Anaerobe 17 (2011) 78–84

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and Toxins

Germination response of spores of the pathogenic bacterium *Clostridium perfringens* and *Clostridium difficile* to cultured human epithelial cells

Daniel Paredes-Sabja [a], Mahfuzur R. Sarker [a,b,*]

[a] Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, 216 Dryden Hall, Corvallis, OR 97331, United States
[b] Department of Microbiology, College of Science, Oregon State University, Corvallis, OR 97331, United States

ARTICLE INFO

Article history:
Received 29 September 2010
Received in revised form
22 January 2011
Accepted 2 February 2011
Available online 12 February 2011

Keywords:
C. perfringens
C. difficile
Spores
Germination

ABSTRACT

Spores of pathogenic *Clostridium perfringens* and *Clostridium difficile* must germinate in the food vehicle and/or host's intestinal tract to cause disease. In this work, we examined the germination response of spores of *C. perfringens* and *C. difficile* upon incubation with cultured human epithelial cell lines (Caco-2, HeLa and HT-29). *C. perfringens* spores of various sources were able to germinate to different extents; while spores of a non-food-borne isolate germinated very well, spores of food-borne and animal isolates germinated poorly in human epithelial cells. In contrast, no detectable spore germination (i.e., loss of spore heat resistance) was observed upon incubation of *C. difficile* spores with epithelial cells; instead, there was a significant ($p < 0.01$) increase in heat-resistant spore titers. In *C. perfringens*, the highest spore germination response observed with the HT-29 cell line, might be in part, due to the expression of germination factor with peptidoglycan cortex hydrolysis activity by HT-29 cells. Collectively, these findings might well have implications in understanding the mechanism of clostridial spore germination *in vivo*.

1. Introduction

The genus *Clostridium* is composed of more than 100 different species, including two enteric pathogens *Clostridium perfringens* and *Clostridium difficile* that are of major concern in human health [1]. *C. perfringens* can produce more than 17 toxins and cause a wide variety of toxico-infections in both humans and animals, which results in a significant economic burden [2–4]. Two important *C. perfringens*-associated human illnesses are *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) gastrointestinal diseases [3]. The majority of FP isolates carry a chromosomal copy of *C. perfringens* enterotoxin gene (*cpe*); while *cpe* is located on a large plasmid in NFB disease isolates [5–8]. The basis for FP isolates' pathogenesis seems not only CPE production, but also other factors that allow FP spores to be better suited to FP environments [9–13]. Recent phylogenetic evidence also indicates that FP isolates belong to a different lineage than NFB and animal isolates [14].

C. difficile is the causative agent of *Clostridium difficile*-associated diseases (CDAD), including a wide spectrum of nosocomial disease that range from mild, self-limiting diarrhoea to serious and life-threatening pseudomembranous colitis and toxic megacolon [15,16]. Two major *C. difficile* toxins, TcdA and TcdB, produced during vegetative growth, are the main virulence factors of CDAD [17–20]. *C. difficile* also can cause significant diseases in animals [21], and similarly as for *C. perfringens*, it can be considered as a zoonotic pathogen [21,22]. Interestingly, *C. difficile* spores have recently been detected in various meat products [23,24] and are able to survive temperatures recommended for cooking ground meats [25].

In both species, spores are recognized as the infectious morphotype [3,26], and can be transmitted to humans via different sources such as, contaminated food products and hospital environments. These spores germinate *in vitro* when they sense the presence of nutrients, termed 'germinants'. *C. perfringens* germination is triggered when spores become in contact with L-valine, L-alanine, L-asparagine, KCl, the mixtures of L-asparagine and KCl (AK), and the co-germinants inorganic phosphate and NaCl (NaPi) [13,27]. For normal spore germination in *C. perfringens* FP isolate SM101, these germinants, especially L-asparagine, KCl and NaPi, require two proteins (GerKA and GerKC) encoded by the *gerK* operon, that might act as a *ger* receptor [13,27]. In contrast, *C. difficile* spores germinate *in vitro* mainly in presence of the bile salt sodium taurocholate [28,29] and the co-germinant glycine [30]. *In vivo* germination of *Clostridium* spores is less clear, although in *C. difficile*, there is evidence that host factors, almost-certainly bile salts, trigger *in vivo* germination [31]. In addition, host factors derived from

* Corresponding author. Department of Biomedical Sciences, Oregon State University, 216 Dryden Hall, Corvallis, OR 97331, United States. Tel.: +1 541 737 6918; fax: +1 541 737 2730.
E-mail address: sarkerm@oregonstate.edu (M.R. Sarker).

1075-9964/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2011.02.001

epithelial cells can also trigger germination of bacterial spores, as germination of *Bacillus cereus* spores induced by cultured enterocytes [32] through the *gerA*-type receptors [33]. Consequently, in this communication, we have investigated the germination response of *C. perfringens* and *C. difficile* spores upon incubation with three different (Caco-2, HeLa and HT-29) cultured human epithelial cell lines. Our results show that germination responses: i) vary between spores of surveyed *Clostridium* species and strains; ii) are induced by epithelial cells; and iii) are highest with HT-29 cells irrespective of *Clostridium* species and strains tested.

2. Materials and methods

2.1. Bacterial stains, human cell lines, and chemicals

C. perfringens and *C. difficile* strains are described in Table 1. HeLa, HT-29 and Caco-2 cells (ATCC, Manassas) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Invitrogen, CA), RPMI-1640 medium (BioWhittaker, Lonza, Walkersville, MD) and Eagle's MEM medium (EMEM) (BioWhittaker, Lonza, Walkersville, MD), respectively. All media were supplemented with 10% (v/v) fetal-calf serum (ATCC, Manassas), penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}). Each cell line was normally harvested with 0.25% trypsin (Gibco), re-suspended in the cell culture medium, and incubated at 37 °C in 5% CO_2 humidified atmosphere.

2.2. Spore preparation and purification

C. difficile spore preparation was done as described [30,34]. Briefly, *C. difficile* strains were grown overnight on Brain Heart Infusion broth (Difco) supplemented with 0.5% yeast extract (Difco) (BHIS) and diluted to an OD_{600} of 0.2 and plated onto BHIS agar. The plates were incubated for 7 days at 37 °C under anaerobic conditions [30,34] in anaerobic jars with a BD GasPak sachet (BD Diagnostic Systems, USA). To recover sporulating cells, BHIS cultured plates were flooded with ice cold sterilized distilled water, suspended and collected cells/spores. Sporulating cells were washed in ice cold water for 10 times by repeated centrifugation and resuspension to release spores from the mother cells. The free spores were separated using 50% HistoDenz and washed five times to eliminate traces of HistoDenz. Spore suspensions were >99% free of vegetative cells, sporulating cells and cell debris, and were stored at −20 °C until use.

Spores of *C. perfringens* isolates were prepared and purified as previously described [27]. Briefly, *C. perfringens* cultures grown overnight on fluid thioglycolate (Difco) were inoculated into Duncan-Strong (DS) sporulation medium [35] and incubated at 37 °C for 24 h. Clean spore preparations were obtained by repeated centrifugation and resuspension with sterile distilled water until spore suspensions were >99% free of sporulating cells, cell debris, and germinated spores and stored at −20 °C until use.

Table 1
Bacterial strains used.

Strain	Relevant characteristics	Source (Reference)
C. difficile		
Strain 630	Epidemic type X, 012 ribotype, toxin A^+ B^+	[50]
Pitt51	CDAD Isolate, toxin A^+ B^+	[54]
Pitt82	CDAD Isolate, toxin A^+ B^+	[54]
Pitt114	CDAD Isolate, toxin A^+ B^+	[54]
Pitt144	CDAD Isolate, toxin A^+ B^+	[54]
Pitt177	CDAD Isolate, toxin A^+ B^+	[54]
C. perfringens		
SM101	FP type A isolates carrying chromosomal *cpe* gene	[55]
F4969	NFB type A isolates carrying plasmid *cpe* gene	[6]
JGS1071	Isolated from diarrheic pig, type C	[56]
06-108654	Isolated from diarrheic bovine, type C	Veterinary Diagnostic lab, CVM, OSU
DPS110	*sleC sleM* (*sleC*::*tetM sleM*::*catP*) mutant of SM101	[41]
DPS117	*cspB* (*cspB*::*tetM*) mutant of SM101	[42]

2.3. Decoating treatment

Spores suspensions at an OD_{600} of 20 were decoated with 1 ml of 50 mM Tris–HCl (pH 8.0)-8 M urea-1% (wt/vol) SDS-50 mM dithiothreitol for 90 min at 37 °C [36]. Decoated spores were washed three times with 150 mM NaCl and twice with water.

2.4. Infection of cultured epithelial cells with spores and germination assay

Although the epithelial cell lines used in this study are derived from carcinomas, these are proven to be valuable models for the study of *Clostridium*-host interactions [37–40]. HeLa and HT-29 cells were seeded into 24-well plates and incubated for 3 days until >99% confluent growth (containing 8×10^5 cells/well). Caco-2 cells were seeded into 24-well plates and incubated until >99% confluent growth and early differentiation (10 days) (containing 8.2×10^5 cells mL^{-1}). Prior to bacterial spore infection, confluent HeLa, HT-29 and Caco-2 cells were washed in pre-warmed PBS (pH 7.4) and then incubated in fresh culture medium overnight. Cells were then washed three times with pre-warmed PBS (pH 7.4) and incubated with 400 μl of culture medium containing heat-activated or untreated *C. difficile* and *C. perfringens* spores but no additives (i.e., no serum or antibiotics) at a multiplicity of infection (MOI) of 10 ($\sim 10^7$ spores/well). Spores were heat-activated by treating at 80 °C for 10 min (*C. perfringens*) or 60 °C for 20 min (*C. difficile*). Infection was carried at 37 °C for 1 h under aerobic conditions. The ability of epithelial cells to trigger germination was first quantified by determining loss of spore heat resistance. Since heat treatment kills the germinated/vegetative cells but not the spores within the sample, loss of spore viability serves as an indicator of the relative amount of spore germination. Spore-infected cultured cells were lysed with 100 μl of 0.06% Triton X-100 for 10 min at 37 °C, and heat treated at 80 °C for 10 min or 60 °C for 20 min to kill germinated spores of *C. perfringens* or *C. difficile*, respectively. Heat treated samples of *C. perfringens* and *C. difficile* spore-infected-epithelial cells were plated onto Brain Heart Infusion agar (BHI) or BHI agar supplemented with 0.5% yeast extract, 2% glucose (BHISG) and 0.1% sodium taurocholate (BHISG + ST) (Himedia Laboratories Pvt. Ltd. Mumbai, India), respectively. The plates were incubated for 24 h at 37 °C under anaerobic conditions and colony forming units (CFU) were counted. For initial CFU counts, heat-activated or untreated *C. perfringens* and *C. diffiicle* spores where first incubated with 0.06% Triton X-100 and tissue culture media for 1 h at 37 °C, plated onto BHI and BHISG + ST plates, respectively, and incubated for 24 h at 37 °C under anaerobic conditions. Loss of spore heat resistance was calculated with the following formula: Log Reduction - (Log_{10}[final CFU] − Log_{10}[initial CFU]). For *C. difficile* spores, loss of heat resistance was measured by the following formula: CFU % of total [(Final CFU)/(Initial CFU)] × 100. Alternatively, in some experiments the latter formula was normalized to log-scale.

To measure germination of *C. difficile* spores through a bile salt independent pathway, a different approach was used. Heat-activated *C. difficile* spores were incubated with human epithelial cells