Journal of Microbiological Methods 91 (2012) 93–100

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

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

Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp.

Simmon Hofstetter [a,*], Christian Denter [b], Roland Winter [b], Lynn M. McMullen [a], Michael G. Gänzle [a]

[a] University of Alberta, Department of Agricultural, Food and Nutritional Science, Edmonton, Alberta, Canada, T6G 2P5
[b] Technische Universität, Biophysikalische Chemie, Fakultät Chemie, Otto-Hahn Str. 6, D-44227 Dortmund, Germany

ARTICLE INFO

Article history:
Received 14 June 2012
Received in revised form 17 July 2012
Accepted 17 July 2012
Available online 3 August 2012

Keywords:
Clostridium
Endospore inner membrane
LAURDAN

ABSTRACT

A method for measuring the fluidity of inner membranes of populations of endospores of *Clostridium* spp. with a fluorescent dye was developed. Cells of *Clostridium beijerinckii* ATCC 8260 and *Clostridium sporogenes* ATCC 7955 were allowed to sporulate in the presence of 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN) on a soil-based media. Labeling of endospores with LAURDAN did not affect endospore viability. Removal of the outer membranes of endospores was done using a chemical treatment and confirmed using transmission electron microscopy (TEM). Two-photon confocal laser scanning microscopy (CLSM), and generalized polarization (GP) measurements were used to assess fluorescence of endospores. Lipid composition analysis of cells and endospores was done to determine whether differences in GP values are attributable to differences in membrane composition. Removal of the outer membranes of endospores did not significantly impact GP values. Decoated, labeled endospores of *C. sporogenes* ATCC 7955 and *C. beijerinckii* ATCC 8260 exhibited GP values of 0.77 ± 0.031 and 0.74 ± 0.027 respectively. Differences in ratios of fatty acids between cells and endospores are unlikely to be responsible for high GP values observed in endospores. These GP values indicate high levels of lipid order and the exclusion of water from within inner membranes of endospores.

1. Introduction

Bacterial endospores are widely distributed within the environment (Carlin, 2011; Nicholson, 2002), and are a significant problem for the food industry. Endospores of *Clostridium* spp. are known to cause food spoilage and foodborne illness (Borge et al., 2001; Brown, 2000; Cortezzo et al., 2004; Salkinoja-Salonen et al., 1999). Endospores resist thermal processes used to extend the storage life of foods (Lee et al., 2006; Reddy et al., 2003). Pressure-assisted thermal sterilization (PATS) has been examined as an alternative to thermal processing for control of endospores (Meyer et al., 2000; Wilson and Baker, 1997). The variability in resistance to PATS amongst different species of endospores means high temperatures are still required for safe food processing (Margosch et al., 2004a, 2004b; Reddy et al., 1999, 2003).

Resistance of endospores to heat and pressure is attributed primarily to low water activity within the core of the endospore (Black et al., 2007; Margosch et al., 2004a, 2004b, 2006). The presence of a calcium–dipicolinic acid (Ca–DPA) matrix is the reason for low water activity within the core (Setlow, 2006). Integrity of the Ca–DPA matrix is maintained by the inner membrane acting as a barrier to the entry of water from the external environment (Paidhungat, 2000; Setlow, 2006). A release of DPA coincides with rehydration of the core, and is known to facilitate the inactivation of endospores by heat and pressure (Black et al., 2007; Margosch et al., 2004a, 2004b, 2006). A better understanding of the inner membranes of endospores during and after physical and chemical treatments will yield information useful in designing specific inactivation strategies for deployment in the food industry.

Fluorescent dyes have been used extensively to examine germination of endospores. Fluorescent dyes used in this manner depend on the extent to which an endospore membrane is permeable. The terbium ion is often used to complex with DPA released during inactivation studies as a measure of thermal resistance and germination (Kort et al., 2005; Reineke et al., 2011). Fluorescent dyes such as SYTO 16, an indicator of germination, and propidium iodide, an indicator of membrane damage in vegetative cells (Bunthof et al., 1999; Ulmer et al., 2000), are only useful for labeling the interior of endospores deficient of Ca–DPA (Baier et al., 2011). The same is true of SYTO 9, Hoechst 33342, and carboxyfluorescein diacetate (CFDA) (Cronin and Wilkinson, 2008).

Sporulation studies typically employ green fluorescent protein (GFP) or an analogue. Such protein fluorophores exhibit great utility

* Corresponding author at: University of Alberta, Dept. of Agricultural, Food and Nutritional Science, 4-10 Ag-For Centre, Edmonton, AB, Canada T6G 2P5. Tel.: +1 780 492 3634; fax: +1 780 492 4265.
E-mail addresses: simmon@ualberta.ca (S. Hofstetter), christian.denter@uni-dortmund.de (C. Denter), roland.winter@tu-dortmund.de (R. Winter), Lynn.McMullen@ualberta.ca (L.M. McMullen), mgaenzle@ualberta.ca (M.G. Gänzle).

0167-7012/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mimet.2012.07.023

in studying protein localization and gene expression during sporulation, specifically in *Bacillus* spp. (Asai et al., 2001; Hilbert et al., 2004; McBride et al., 2005). Green fluorescent protein and its analogues require oxygen for maturation and subsequent fluorescence (Reid and Flynn, 1997). As such, GFP can only be expressed in aerobic, endospore-forming bacteria such as *Bacillus* spp. and not *Clostridium* spp. The oxygen requirement of this probe precludes its use to examine membrane properties of endospores of *Clostridium* spp.

The membrane dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide has been successfully used to dye *Bacillus subtilis* cells during sporulation (Pogliano et al., 1999). This dye preferentially labels membranes, and is able to reveal septal biogenesis and engulfment mechanisms of the sporulation process (Pogliano et al., 1999). Following these observations, endospores of *B. subtilis* and *Bacillus megaterium* containing membrane-specific fluorescent dyes were successfully generated without adversely affecting the sporulation process (Cowan et al., 2004). The fluorescent probes dibromide (FM-4-64) and di-4-ANEPPS insert into inner membranes of endospores and have been used to examine inner membrane fluidity during germination (Cowan et al., 2004). Fluorescence recovery after photobleaching (FRAP) of di-4-ANEPPS, in addition to volumetric measurements using FM-4-64, established that the inner membranes of endospores are immobile, with fluidity returning upon germination (Cowan et al., 2004). The dye FM-4-64 is useful for volumetric assessment of individual endospores, but its spectra does not directly elicit any information about the state of the inner membrane state. Analysis of di-4-ANEPPS using FRAP, in contrast, is exquisitely powerful in that it allows measurement of membrane fluidity of individual endospores (Cowan et al., 2004; Koppel, 1985). However, FRAP analysis cannot rapidly assess inner membrane fluidity of endospore populations during high pressure, high temperature processing. A fluorescence protocol for assessing inner membrane properties of populations of endospores during high pressure, high temperature processing has not been established at this time.

The goal of this study was to develop a method to measure inner membrane fluidity of bacterial endospore populations of *Clostridium* spp. Endospore membranes were integrated with 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN). LAURDAN has been used to assess membrane fluidity of vegetative cells, but not endospores (Sanchez et al., 2007; Ulmer et al., 2002). Shifts in the fluorescence spectrum of LAURDAN accurately indicate differences in membrane fluidity (Nicolini et al., 2006), and its use in this respect has been well established (Harris et al., 2002; Parasassi et al., 1991, 1998). *Clostridium sporogenes* ATCC 7955 was chosen for its ability to generate heat-resistant endospores, and genetic similarity to *Clostridium botulinum* Group I (Collins and East, 1998). *Clostridium beijerinckii* ATCC 8260 was chosen due to potential involvement in blown-pack meat spoilage and growth at 4 °C (Broda et al., 1996).

2. Materials and methods

2.1. Bacterial strains and growth conditions

C. beijerinckii ATCC 8260, and *C. sporogenes* ATCC 7955 cells were inoculated from frozen stocks, held at −80 °C in 60% glycerol, into Reinforced Clostridial Media (RCM; Difco, Sparks, USA) broth and incubated anaerobically overnight at 37 °C.

2.2. Sporulation and harvesting endospores

Weihenstephan Sudhang (WSH) media was prepared as described by Margosch et al.(2006). Endospores containing LAURDAN (Invitrogen, Cat. No. D-250) (WSH-L endospores) were generated by plating 400 μL of 0.1 M LAURDAN suspended in ethanol onto individual WSH agar plates, allowing the ethanol to evaporate in the absence of light. Aliquots (100 μL) of a fully grown culture of *Clostridium* spp. were plated on LAURDAN-containing media in an anaerobic hood. Plates were incubated anaerobically at 37 °C, in the absence of light, for 14 d. Sporulation was confirmed using phase-contrast microscopy in which ~95% endospore purity was observed. Endospores were harvested from the surface of WSH agar by washing with sterile 0.9% saline solution. Harvested endospores were centrifuged at 2700 ×*g* for 5 min and suspended in sterile 0.9% saline. Care was taken to discard the top layer of each cell pellet. Stock solutions of endospores were standardized to OD_{600} values of 0.5 using sterile 0.9% saline. Control endospore stock solutions were generated as above, omitting addition of LAURDAN-saturated ethanol to WSH agar plates. Standardized endospore stocks were stored at −20 °C. Sterile 0.9% saline was used as the endospore suspension medium in subsequent manipulations.

2.3. Removal of endospore coat and outer membrane

Aliquots (1 mL) of stock solutions of WSH-L endospores were centrifuged at 2700 ×*g* for 10 min and the supernatant was removed. Pellets were suspended in a solution of 0.5% SDS, 0.1 M dithiothreitol, 0.1 M NaCl (pH 10, adjusted with 1 M NaOH) for 3 h at 37 °C as described previously (Fitz-James, 1971). Following incubation, endospores were centrifuged at 2700 ×*g* for 10 min and pellets were suspended in sterile 0.9% saline to an OD_{600} of 0.5. Samples were kept on ice until used.

2.4. Lipid extraction of Clostridium cells and endospores

Lipid extraction was done according to a modified protocol applicable to endospores (Bertsch et al., 1969). Pellets (0.25 g) of cells and decoated endospores of *C. sporogenes* ATCC 7955 and *C. beijerinckii* ATCC 8260 were mixed with 3.75 mL of chloroform, 7.5 mL of methanol, and 3 mL of 50 mM K_2HPO_4 buffer solution (pH 7.4) in a screw cap vial. Samples were homogenized on a tissue-tearor for 3 min until pellets were dispersed in the solvent. Samples were subjected to brief sonication in a water bath before homogenization to prevent formation of aggregates. Samples were agitated on a shaker for 1 h at 200 rpm. Equal volumes of chloroform and 50 mM K_2HPO_4 buffer solution (pH 7.4) were added to each sample to generate a biphasic system. Sample containers were placed on a shaker for 30 min at 200 rpm, and centrifuged at 4000 ×*g* for 20 min. In each sample, the upper aqueous, non-lipid contaminants were carefully aspirated and the lower organic lipid phase transferred to a separate Teflon-lined, screw cap vial. These samples were evaporated to dryness under nitrogen gas, and the lipid film suspended in a small volume of 2:1 chloroform: methanol (v/v). Samples were stored at −20 °C until needed.

2.5. Preparation of fatty acid methyl esters (FAMEs)

Preparation of FAMEs was done as previously described (Christie, 2003). A known amount of total lipids or triacylglycerol in 2:1 chloroform: methanol (v/v) was mixed with heptadecanoic acid (17:0, internal standard). The mixture was evaporated under nitrogen gas, and 1 ml of 2% sulphuric acid in methanol (v/v, methylating agent) was added. The mixture was incubated at 80 °C for 1 h, cooled on ice for 10 min, and neutralized with 0.5 mL of 0.5% sodium chloride solution. Fatty acid methyl esters were extracted by addition of 2×2 mL aliquots of hexane and vortexing. The two layers were allowed to separate. The upper hexane layer was recovered. Samples were sent for quantification of fatty acids by gas chromatography, by the Wishart Lab Group (University of Alberta, Department of Biological Sciences, Edmonton, Alberta, Canada).

2.6. Transmission electron microscopy (TEM) of endospores

Control and decoated WSH-L endospore stock solutions were centrifuged at 2700 ×*g* for 5 min. Pellets were prefixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) at room temperature for