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L-lysine (pH 6.0) induces germination of spores of *Clostridium perfringens* type F isolates carrying chromosomal or plasmid-borne enterotoxin gene

Saeed Banawas[a,b,c], Mahfuzur R. Sarker[a,b,*]

[a] Department of Biomedical Sciences, Oregon State University, Corvallis, OR 97331, USA
[b] Microbiology, Oregon State University, Corvallis, OR 97331, USA
[c] Department of Medical Laboratories, College of Applied Medical Science, Majmaah University, 11952, Saudi Arabia

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ABSTRACT

C. perfringens type F isolates carrying enterotoxin gene (*cpe*) on the chromosome (C-*cpe* isolates) are mostly associated with food poisoning, while isolates carrying plasmid-borne *cpe* (P-*cpe* isolates) with non-food-borne gastrointestinal diseases. Spore germination is considered the most essential step for initiation of these diseases. Identifying the most effective germinants for spores of C-*cpe* and P-*cpe* isolates should help developing novel strategies involving induction of spore germination followed by inactivation of germinated spores with mild treatments. In this study, we showed that (i) L-lysine (pH 6.0) triggered germination of spores of all tested C-*cpe* and P-*cpe* isolates; although extremely low concentration of L-lysine (5–10 mM) induced germination of C-*cpe* spores, 10-fold higher concentration (50 mM) was required for P-*cpe* spore germination; (ii) P-*cpe* strain F4969 *gerKC* spores did not germinate, C-*cpe* strain SM101 *gerKC* spores germinated extremely poorly and these *gerKC* spores released significantly less DPA as compared to wild type spores; and these defects were restored to a nearly wild-type level by complementing *gerKC* spores with wild-type *gerKC*; and (iii) F4969 *gerAA* spores also did not germinate, and released less DPA than wild-type spores in presence of L-lysine (pH 6.0); and these defects were restored partially (germination) and fully (DPA release) by complimenting *gerAA* spores with wild-type *gerAA*. Collectively, our current study identified L-lysine as a universal germinant for spores of both C-*cpe* and P-*cpe* isolates and provided evidence that GerKC (from SM101 or F4969) and F4969 GerAA play major roles in L-lysine-induced germination.

1. Introduction

Clostridium perfringens is a spore-forming, gram-positive, rod-shaped, anaerobic bacterium that capable of causing a wide spectrum of diseases in both humans and animals [1]. However, the two most common *C. perfringens*-associated illnesses in humans are *C. perfringens* type F food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) diseases [1–3]. *C. perfringens* can be classified into seven toxinotypes (A through G) [4]. *C. perfringens* type F isolates producing *C. perfringens* enterotoxin (CPE) are mainly responsible for causing FP and NFB GI diseases [1,3,5]. Interestingly, FP are mostly caused by type F isolates carrying CPE-encoding gene (*cpe*) on the chromosome (C-*cpe* isolates), while NFB GI diseases (i.e., sporadic diarrhea and antibiotic-associated diarrhea) by type F isolates carrying plasmid-borne *cpe* (P-*cpe* isolates) [6–8]. However, some studies found that *C. perfringens* FP outbreaks also can occur by P-*cpe* isolates [9,10]. Both C-*cpe* and P-*cpe* isolates can form metabolically dormant spores that are much more resistant than their vegetative cells to a variety of lethal factors such as heat, chemicals, radiation, osmotic stress, sodium nitrite, and pH [11–15]. These dormant *C. perfringens* spores can return to actively growing cells through the process of germination once growth-promoting food sources are available [1]. Upon ingestion of contaminated foods, *C. perfringens* vegetative cells undergo sporulation followed by CPE production, leading to CPE-mediated cramping and diarrhea in the human gut [1,3].

In *Bacillus* and *Clostridium* species, germination is initiated when bacterial spores sense the specific nutrient compounds termed germinants (i.e. amino acids and salts) by specific germinant receptors (GRs). Upon binding of the specific germinant to its cognate GR located in spore inner membrane [16–20] several biophysical and biochemical

Abbreviations: CPE, *C. perfrongens* enterotoxin; GI, Gastrointestinal; FP, Food Poisoning; NFB, Non-food-borne; P-*cpe*, *C. perfringens* carrying *cpe* on the chromosome; P-*cpe*, *C. perfringens* carrying plasmid-borne *cpe*; FTG, Fluid Thioglycolate; TGY, Trypticase-Glucose-Yeast extract; DS, Duncan Strong

* Corresponding author. Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, 216 Dryden Hall, Corvallis, OR 97331, USA.
E-mail addresses: S.Banawas@mu.edu.sa (S. Banawas), sarkerm@oregonstate.edu (M.R. Sarker).

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reactions take place. These serial reactions lead to degrade the spores' peptidoglycan (PG) cortex by cortex-lytic enzymes, which allows the rehydration of the spore core and resumption of enzymatic activities and metabolism [19,21–24]. Our previous studies identified several germinants for spores of C-*cpe* and P-*cpe* isolates. For example, KCl and L-asparagine at pH 7.0, and L-glutamine and sodium phosphate (NaPi) at pH 6.0, are good germinants for C-*cpe* isolates [25–27]. However, L-cysteine, L-serine, L-threonine, L-asparagine all at pH 6.0 and a mixture of KCl and L-asparagine (AK) at pH 7.0 are universal germinants for both C-*cpe* and P-*cpe* isolates [27]. Our recent study also identified amino acids and bicarbonate (pH 7.0) are co-germinants for P-*cpe* isolates [28]. *C. perfringens* has four GR genes including a monocistronic *gerAA*, a bicistronic *gerKA-KC* operon, and *gerKB* located in upstream and opposite orientation of *gerK* locus [24,26,29]. Interestingly, these four GR genes are well conserved in *C. perfringens* isolates [24,26,30]. Our previous studies showed that most, if not all, identified nutrient germinants act through the main GR protein GerKC in spores of both C-*cpe* strain SM101 and P-*cpe* strain F4969 [16,30]. However, GerKB and GerAA proteins play minor roles in SM101 spore germination [26]. In striking contrast to SM101 GerAA, F4969 GerAA plays a major role in spore germination with AK and L-cysteine [30].

As spore germination plays a critical role in the pathogenesis of *C. perfringens*, identifying the most effective germinants for spores of a wide range of C-*cpe* and P-*cpe* isolates should led to the development of novel strategies involving induction of spore germination followed by inactivation of germinated spores with mild treatments [31,32]. In our previous study, we showed that only 3 out of 20 amino acids (L-cysteine, L-glutamine, and L-lysine), all at pH 7.0, could trigger germination of spores of both C-*cpe* and P-*cpe* isolates, with lysine being the least effective [27]. However, at pH 6.0, L-cysteine was an effective germinant for spores of both C-*cpe* and P-*cpe* isolates, while L-glutamine was a unique germinant for spores of C-*cpe*, but not for those of P-*cpe*, isolates [27]. These discrepancies between spore germination results at pH 7.0 versus 6.0 suggest that L-lysine at pH 6.0 might be an effective germinant for both C-*cpe* and P-*cpe* spores. Consequently, in the current study, we characterized L-lysine (pH 6.0)-triggered germination of spores of C-*cpe* and P-*cpe* isolates. Our current results showed that, i) indeed, L-lysine at pH 6.0 is an effective germinant for spores of both C-*cpe* and P-*cpe* isolates; and further ii) GerKC is the main GR protein required for L-lysine germination of spores of C-*cpe* strain SM101 and P-*cpe* strain F4969; although F4969 GerAA protein also plays a role in L-lysine germination of F4969 spores.

2. Material and methods

2.1. Bacterial strains

C. perfringens strains used in this study are listed in Table 1.

2.2. Spore preparation and purification

C. perfringens cooked-meat stock culture (0.1 ml) was inoculated into 10 ml Thioglycolate (FTG) broth (Difco, USA) and incubated at 37 °C for 16–18 h as described previously [26,33]. Then ~0.4 ml FTG-grown culture was transferred into a new 10 ml FTG broth and incubated at 37 °C for 8–12 h. Next, 0.4 ml of actively growing FTG culture was inoculated into 10 ml Duncan-Strong (DS) broth [34] and incubated at 37 °C for 18–24 h and spore formation was confirmed by phase-contrast microscopy (Leica MDLS, Leica microsystems). For large-scale spore preparation, *C. perfringens* spores was obtained by scaling up the aforementioned procedure in 800 ml DS, and spores were purified by repeated washing with cold sterile distilled water until spore suspensions were > 99% free of cell debris, sporulating and germinating cells, as determined by phase-contrast microscopy. The spore suspensions were adjusted with sterile distilled water to a final optical density at 600 nm (OD_{600}) ~ 6.0 that corresponds to approximately 10^8 CFU/ml and stored at −80 °C until used [26].

Table 1
C. perfringens strains used in this study.

Strain or mutant	Relevant characteristic	Source or reference
C. perfringens strains		
SM101	Electroporatable derivative of type F isolate NCTC8798; carries a chromosomal *cpe* gene	[39]
E13	Type F isolate; carries chromosomal *cpe*	[8]
6239	Type F isolate; carries chromosomal *cpe*	[40]
NCTC10239	Type F isolate; carries chromosomal *cpe*	[6]
F4969	Type F isolate; carries plasmid-borne *cpe*	[6,41]
NB16	Type F isolate; carries plasmid-borne *cpe*	[8]
B40	Type F isolate; carries plasmid-borne *cpe*	[6,41]
DPS122	*gerKC* mutant of SM101	[16]
DPS119	*gerKA* mutant of SM101	[16]
DPS122(pSB18)	SM101 *gerKC* mutant expressing wild-type *gerKA-KC*	[16]
SB106	*gerKC* mutant of F4969	[30]
SB110	*gerKA* mutant of F4969	[30]
SB103	*gerAA* mutant of F4969	[30]
SB106(pSB18)	F4969 *gerKC* mutant expressing wild-type *gerKA-KC*	[30]
SB103(pSB23)	F4969 *gerAA* mutant expressing wild-type *gerAA*	[30]

2.3. Preparation of germinant solutions

L-lysine used in this study was purchased from Sigma (Sigma-Aldrich, Co., St. Louis, MO, USA) and L-lysine solution was prepared with 25 mM Tris-HCl buffer (pH 7.0). To examine the effect of pH, L-lysine solution was prepared at 100 mM with 25 mM Tris-HCl buffer (pH 7.0) and then adjusted to various pHs, ranging from pH 4.0 to 9.0 in 1.0 unit increments, using 1 M HCl or 1 M NaOH. To examine the concentration effect of L-lysine, spore germination was assessed using various concentrations of L-lysine ranging from 5 to 250 mM in 25 mM Tris-HCl buffer (pH 7.0) and adjusted to the final pH of 6.0.

2.4. Spore germination

Spore germination of *C. perfringens* was done as previously described [16,26,30]. Briefly, spore suspensions were heat activated for 10 min at 75 °C (P-*cpe* spores) or 80 °C (C-*cpe* spores) and then cooled to 37 °C in water bath. These heat-activated spores (OD_{600} of ~1.0) were incubated with pre-warmed L-lysine solution at 37 °C for 60 min in a total volume of 0.2 ml in 96-well microtiter plate. Spore germination was routinely measured by monitoring in change in OD_{600} of spores-lysine solution using a Synergy™ MX multi-mode microplate reader (BioTek® Instruments, Inc., Winooski, VT, USA). The ~60% decrease in OD_{600} indicates complete spore germination as found in our previous studies [16,26–28,30]. The extent of germination was expressed as the percentage of decrease in OD_{600} relative to initial value. Level of spore germination was also confirmed by phase-contrast microscopy after 60 min post-inoculation, as fully germinated spores changes from phase-bright to phase-dark. The rate of Germination was determined by measuring the OD_{600} of germinating spores every 2.5 min and the maximum rate was expressed as a percentage of maximal loss of OD_{600} per minute, relative to the initial value of spore suspension. All values reported are average of three experiments performed with at least three independent spore preparations.

2.5. DPA release

To measure DPA release during L-lysine-triggered spore germination, heat activated (80 °C, 10 min for C-*cpe* and 75 °C, 10 min for P-*cpe*) spores (OD_{600} ~ of 1.5) were incubated with pre-warmed 100 mM L-