1 **Use of the Fluorescent Probe LAURDAN to Label and Measure Inner**
2 **Membrane Fluidity of Endospores of *Clostridium* spp.**
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1 ## Abstract

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

19

20 **Keywords:**

21 *Clostridium*, endospore inner membrane, LAURDAN

22

1. Introduction

Bacterial endospores are widely distributed within the environment (Carlin, 2011; Nicholson, 2002). Endospores of *Clostridium* spp. cause food spoilage and foodborne illness (Borge et al., 2001; Brown, 2000; Cortezzo et al., 2004; Salkinoja-Salonen, 1999). Endospores resist pasteurization and processing at 121°C is required to to extend the storage life of low-acid foods stored at ambient temperature (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 resistance to PATS of endospores relevant for food safety means high temperatures are still required for safe food processing (Margosch et al., 2004a; Margosch et al., 2004b; Reddy et al., 1999; Reddy et al., 2003).

Resistance of endospores to heat and pressure is attributed primarily to low water activity within cores of endospores (Black et al., 2007). 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). The release of DPA coincides with rehydration of the core, and facilitates the inactivation of endospores by heat and pressure (Black et al., 2007; Margosch et al., 2004a; Margosch et al., 2004b; Margosch et al., 2006). A better understanding of the behavior of the inner membranes of endospores during and after physical and chemical treatments will yield information useful in designing specific inactivation strategies for the food industry.

Fluorescent dyes have been used extensively to examine germination of endospores. Fluorescent dyes used in this manner probe the permeability of the endospore membrane.

1 The terbium ion is often used to complex with DPA released during inactivation studies
2 as a measure of thermal resistance and germination (Kort et al., 2005; Reineke et al.,
3 2011). Fluorescent dyes such as SYTO 16, an indicator of germination, and propidium
4 iodide, an indicator of membrane damage in vegetative cells (Bunthof et al., 1999; Ulmer
5 et al., 2000), are only useful for labeling the interior of endospores deficient of Ca-DPA
6 (Baier et al., 2011). The same is true of SYTO 9, Hoechst 33342, and carboxyfluorescein
7 diacetate (CFDA) (Cronin and Wilkinson, 2008).

8 Sporulation studies have employed green fluorescent protein (GFP) or analogues. GFP
9 exhibits utility in studying protein localization and gene expression during sporulation,
10 specifically in *Bacillus* spp. (Asai et al., 2001; Hilbert et al., 2004; McBride et al., 2005).
11 However, green fluorescent protein and its analogues require oxygen for maturation and
12 subsequent fluorescence (Reid and Flynn, 1997) and can be functionally expressed in
13 aerobic endospore-forming bacteria only. The oxygen requirement of GFP precludes its
14 use in endospores of *Clostridium* spp.

15 The membrane dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-
16 hexatrienyl) pyridinium dibromide has been successfully used to dye *Bacillus subtilis*
17 cells during sporulation (Pogliano et al., 1999). This dye preferentially labels membranes,
18 and is able to reveal septal biogenesis and engulfment mechanisms of the sporulation
19 process (Pogliano et al., 1999). Endospores of *Bacillus subtilis* and *Bacillus megaterium*
20 containing the membrane-specific fluorescent dyes dibromide (FM-4-64) and di-4-
21 ANEPPS were successfully generated without adversely affecting the sporulation process
22 (Cowan et al., 2004). The dye FM-4-64 is useful for volumetric assessment of individual
23 endospores (Cowan et al., 2004). Analysis of di-4-ANEPPS using fluorescence recovery

after photobleaching (FRAP) allows measurement of membrane fluidity of individual endospores (Cowan et al., 2004; Koppel, 1985). FRAP of di-4-ANEPPS established that the inner membranes of endospores are immobile, with fluidity returning upon germination (Cowan et al., 2004). However, FRAP analysis cannot rapidly assess inner membrane fluidity of endospore populations during processing under high pressure and / or high temperature conditions. 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 the fluidity of the inner membrane of bacterial endospores of *Clostridium* spp. Endospore membranes were integrated with 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN). LAURDAN has been used in assessing 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; Parasassi et al., 1998). To ascertain the localization of the probe in the inner membrane, LAURDAN-labeled endospores were compared to decoated endospores lacking an outer membrane. *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 refrigeration temperature (4°C) (Broda et al., 1996).

2. Materials and Methods

1 *2.1 Bacterial strains and growth conditions.*

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

6 *2.2 Sporulation and harvesting endospores.*

7 Weihenstephan Südhang (WSH) media was prepared as described (Margosch et al.,
8 2006). Endospores containing LAURDAN (Invitrogen, Cat. No. D-250) (denoted WSH-
9 L endospores in this manuscript) were generated by plating 400 µL of LAURDAN-
10 saturated ethanol onto individual WSH agar plates, allowing ethanol to evaporate in the
11 absence of light, and then plating 100 µL of fully grown RCM broth culture in an
12 anaerobic hood. Plates were incubated anaerobically at 37°C, in the absence of light, for
13 14 days. Sporulation was confirmed using phase-contrast microscopy in which ~95%
14 endospore purity was observed. Endospores were harvested from the surface of WSH
15 agar plates by washing with sterile 0.9% saline solution. Harvested endospores were
16 centrifuged at 2,700 x*g* for 5 min, and suspended in sterile 0.9% saline. Care was taken to
17 discard the top layer of each cell pellet. Endospore stock solutions were standardized to
18 OD_{600} values of 0.5 using sterile 0.9% saline. Control endospore stock solutions were
19 generated as above, omitting addition of LAURDAN-saturated ethanol to WSH agar
20 plates. Standardized endospore stocks were stored at -20°C. Sterile 0.9% saline was used
21 as the endospore suspension medium in subsequent manipulations.

22 *2.3 Removal of endospore coat and outer membrane.*

1 Aliquots (1 mL) of WSH-L endospore stock solutions were centrifuged at 2,700 x*g* for 10
2 min and supernatant removed. Pellets were suspended in a solution of 0.5% SDS, 0.1 M
3 dithiothreitol, 0.1 M NaCl (pH 10, adjusted with 1 M NaOH) for 3 hours at 37°C as
4 described previously (Fitz-James, 1971). Following incubation, endospores were
5 centrifuged at 2,700 x*g* for 10 min and pellets suspended in sterile 0.9% saline to an
6 OD_{600} of 0.5. Samples were kept on ice until used.

7 *2.4 Lipid extraction of Clostridium cells and endospores.*

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

23 *2.5 Preparation of fatty acid methyl esters (FAMEs).*

Preparation of FAMEs was done as described previously (Christie, 2003). A known amount of total lipids or triacylglycerol in 2:1 chloroform: methanol (v/v) was taken together 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 by 0.5 mL of 0.5% sodium chloride solution. Fatty acid methyl esters were extracted by addition of 2 x 2 mL aliquots of hexane and vortexing. The two layers were allowed to separate. The upper hexane layer was recovered. Analysis of membrane fatty acids was performed by service of the Pan Alberta Metabolomics Platform facility (PANAMP, 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 2,700 x*g* for 5 min. Pellets were prefixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) at room temperature for 1 h. Prefixed samples were centrifuged at 2,700 x*g* for 5 min and washed in cacodylate buffer (pH 7.2) for 15 min three times. Samples were then fixed in 1-2% osmium tetroxide (OsO_4) in cacodylate buffer (pH 7.2) at room temperature for 1-3 hs, and washed in distilled water. Washed samples were dehydrated in series using 50%, 70%, 80%, 90%, and absolute ethanol for 15 min each. Two additional dehydration steps using absolute ethanol were done for 10 min each. Absolute ethanol was replaced with propylene oxide, incubated at room temperature for 10 min, followed by two more propylene oxide washes of 10 min each. Samples were then embedded in Araldite CY212 / propylene oxide mixture for 1 h, followed by a pure Araldite CY212 mixture overnight in a vacuum desiccator. Polymerization was done at 60˚C for 2 days. Samples were

observed using a Philips – FEI transmission electron microscope (Morgagni 268, Hillsboro, USA) operating at 80 kV.

2.7 Two-photon confocal laser scanning microscopy (CLSM) of WSH-L endospores.

Control WSH-L and decoated WSH-L endospores (10 µL) were allowed to dry onto the surface of quartz glass slides. Two-photon excitation microscopy experiments were recorded by a confocal laser-scanning microscope (Biorad MRC 1024, extended for multi-photon excitation) coupled via a side-port to an inverted microscope (Nikon, Eclipse TE-300 DV, infinity corrected optics) enabling fluorescence excitation in the focal plane of an objective lens (Nikon Plan Apochromat 60x, water immersion, NA = 1.2, collar rim corr.). By focusing a high peak power laser light source on a diffraction-limited spot, through a high numerical aperture objective, the high photon densities required for two-photon absorption were achieved. Only fluorophores in areas with high photon flux were excited. This offered the advantage that two-photon absorption did not occur in areas above and below the focal plane because of insufficient photon flux densities, and allowed a sectioning effect without the use of emission pinholes as in confocal microscopy. Fluorescence in the PMT-channels (emission bandpass filter at 405 nm/(FWHM 35 nm) and at 460 nm, respectively) was acquired simultaneously using a Ti-Sap-Laser (Coherent, Mira 900-F, 76 MHz repetition rate, ca. 250 fs pulse width, pumped by a 5 W Verdi) tuned to 800 nm for two-photon excitation of LAURDAN. Image acquisition was controlled by LaserSharp2000 software (formerly Biorad, now Zeiss). Images were monitored with a frame rate of ca. 1 Hz, at a resolution of 512 x 512 pixels, and at lowest average laser power of about 10 mW.

2.8 Fluorescence measurement of WSH-L endospores and cells.

1 Vegetative cells and control endospores were labeled with LAUDRAN as described
2 previously (Molina-Höppner et al., 2004). Overnight cell cultures grown in RCM broth
3 were centrifuged, at 2,700 x*g* for 5 min, and suspended in sterile 0.9% saline twice.
4 Ethanol containing LAURDAN was added to a suspension of cells, and unlabeled
5 endospores, to a final concentration of 40 μM. Staining of vegetative cells with
6 LAURDAN was done in the dark at 30°C for 30 min. Suspensions of vegetative cells,
7 labeled endospores, and WSH-L endospores were washed twice by centrifuging at 2,700
8 x*g* for 5 min and suspending in sterile 0.9% saline. Suspensions (100 μL) were aliquotted
9 into 96-well microtitre plates in triplicate. Generalized polarization (GP) values of
10 solutions were calculated as GP = $(I_{440}-I_{490})/(I_{440}+I_{490})$ (Bagatolli et al., 2003) using the
11 relative fluorescence intensity at 440 nm and 490 nm measured at an excitation
12 wavelength of 360 nm with a spectrofluorometer (Varioskan Flash, Thermo Electron
13 Corporation, Nepean, Canada).

14 Fluorescence emission spectra of labeled, unlabeled, and WSH-L endospores were
15 measured following excitation at 360 nm using a specrtrofluorometer (SynergyMx,
16 BioTek, Winooski, USA). Fluorescence emission spectra of labeled and unlabeled cells
17 and WSH-L endospores were measured following excitation at 360 nm using a different
18 spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada).
19 The fluorescence emission spectra of 10 mM LAURDAN suspended in 95% ethanol was
20 measured as above.

21 3. Results

22 *3.1 Viability of endospores following sporulation in LAURDAN.*

1 Viability of endospores labeled with LAURDAN was assessed to determine whether
2 LAURDAN labeling adversely affected physiology. Control and LAURDAN-labeled
3 endospore stock solutions were plated on RCM agar and incubated at 37°C, anaerobically
4 for 6 days. No difference was observed between colony counts of control and
5 LAURDAN-labeled endospores (data not shown).

6 *3.2 Removal of outer membranes of WSH-L endospores.*

7 Endospores contain two membranes, both of which can bind fluorescent dyes. The outer
8 membrane was removed to examine differences in endospores containing both
9 membranes, and those retaining only the inner membrane (Pogliano et al., 1999).
10 Transmission electron microscopy verified the removal of the outer membrane in WSH-L
11 endospores. Endospores of *Clostridium* spp. that contained LAURDAN were subjected to
12 a decoating protocol and compared to untreated WSH-L endospores using TEM. The
13 decoating protocol successfully removed the outer membrane and protein coat of
14 endospores of both *C. beijerinckii* and *C. sporogenes* (Fitz-James, 1971) (Figure 1). The
15 exosporium of endospores of *C. sporogenes* was severely disrupted, but not entirely
16 removed (Figure 1). Little to no exosporium was observed surrounding endospores of *C.*
17 *beijerinckii* before or after decoating (Figure 1). Decoating endospores did not appear to
18 alter their size relative to untreated endospores (Figure 1).

19 *3.3 Lipid composition of Clostridium spp. cells and endospores.*

20 Lipid composition of cells, endospores, and decaoted endospores was analyzed to
21 determine whether differences in the fatty acid composition accounts for differences in
22 LAURDAN GP. Cells of *C. beijerincki* ATCC 8260 and *C. sporogenes* ATCC 7955
23 contained significantly greater quantities of short chain fatty acids (SCFAs) than

1 respective control and decoated endospores (Table 1). Endospores of *C.* sporogenes
2 ATCC 7955 contained significantly more SCFAs and saturated fatty acids (SFAs) than
3 those of *C. beijerinckii* ATCC 8260 (Table 1). No significant differences were observed
4 between control and decoated endospores for both *Clostridium* spp. (Table 1).

5 *3.4 Two-photon CLSM of WSH-L endospores.*

6 Two-photon CLSM of control and decoated WSH-L endospores of *Clostridium* spp. were
7 used to qualitatively examine the fluorescence of individual endospores. Two-photon
8 CLSM visualized individual WSH-L endospores (Figure 2). Fluorescence was observed
9 for both control and decoated WSH-L endospores of both *Clostridium* spp. when a 405
10 nm bandpass filter was used (Figure 2). The 405 nm bandpass filter allowed high (440
11 nm) and low (490 nm) energy emissions to be detected. Fluorescence observed using this
12 filter confirms WSH-L endospores fluoresce individually. No fluorescence was observed
13 when a 460 nm bandpass filter was applied (Figure 2). The 460 nm bandpass filter
14 excludes the low energy emission (490 nm) of LAURDAN from being detected.
15 Decoating did not adversely affect fluorescence of WSH-L endospores.

16 *3.5 Fluorescence emission spectra.*

17 The fluorescence emission spectra of WSH-L endospores (formed during sporulation)
18 and endospores labeled with LAURDAN after sporulation were recorded, before and
19 after washing with 0.9% saline, for comparison of dye retention. Suspension of
20 LAURDAN in 95% ethanol exhibited a characteristic emission peak between 450 and
21 500 nm (data not shown). Unlabeled cells of *Clostridium* spp. did not exhibit detectable
22 fluorescence emission spectra when excited using 360 nm (data not shown); this
23 background fluorescence was subtracted from fluorescence spectra of WSH-L

endospores. Endospores of both *Clostridium* spp. exhibited a higher overall fluorescence intensity when labeled after sporulation in comparison to WSH-L endospores (Figures 3 and 4), which take up LAURDAN during sporulation. However, endospores labeled with LAURDAN after sporulation also exhibited variable retention of fluorescence intensity after being washed in 0.9% saline. Endospores of *C. sporogenes* exhibited a substantial decrease (~50%) in fluorescence intensity (Figure 4B) after washing, whereas endospores of *C. beijerinckii* did not (Figure 3B). The WSH-L endospores for both *Clostridium* spp. retained their original fluorescence intensity after being washed with 0.9% saline (Figures 3D and 4D). The WSH-L endospores were selected for use in subsequent experiments due to this favorably consistent retention of fluorescence after washing.

3.6 GP values of WSH-L endospores and LAURDAN-labeled cells.

GP values were calculated to quantitatively compare fluorescence of LAURDAN-labeled cells to WSH-L endospores, and to determine whether decoating affected fluorescence of endospores. GP values of control WSH-L endospores were similar to those of decoated WSH-L endospores for both strains of *Clostridium* spp. (Table 2). The GP values of both WSH-L- and decoated WSH-L endospores were substantially higher than those of labeled cells (Table 2).

4. Discussion

This study established a protocol to label and measure inner membrane fluidity of populations of endospores of *Clostridium* spp. To verify that LAURDAN fluorescence of WSH-L endospores is not influenced by the outer membrane, intact endospores were compared to decoated endospores. The effect of removing the outer membrane of

1 endospores on LAURDAN fluorescence was measured using two-photon CLSM, GP
2 values and comparison with fatty acid composition. Measuring the fluidity of the inner
3 membrane has potential utility for determining the effects of membrane-active
4 antimicrobials, high pressure, and heat on endospores during and after exposure.

5 The fluorophore LAURDAN preferentially gathers within hydrophobic regions (Parasassi
6 et al., 1998), and the outer membrane of endospores may sequester enough of the
7 fluorophore to influence GP measurements. The core, inner membrane, germ cell wall,
8 cortex, outer membrane and protein coat observed with endospores of *C. sporogenes*
9 ATCC 7955 and *C. beijerinckii* ATCC 8260 are comparable to endospores of *Bacillus*
10 *cereus* and *Bacillus megaterium* (Belliveau et al., 1992; Fitz-James, 1971). Endospores of
11 *C. sporogenes* ATCC 7955 possess an exosporium, whereas those of *C. beijerinckii*
12 ATCC 8260 do not. The presence of an exosporium is species-dependent, and not an
13 essential component of endospore resistance (Koshikawa et al., 1984). Transmission
14 electron microscopy confirmed that decoating WSH-L endospores of *Clostridium* spp.
15 removes the outer membrane, and alters spore morphology consistent with decoating of
16 other endospores (Belliveau et al., 1992; Fitz-James, 1971). Decoating of endospores
17 allowed examination of the influence of the outer membrane on LAURDAN
18 fluorescence.

19 Fluorescence visualization using two-photon confocal excitation is preferable to single
20 photon excitation because it does not photobleach samples (Nicolini et al., 2006). Two-
21 photon CLSM revealed that WSH-L endospores of *Clostridium* spp. integrate and retain
22 LAURDAN after sporulation. Decoating did not alter WSH-L endospore fluorescence.
23 The absence of fluorescence in WSH-L endospores when using a 460 nm bandpass filter,

1 and the fluorescence observed when using a 405 nm bandpass filter suggest LAURDAN
2 is in a highly ordered environment.

3 The LAURDAN emission spectra measured in cells and endospores of labeled clostridia
4 are consistent with the spectra of LAURDAN, confirming membranes of cells and
5 endospores of *Clostridium* spp. were labeled with LAURDAN. Endospores that are
6 labeled during sporulation (WSH-L endospores) retain LAURDAN consistently after
7 washing but endospores labeled after sporulation did not. The WSH-L endospores are
8 preferable for experimental use because consistent dye retention will prevent handling
9 and washing steps involved in experimental preparation from influencing fluorescence
10 readings. Generalized polarization values for LAURDAN in membranes of liquid-
11 crystalline phases typically range from -0.3 to 0.3, and those in the gel phase from 0.5 to
12 0.6 (Sanchez et al., 2007). Experimentally, however, values for cells typically range from
13 -0.3 to 0.6 (Parasassi et al., 1998). The highest GP values are usually observed in pure
14 lipid suspensions during gel state measurements (Parasassi et al., 1998). GP values of
15 decoated WSH-L endospores of *Clostridium* spp. do not differ substantially from those
16 containing the outer membrane. This suggests the outer membrane of endospores does
17 not sequester LAURDAN to an extent capable of adversely affecting accurate GP
18 calculations for endospore populations.

19 The GP values we report for clostridial endospores indicate a high level of order
20 consistent with a gel phase membrane. These high GP values agree with lipid immobility
21 observed in inner membranes of endospores of *Bacillus* spp. (Cowan et al., 2004). Inner
22 membranes exist in a compressed state, with surface areas expanding 1.5-fold upon
23 germination in the absence of lipid synthesis (Cowan et al., 2004). Membrane lipids of

1 endospores of *Bacillus* spp. are similar in both quality and quantity to those of vegetative
2 cells (Bertsch, 1969; Matches, 1964; Nikolopoulou, 1987; Racine, 1980). Endospores of
3 *Clostridium* spp. in this study differ significantly from vegetative cells in content of
4 SCFA and SFAs. However, the membrane fatty acid composition is unlikely to be
5 responsible for the magnitude of differences in GP values observed between cells and
6 endospores of *Clostridium* spp. Shifts in the growth temperature altered the membrane
7 composition in cells of *Lactobacillus plantarum* grown far more substantially than
8 differences observed here between cells and endospores of *Clostridium* spp. (Ulmer et al.,
9 2002). However, the in GP values of *L. plantarum* grown at different temperatures
10 differed by a maximum of 0.2 (Ulmer et al., 2002). The compressed state of inner
11 membranes of endospores reported for *Bacillus* spp. (Cowan et al., 2004) is therefore a
12 more likely contributor to differences in GP values between cells and endospores.

13 In conclusion, endospores containing LAURDAN can successfully be generated on WSH
14 agar, harvested, and GP values measured. Two-photon CLSM and fluorescence
15 spectroscopy are suitable to assess fluorescence of individual endospores and
16 populations, respectively. Outer membranes of WSH-L endospores can be removed
17 without adversely affecting fluorescence, and do not appear to sequester LAURDAN in
18 amounts that significantly interfere with readings. The WSH-L endospores exhibit high
19 GP values consistent with a high degree of inner membrane lipid packing and immobility.
20 The WSH-L endospores may provide a means of assessing endospore changes in inner
21 membrane fluidity during and after a variety of food-processing parameters, including
22 exposure to high temperature, pressure, and antimicrobials, for further the understanding
23 of mechanisms of spore inactivation at these conditions.

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7 ## References

8 Asai, K., Takamatsu, H., Iwano, M., Kodama, T., Watabe, K., and Ogasawara, N. 2001.
9 The *Bacillus subtilis* yabQ gene is essential for formation of the spore cortex.
10 Microbiol.-UK. 147:919-927.
11 Bagatolli, L.A., Sanchez, Hazlett, T., and Gratton, E. 2003. Giant vesicles, laurdan, and
12 two-photon fluorescence microscopy: Evidence of lipid lateral separation in
13 bilayers. Meth. Enzym. 360:481-500.
14 Baier, D., Reineke, K., Doehner, I., Mathys, A., and Knorr, D. 2011. Fluorescence-based
15 methods for the detection of pressure-induced spore germination and inactivation.
16 High Pressure Research. 31:110-115.
17 Belliveau, B.H., Beaman, T.C., Pankratz, H.S., and Gerhardt, P. 1992. Heat killing of
18 bacterial spores analyzed by differential scanning calorimetry. J. Bacteriol.
19 174:4463-4474.
20 Bertsch, L.L., Bonsen, P.P., and Kornberg, A. 1969. Biochemical studies of bacterial
21 sporulation and germination . XIV. Phospholipids in *Bacillus megaterium*. J.
22 Bacteriol. 98:75-81.
23 Black, E.P., Setlow, P., Hocking, A.D., Stewart, C.M., Kelly, A.L., and Hoover, D.G.

1 2007. Response of Spores to High-Pressure Processing. Com. Rev. Food Sci. Food
2 Safety. 6:103-119.
3 Borge, G.I.A., Skeie, M., Sorhaug, T., Langsrud, T., and Granum, E. 2001. Growth and
4 toxin profiles of *Bacillus cereus* isolated from different food sources. Int. J. Food
5 Microbiol. 6:237-246.
6 Broda, D.M., DeLacy, K.M., Bell, R.G., Braggins, T.J., and Cook, R.L. 1996.
7 Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled
8 vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Intl. J.
9 Food Microbiol. 29:335-352.
10 Brown, K. 2000. Control of bacterial spores. Brit. Med. Bulletin. 56:58-71. Bunthof, C.J.,
11 van den Braak, S., Breeuwer, P., Rombouts, F.M., and Abee, T. 1999. Rapid
12 fluorescence assessment of the viability of stressed *Lactococcus lactis*. Appl.
13 Environ. Microbiol. 65:3681-3689.
14 Carlin, F. 2011. Origin of bacterial spores contaminating foods. Food Microbiol. 28:177-
15 182.
16 Christie, W. 2003. *Lipid Analysis – 3rd Edition*. Bridgewater: The Oily Press. Collins,
17 M.D., and East, A.K. 1998. Phylogeny and taxonomy of the food-borne pathogen
18 *Clostridium botulinum* and its neurotoxins. J. Appl. Microbiol. 84:5-17.
19 Cortezzo, D.E., Setlow, B., and Setlow, P. 2004. Analysis of the action of compounds
20 that inhibit the germination of spores of *Bacillus* species. J. Appl. Food Microbiol.
21 96:725-741.

1 Cowan, A.E, Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., and Setlow, P.
2 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are
3 largely immobile. Proc. Natl. Aca. Sci. USA. 101:7733-7738.
4 Cronin, U.P. and Wilkinson, M.G. 2008. Monitoring changes in germination and
5 permeability of *Bacillus cereus* endospores following chemical, heat and enzymatic
6 treatments using flow cytometry. J Rapid Methods Automation Microbiol. 16:164-
7 184.
8 Fitz-James, P.C. 1971. Formation of protoplasts from resting spores. J. Bacteriol.
9 105:1119-1136.
10 Harris, F.M., Best, K.B., and Bell, J.D. 2002. Use of laurdan fluorescence intensity and
11 polarization to distinguish between changes in membrane fluidity and phospholipid
12 order. Biochim. Biophys. Acta. Biomembranes. 1565:123-128.
13 Hilbert, D.W., Chary, V.K., and Piggot, P.J. 2004. Contrasting effects of sigma(E) on
14 compartmentalization of sigma(F) activity during sporulation of *Bacillus subtilis*. J.
15 Bacteriol. 186:1983-1990.
16 Koppel, D.E. 1985. Normal-mode analysis of lateral diffusion on a bounded membrane
17 surface. Biophys. J. 47:337-347.
18 Kort, R., O'Brien, A.C., van Stokkum, I.H.M., Oomes, S.J.C.M., Crielaard, W.,
19 Hellingwerf, K.J., and Brul, S. 2005. Assessment of heat resistance of bacterial
20 spores from food isolates by fluorescence monitoring of dipicolinic acid release.
21 Appl. Environ. Microbiol. 71:3556-3564.
22 Koshikawa, T., Beaman, T.C., Pankratz, H.S., Nakashio, S., Corner, T.R., and Gerhardt,
23 P. 1984. Resistance, germination, and permeability correlates of *Bacillus*

1 *megaterium* spores successively divested of integument layers. J. Bacteriol. 159:
2 624-632.
3 Lee, M.J., Dong-Ho, B., Dong-Ha, L., Ki-Hyo, J., Deog-Hwan, O., and Sang-Do, H.
4 2006. Reduction of *Bacillus cereus* in Cooked Rice Treated with Sanitizers and
5 Disinfectants. J. Microbiol. Biotech. 16:639-642.
6 Margosch, D., Ehrmann, M.A., Buckow, R., Heinz, V., Vogel, R.F., and Gänzle, M.
7 2006. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus*
8 *amyloliquefaciens* endospores at high temperature. Appl. Environ. Microbiol.
9 72:3476-3481.
10 Margosch, D., Ehrmann, M.A., Gänzle, M.G., and Vogel, R.F. 2004a. Comparison of
11 pressure and heat resistance of *Clostridium botulinum* and other endospores in
12 mashed carrots. J. Food Protection. 67:2530-2537.
13 Margosch, D., Gänzle, M.G., Ehrmann, M.A., and Vogel, R.F. 2004b. Pressure
14 inactivation of *Bacillus* endospores. Appl. Environ. Microbiol. 70:7231-7238.
15 Matches, J.W. 1964. Phospholipids in vegetative cells and spores of *Bacillus polymyxa*.
16 J. Bacteriol. 87:16-23.
17 McBride, S.M., Rubio, A., Wang, L., and Haidenwang, W.G. 2005. Contributions of
18 protein structure and gene position to the compartmentalization of the regulatory
19 proteins sigma(E) and SpoIIE in sporulating *Bacillus subtilis*. Molec. Microbiol.
20 57:434-451.
21 Meyer, R.S., Cooper, K.L., Knorr, D., Lelieveld, H.L.M. 2000. High-pressure
22 sterilization of foods. Food. Tech. 54:67-72.

1 Molina-Höppner, A., Doster, W., Vogel, R.F., and Gänzle, M.G. 2004. Protective effect
2 of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal
3 high-pressure treatments. Appl. Env. Microbiol. 70:2013-2020.
4 Nicholson, W.L. 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life
5 Sci. 59:410-416.
6 Nicolini, C., Celli, A., Gratton, E., and Winter, R. 2006. Pressure tuning of the
7 morphology of heterogeneous lipid vesicles: a two-photon-excitation fluorescence
8 microscopy study. Biophys. J. 91:2936-2942.
9 Nikolopoulou, M. 1987. Lipid composition of *Bacillus megaterium* spores and spore
10 membranes. Lipids. 22:698-703.
11 Paidhungat, M.S. 2000. Characterization of spores of *Bacillus subtilis* which lack
12 dipicolinic acid. J. Bacteriol. 182: 5505-5512.
13 Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R.M., and Gratton, E. 1991.
14 Quantitation of lipid phases in phospholipid vesicles by the generalized polarization
15 of Laurdan fluorescence. Biophys. J. 60:179-189.
16 Parasassi, T., Krasnowska, E.K., Bagatolli, L., and Gratton, E. 1998. Laurdan and Prodan
17 as polarity-sensitive fluorescent membrane probes. J. Fluor. 8:365-373.
18 Pogliano, J., Osborne, N., Sharp, M.D., Abanes-De Mello, A., Perez, A., Sun, Y.L., and
19 Pogliano, K. 1999. A vital stain for studying membrane dynamics in bacteria: a
20 novel mechanism controlling septation during *Bacillus subtilis* sporulation. Mol.
21 Microbiol. 31:1149-1159.
22 Racine, F. 1980. Isolation and properties of membranes from *Bacillus megaterium*
23 spores. J. Bacteriol. 143:1208-1214.

1 Reddy, N.R., Solomon, H.M., Fingerhut, G.A., Balasubramaniam, V.M., and
2 Rhodehamel, E.J. 1999. Inactivation of *Clostridium botulinum* Type E spores in a
3 model buffer or food system by high pressure processing. Presented at the Annual
4 Meeting of the Institute of Food Technologists. J. Food Saf. 19:277-288.
5 Reddy, N.R., Solomon, H.M., Tetzloff, R.C., and Rhodehamel, E.J. 2003. Inactivation of
6 *Clostridium botulinum* Type A spores by high-pressure processing at elevated
7 temperature. J. Food Prot. 66:1402-1407.
8 Reid, B.G. and Flynn, G.C. 1997. Chromophore formation in green fluorescent protein.
9 Biochemistry. 36:6786-6791.
10 Reineke, K., Mathys, A., and Knorr, D. 2011. The impact of high pressure and
11 temperature on bacterial spores: Inactivation mechanisms of *Bacillus subtilis* above
12 500 MPa. J. Food Sci. 76:M189-M197.
13 Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kampfer, P., Andersson, M.C.,
14 Honkanen-Buzalski, T., and Scoging, A.C. 1999. Toxigenic strains of *Bacillus*
15 *licheniformis* related to food poisoning. Appl. Environ. Microbiol. 65:4637-4645.
16 Sanchez, S.A., Tricerri, M.A., Gunther, G., and Gratton, E. 2007. Laurdan generalized
17 polarization: from cuvette to microscope (Ed: Mendez-Vilas, A., and Diaz, J.).
18 Modern Research and Educational Topics in Microscopy. Pg. 1007-1014.
19 Setlow, P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation,
20 heat and chemicals. J. Appl. Microbiol. 101:514-525.
21 Ulmer, H.M., Gänzle, M.G., and Vogel, R.F. 2000. Effects of high pressure on survival
22 and metabolic activity of *Lactobacillus plantarum* TMW1.460. Appl. Environ.
23 Microbiol. 66:3966-3973.

1 Ulmr, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R., and Vogel, R.F. 2002.
2 Effects of pressure-induced membrane phase transitions on inactivation of HorA,
3 on ATP-Dependant Multidrug Resistance Transporter, in *Lactobacillus plantarum*.
4 Appl. Environ. Microbiol. 68:1088-1095.
5 Wilson, M.J., and Baker, R. 1997. High temperature/ulta-high pressure sterilization of
6 low acid foods. PCT IB9601500.
7

1 **Figure Captions**

2 **Figure 1.** Transmission electron microscopy (TEM) photos of endospores of *C.*
3 *sporogenes* and *C. beijerinckii* with outer membrane and protein coat removed. For each
4 *Clostridium* spp., twenty endospores from four photos were assessed. Photos presented in
5 Figure 1 are representative of observations noted for endospores of respective
6 *Clostridium* spp.

7 **Figure 2.** Two-photon confocal laser scanning microscopy photos of WSH-L endospores
8 of *Clostridium* spp. before and after decoating. Samples were visualized at (A) 405 nm
9 and (B) 460 nm, using bandpass filters, following two-photon excitation.

10 **Figure 3.** Fluorescence emission spectra of endospores of *C. beijerinckii* labeled after
11 sporulation or during sporulation (WSH-L endospores) with LAURDAN. Samples were
12 visualized following excitation at 360 nm using a spectrofluorometer. (A) endospores
13 labeled after sporulation; (B) endospores labeled after sporulation and washed with 0.9%
14 saline; (C) WSH-L endospores; (D) WSH-L endospores washed with 0.9% saline.

15 **Figure 4.** Fluorescence emission spectra of endospores of *C. sporogenes* labeled after
16 sporulation or during sporulation (WSH-L endospores) with LAURDAN. Samples were
17 visualized following excitation at 360 nm using a spectrofluorometer. (A) endospores
18 labeled after sporulation; (B) endospores labeled after sporulation and washed with 0.9%
19 saline; (C) WSH-L endospores; (D) WSH-L endospores washed with 0.9% saline.

20

Table 1. Total fatty acid content in cells and endospores of *Clostridium sporogenes* ATCC 7955 and *Clostridium beijerinckii* ATCC 8260.[a] A comparison of ratios of short chain (SCFA) and long chain fatty acids (LCFA), as well as saturated (SFA) and unsaturated fatty acids (USFA), between cells and endospores.

	Clostridium sporogenes ATCC 7955			*Clostridium beijerinckii* ATCC 8260		
Fatty Acid	Cells	Control Endospores	Decoated Endospores	Cells	Control Endospores	Decoated endospores
	Mol% FA /mg lipid ± Std. Dev.	Mol% FA /mg lipid ± Std. Dev.	Mol% FA /mg lipid ± Std. Dev.	Mol% FA /mg lipid ± Std. Dev.	Mol% FA /mg lipid ± Std. Dev.	Mol% FA /mg lipid ± Std. Dev.
12:0	0.208 ± 0.046	0.0633 ±0.017	0.0765 ± 0.017	0.287 ± 0.038	0.164 ± 0.038	0.243 ± 0.12
13:0	0.110 ± 2.3x10^-3	0.0662 ± 4.7x10^-3	0.0684 ± 7.2x10^-3	0.0869 ± 0.012	0.0789 ± 4.7x10^-3	0.0977 ± 0.019
14:0	28.4 ± 1.3	27.2 ± 0.17	29.8 ± 2.4	28.6 ± 5.3	23.7 ± 1.5	22.1 ± 0.83
i15:0	0.124 ± 0.024	0.182 ± 2.9x10^-3	0.152 ± 2.0x10^-3	0.0977 ± 0.018	0.162 ± 0.014	0.171 ± 0.012
a15:0	2.85 ± 0.26	0.371 ± 0.087	0.268 ± 0.016	1.73 ± 0.22	0.541 ± 0.18	0.926 ± 0.30
15:0	2.01 ± 0.098	2.02 ± 0.050	1.85 ± 0.093	1.64 ± 0.24	1.40 ± 0.089	1.42 ± 0.082
i16:0	5.22 ± 0.34	4.89 ± 0.24	3.57 ± 0.33	6.67 ± 1.0	3.85 ± 0.35	3.30 ± 0.10
16:0	44.6 ± 1.6	47.2 ± 0.66	45.5 ± 1.3	44.2 ± 1.1	48.3 ± 2.8	47.1 ± 0.95
16:1	4.95 ± 0.90	4.83 ± 0.37	4.96 ± 0.74	4.72 ± 0.67	3.83 ± 0.39	4.97 ± 0.75
i17:0	0.097 ± 0.033	0.123 ± 0.025	0.155 ± 3.0x10^-3	0.430 ± 0.089	0.132 ± 0.015	0.143 ± 0.053
17:0cy	1.34 ± 0.23	1.61 ± 0.24	1.98 ± 0.54	2.14 ± 0.63	1.62 ± 0.56	2.42 ± 0.52
18:0	3.88 ± 0.53	4.76 ± 0.23	4.59 ± 0.74	3.45 ± 0.34	6.22 ± 0.41	6.22 ± 1.4
18:1	1.36 ± 0.39	1.49 ± 0.16	1.68 ± 0.15	2.63 ± 0.044	1.70 ± 0.13	2.18 ± 0.31
18:1(9E)	2.80 ± 0.25	3.59 ± 0.35	2.79 ± 0.31	2.93 ± 0.32	6.56 ± 0.61	6.03 ± 0.33
18:2	0.250 ± 0.090	0.264 ± 0.079	0.313 ± 0.067	0.0836 ± 9.4x10^-3	0.347 ± 0.097	0.530 ± 0.090
19:0cy	0.463 ± 0.083	0.613 ± 0.084	0.723 ± 0.11	0.209 ± 0.046	0.431 ± 0.10	0.947 ± 0.21
19:0	0.858 ± 0.25	0.352 ± 0.038	0.995 ± 0.20	0.226 ± 0.016	0.380 ± 0.072	0.722 ± 0.25
20:0	0.139 ± 0.099	0.134 ± 0.011	0.170 ± 0.046	0.0535 ± 0.027	0.162 ± 0.040	0.162 ± 0.078
22:0	0.0550 ± 0.020	0.0617 ± 0.010	0.0766 ± 0.034	0.0208 ± 5.3x10^-3	0.0706 ± 6.0x10^-3	0.0760 ± 0.040
22:1(13)	0.300 ± 0.085	0.179 ± 0.096	0.241 ± 0.021	0.0693 ± 0.021	0.328 ± 0.048	0.330 ± 0.21

	P-Values of T-Tests Comparing Fatty Acid Ratios Within *Clostridium* spp.			
	Clostridium sporogenes ATCC 7955		*Clostridium beijerinckii* ATCC 8260	
Ratios Compared	SCFA / LCFA	SFA / USFA	SCFA / LCFA	SFA / USFA
Cells:Control Endospores	0.053	0.293	<0.001*	0.037*
Cells:Decoated Endospores	0.063	0.595	<0.001*	0.009*
Control:Decoated Endospores	0.795	0.543	0.323	0.168

	P-Values of T-Tests Comparing Fatty Acid Ratios Between *Clostridium* spp.	
	SCFA / LCFA	SFA / USFA
Control : Control Endospores	<0.001*	0.014*
Decoated : Decoated Endospores	0.002*	0.005*

[a] Values represent mean ± standard deviation of three replicates.
* Statistically significant.

1 **Table 2.** Generalized polarization values of LAURDAN-labeled cells and WSH-L

2 endospores of *Clostridium* spp.[a] suspended in 0.9% saline.

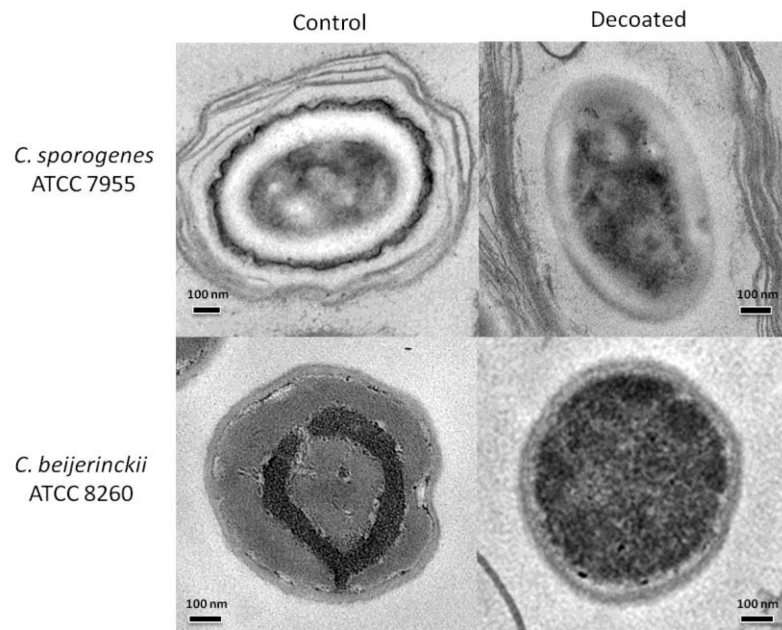
	Cells	Control Endospores	Decoated Endospores
C. beijerinckii	0.35 ± 0.00	0.73 ± 0.00	0.74 ± 0.027
C. sporogenes	0.31 ± 0.012	0.77 ± 0.010	0.77 ± 0.031

3 [a] Values represent mean ± standard deviation of three replicates.

4

5

1 **Figure 1.**



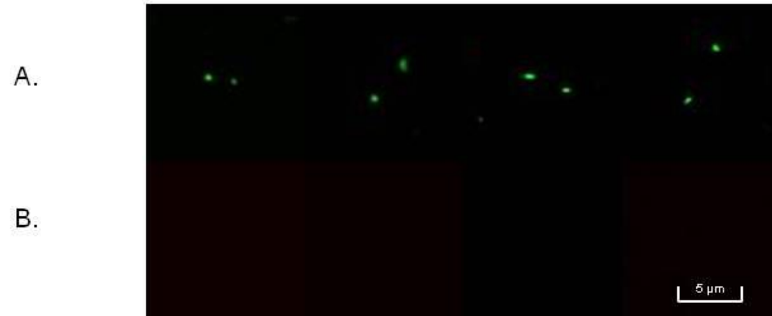
2

3

1 **Figure 2.**

C. beijerinckii ATCC 8260 | *C. sporogenes* ATCC 7955

Control Decoated Control Decoated



2

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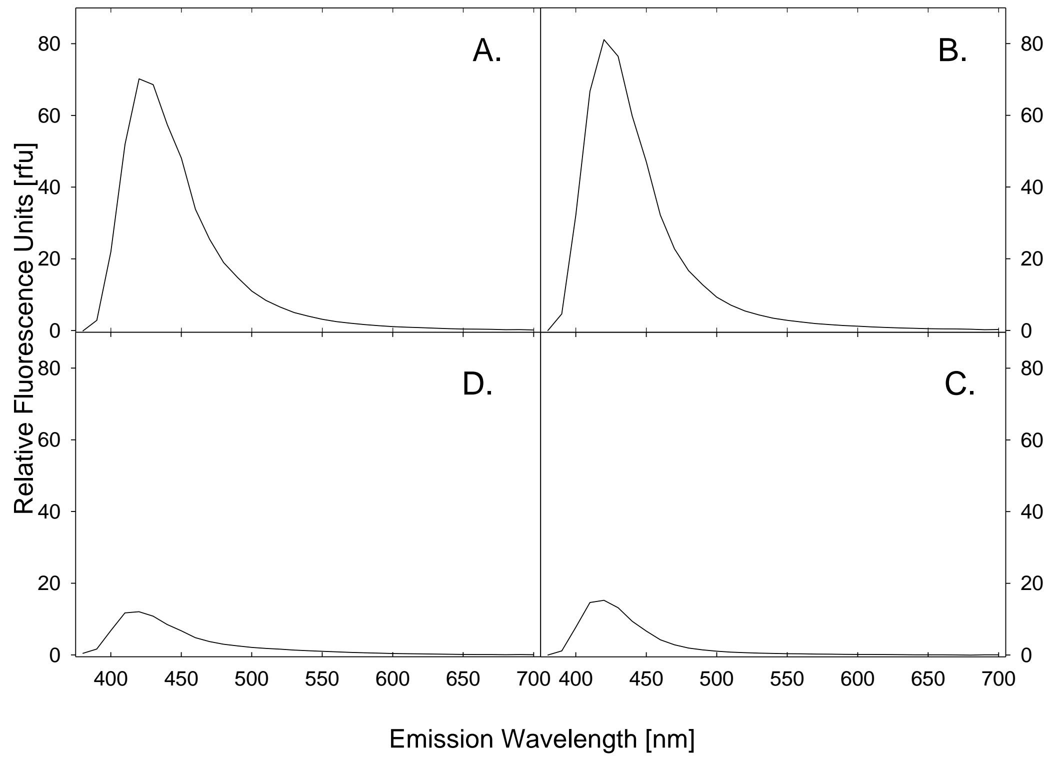
1 **Figure 3.**



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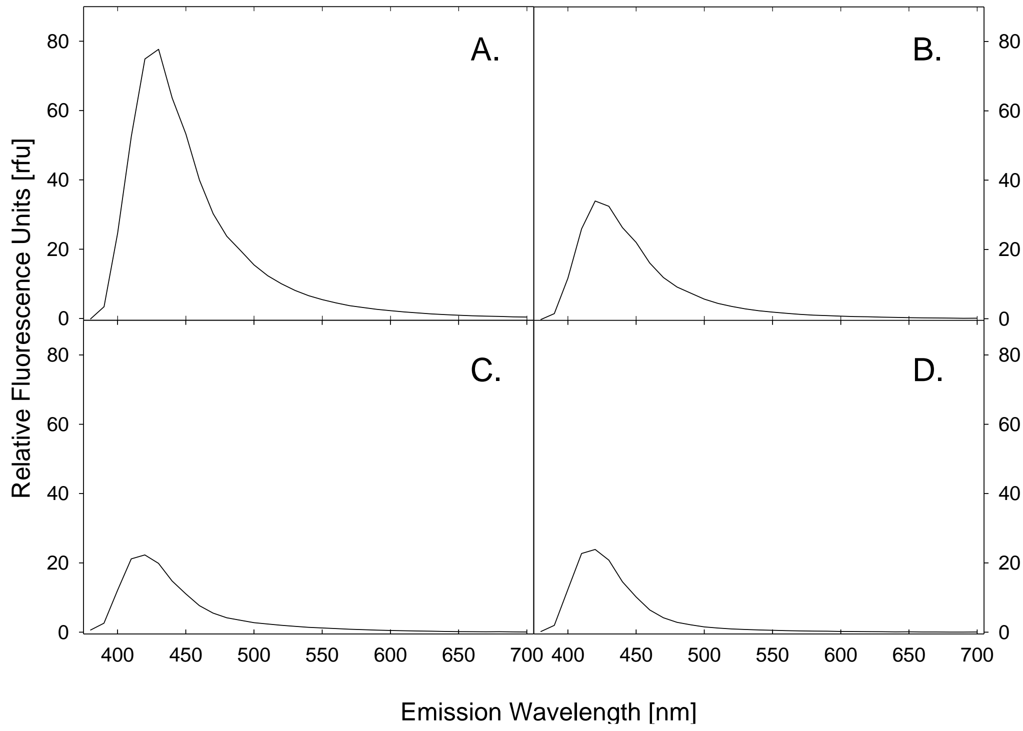
1 **Figure 4.**



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