL. Co-culture with potentially probiotic microorganisms antagonises virulence factors of *Clostridium difficile* in vitro. Antonie van Leeuwenhoek. 2010;98:19–29.
 45. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366:1079–84.