Journal of Microbiological Methods 77 (2009) 29–36

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

# Permeabilization and hybridization protocols for rapid detection of *Bacillus* spores using fluorescence *in situ* hybridization

Geneviève Filion [a,b], Christian Laflamme [a,b], Nathalie Turgeon [a,b], Jim Ho [c], Caroline Duchaine [a,b,*]

[a] Centre de recherche de l'Hôpital Laval, Institut universitaire de cardiologie et de pneumologie de l'Université Laval, 2725 Chemin Ste-Foy, Québec city, Québec, Canada G1V 4G5
[b] Département de biochimie et de microbiologie, Faculté des sciences et de génie, Université Laval, 1045 avenue de la médecine, Québec city, Québec, Canada G1V 0A6
[c] Biological Detection and Identification Section, Defence Research & Development Suffield, (DRDC-S) Box 4000, Medicine Hat, Alberta, Canada T1A 8K6

ARTICLE INFO

*Article history:*
Received 17 December 2008
Received in revised form 19 December 2008
Accepted 19 December 2008
Available online 31 December 2008

*Keywords:*
Fluorescence *in situ* hybridization
*Bacillus* spores
Permeabilization
Fluorescence microscopy
Electron microscopy

ABSTRACT

*Background*: Fluorescence *in situ* hybridization (FISH) is not adapted for the detection of bacterial spores because of their resistance to conventional permeabilization treatments. Since spore-forming bacteria have important ecological, economical, and medical roles, their *in situ* detection needs to be improved. The aim of this study was to develop rapid and effective protocols to permeabilize *Bacillus* spores in order to apply the FISH technique. *Methods*: Permeabilization protocols were developed for three species of *Bacillus* spores. Hybridization was performed using universal and specific probes. Surface structural analysis of the permeabilization treatments was performed using scanning electron microscopy. *Results*: With the proposed protocols, *Bacillus* spores can be labeled in less than 1 h. The scanning microscopy showed some visible structural differences between the permeabilized spores compared to intact ones. *Conclusion*: For the first time, rapid and effective protocols to detect *Bacillus* spores by FISH were developed and can be applied to study *Bacillus* spores using *in situ* labeling within 1 h. Previously published *in situ* hybridization protocols have never reached or been close to the currently described rapidity. This work will contribute to the possibility of near real time detection of biological threats that may be present as spores.


## 1. Introduction

To facilitate the study of microorganisms in mixed environments, molecular methods were developed to allow the detection and the characterization of the microbial population without culturing. Amongst these techniques, fluorescence *in situ* hybridization (FISH) is widely used for the analysis of bacteria in complex environmental samples including activated sludge (Lenaerts et al., 2007), marine environments (Pernthaler et al., 2002; Sekar et al., 2004), biofilms (Bottari et al., 2006), soil (Bertaux et al., 2007), agricultural environments (Lange et al., 1997) and in medical diagnosis (Bottari et al., 2006).

The FISH technique employs oligonucleotide probes targeted at specific regions of the 16S ribosomal RNA in the bacteria (Amann et al., 2001; Glockner et al., 1999). Its advantages being rapid, sensitive, specific, reliable and adaptable for heterogeneous environments (Bottari et al., 2006). However, the procedure does not work well with spores due to their inherent resistance to probe penetration. In natural environments, microorganisms such as *Bacillus* and *Clostridium* form spores, providing them with survival potential in unfavourable conditions such as dryness, UV light, extreme pH and temperatures. Spores can survive for several years, even decades (Castanha et al., 2006; Driks, 2007; Oncu et al., 2003) contributing to problems such as food-borne diseases, bioterrorism (*B. anthracis*) and many others (Panessa-Warren et al., 1997).

Another limitation of the FISH approach is that to achieve effective nucleic acid labeling and obtain detectable signal, high number of intracellular target sequences are required. Targeting 16S rRNA overcomes this limitation and offers wide possibilities of specific and universal probe design (Bottari et al., 2006). According to Chambon et al. (1968), a bacterial spore contains the same amount of rRNA as a vegetative cell in log phase. Therefore, if spores could be permeabilized, it would be possible to perform successful *in situ* labelling with fluorescent probes and detect the resulting fluorescent signal.

Some of the challenges in spore permeabilization to facilitate FISH labeling and detection are due to the various spore coat layers that include the exosporium, the coat, the cortex, and the inner membrane. The exosporium is the most external layer and is present in some species including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus megaterium*, *Clostridium sporogenes* and *Clostridium difficile* but not in others such as *Bacillus atrophaeus* (Driks, 2007; Panessa-Warren et al., 1997). This external layer is made up of a majority of glycoproteins, proteins and lipids. The coat is critical to spore resistance (Driks, 2007; Setlow, 2006) and is mostly composed of proteins. Peptidoglycan is the major

* Corresponding author. Centre de Recherche de l'Hôpital Laval, 2725 Chemin Ste-Foy, Quebec city, Quebec, Canada G1V 4G5. Tel.: +1 418 656 8711x5837; fax: +1 418 656 4509.
*E-mail address:* caroline.duchaine@bcm.ulaval.ca (C. Duchaine).

0167-7012/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2008.12.009

**Table 1**
Probes sequence

| Probes | Sequences 5′-3′ | Target organisms | Fluorochrome-coupled | References |
|---|---|---|---|---|
| EUB338 | GCT GCC TCC CGT AGG AGT | Eubacteria | FITC or Alexa 546 | Amann et al. (1990) |
| *Not*EUB338 | CGA CGG AGG GCA TCC TCA | Control | FITC or Alexa 546 | |
| pB394 | ATG CGG TTC AAA ATG TTA TCC GG | *B. cereus* | Alexa 488 | Liu et al. (2001) |
| *Not*pB394 | TAC GCC AAG TTT TAC AAT AGG CC | Control | Alexa 488 | |

component of the cortex whereas the inner membrane, responsible for the extremely low permeability to several compounds, surrounds the core that contains the DNA and other genetic materials (Driks, 2007; Setlow, 2003). A mixture of various chemicals and enzymes, applied sequentially, would be required to weaken each layer. For FISH experiments, the permeabilization treatments must allow the probes to reach the rRNA within while maintaining the structural integrity of the spore in order to prevent the loss of the intracellular content. Although two papers describing FISH on spores have been published in the literature, these protocols allowed only the detection of *B. megaterium* spores and *Bacillus* ribotype DA001 endospores and required days (2–3 days) to obtain the results (Felske et al., 1998; Fischer et al., 1995).

The aim of this study was to develop a rapid permeabilization and hybridization protocol to facilitate labelled probe entry into *Bacillus* spores in pure culture and in heterogeneous cultures making FISH detection applicable as a viable near real time method.

Strains of three spores forming bacteria (*B. megaterium*, *B. atrophaeus* and *B. cereus*) were used as models. The permeabilization treatments involved various chemicals and enzyme treatments sequentially with the composition of the spore membranes. The protocol was successfully designed and it allows the detection *Bacillus* spores within 1 h using universal probes as well as specific probes for spores in mixed cultures. We also used scanning electron microscopy to demonstrate the effects of the permeabilization treatments on spore surfaces.

## 2. Materials and methods

### 2.1. Bacterial species, media, and spore purification

Different *Bacillus* sp. strains were used in this study: *B. megaterium* ATCC 14581, *B. atrophaeus* (also known as *B. globigii* or *B. subtilis* var *niger*-provided by DRDC Suffield, Canada) and *B. cereus* ATCC 14579. The spores of *B. megaterium* and *B. atrophaeus* were obtained on a solid sporulation medium containing nutrient agar (Difco, Sparks, MD, USA) with 0.5% yeast extract, 0.7 mM $CaCl_2$, 1 mM $MgCl_2$•$6H_2O$, 50 µM $MnCl_2$•$4H_2O$, final pH of 6.8, incubated at 37 °C for a week (Laflamme et al., 2005). The spores were purified in a sodium bromide density gradient as previously described (Laflamme et al., 2005). The spores were kept in PBS at 4 °C until used. *B. cereus* spores were obtained in a define liquid sporulation media YLHG without glucose but containing 20 mM L-glutamic acid, 6 mM L-leucine, 2.6 mM L-valine, 1.4 mM L-threonine, 0.47 mM L-methionine, 0.32 mM L-histidine, 25 mM D/L-lactic acid, 1 mM acetic acid, 50 mM $FeCl_3$, 2.5 µM $CuCl_2$, 12.5 µM $ZnCl_2$, 66 µM $MnSO_4$, 1 mM $MgCl_2$, 2.5 mM $(NH_4)_2SO_4$, 2.5 µM $Na_2MoO_4$, 2.5 µM $CoCl_2$, 1 mM $Ca(NO_3)_2$ buffered at pH 7.2 with 100 mM of potassium phosphate (de Vries et al., 2005). The culture was incubated at 30 °C and shaken at 225 RPM for a week. The spores were harvested by centrifugation (4000 *g*, 10 min) and washed twice in PBS (13,400 *g*, 2 min) and kept in PBS at 4 °C until their used. *Escherichia coli* DH5α and *B. cereus* ATCC 14579 vegetative cells were cultured both in tryptic soy broth (TSB) (Difco) at 37 °C and shaken at 100 RPM overnight. *Lactococcus lactis* MG1363 (formerly *Streptococcus lactis*) was cultured overnight at room temperature without shaking in M17 media (Difco) supplemented with 0.5% glucose. *E. coli*, *B. cereus* vegetative cells and *L. lactis* were washed twice in PBS and used immediately.

### 2.2. Spore permeabilization

The permeabilization treatments were optimized for each strain in order to obtain higher percentage of positive spores and stronger fluorescence signal after the hybridization. Approximately $10^8$ spores were used for the permeabilization treatments.

#### 2.2.1. B. megaterium

The spores were permeabilized in two steps. First, they were suspended in 1 mL of 10 mg/mL SDS (sodium dodecyl sulfate) and 50 mM DTT (1,4-dithiothreitol) (Fluka, St-Louis, MO, USA) and incubated for 15 min at 65 °C. Samples were shaken every 5 min during the incubation. Spores were harvested by centrifugation at 13,400 *g* for 2 min and washed once with 1 mL of PBS. Then they were resuspended in 1 mL of 1 mg/mL lysozyme (Sigma-Aldrich, St-Louis, MO, USA) in 100 mM Tris/HCl and 5 mM EDTA buffer and incubated for 15 min at 37 °C. This was followed by two washes in 1 mL of PBS.

#### 2.2.2. B. atrophaeus

The spores were permeabilized in two steps. First, they were suspended in 0.5 mL of 8 M urea, 20 mg/mL SDS and 100 mM DTT in 500 mM Tris pH 10 buffer and incubated for 15 min at 60 °C and shaken every 5 min. Then they were washed once in 1 mL of PBS and resuspended in 1 mL of 7 mg/mL lysozyme and 7 U/mL mutanolysin (Yokogawa et al., 1974) in 100 mM Tris/HCl and 5 mM EDTA buffer and incubated for 15 min at 37 °C. The permeabilized spores were then washed twice in 1 mL of PBS.

#### 2.2.3. B. cereus

The spores were treated with 0.5 mL of 30 ng/mL proteinase K (Sigma-Aldrich) and 6 mg/mL SDS in 10 mM Tris and 1 mM EDTA pH 8 buffer for 5 min at 37 °C then washed twice in 1 mL of PBS. The permeabilization was continued as described for *B. atrophaeus*.

**Table 2**
Comparison of the optimized protocols for all the bacterial models

| Treatments | *B. megaterium* | *B. atrophaeus* | *B. cereus* | Universal protocol |
|---|---|---|---|---|
| Proteinase K (30ng/mL) | – | – | 5 min, 37 °C | – |
| SDS (10 mg/mL) + DTT (50 mM) | 15 min, 65 °C | – | – | – |
| Urea (8M) + SDS (20 mg/mL) + DTT (100 mM) | – | 15 min, 60 °C | 15 min, 60 °C | 15 min, 60 °C |
| PBS washing (1) | Yes | Yes | Yes | Yes |
| Lysozyme (1 mg/mL) | 15 min, 37 °C | – | – | – |
| Lysosyme (7 mg/mL) + Mutanolysin (7 U/mL) | – | 15 min, 37 °C | 15 min, 37 °C | 15 min, 37 °C |
| PBS washing (2) | Yes | Yes | Yes | Yes |
| Hybridization | 15 min | 15 min | 15 min | 15 min |
| Buffer number | 1 | 1 | 2 | 2 |
| Temperature | 42 °C | 42 °C | 46 °C | 46 °C |
| Total time | 1 h | 1 h | 1 h | 1 h |

### 2.3. Fluorescent in situ hybridization (FISH)

Universal probes (EUB338 and *not*EUB338) coupled with FITC were used (Table 1). For optimize the fluorescent signal, different buffers were used depending on the bacterial models. The final concentration of probes was 1.62 ng/μL for all the models. For *B. megaterium* and *B. atrophaeus* spores, the hybridization was performed in buffer 1 (630 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, 0.01% SDS, pH 7.2, 30% formamide (Sigma-Aldrich)) for 15 min at 42 °C. For *B. cereus*, the hybridization was performed in buffer 2 (0.9 M NaCl, 0.01% SDS, 20 mM Tris/HCl, pH 7.6) for 15 min at 46 °C. The hybridization reactions were stopped in 300 μL of ice-cold PBS.

### 2.4. Universal protocol

A universal protocol was done in order to detect spores from *Bacillus*. The permeabilization was done as described for *B. atrophaeus* spores. Each *Bacillus* strains were permeabilized and hybridized individually. Universal probes (EUB338 and *not*EUB338) coupled with Alexa 546 were used at final concentration of 2,46 ng/μL. The

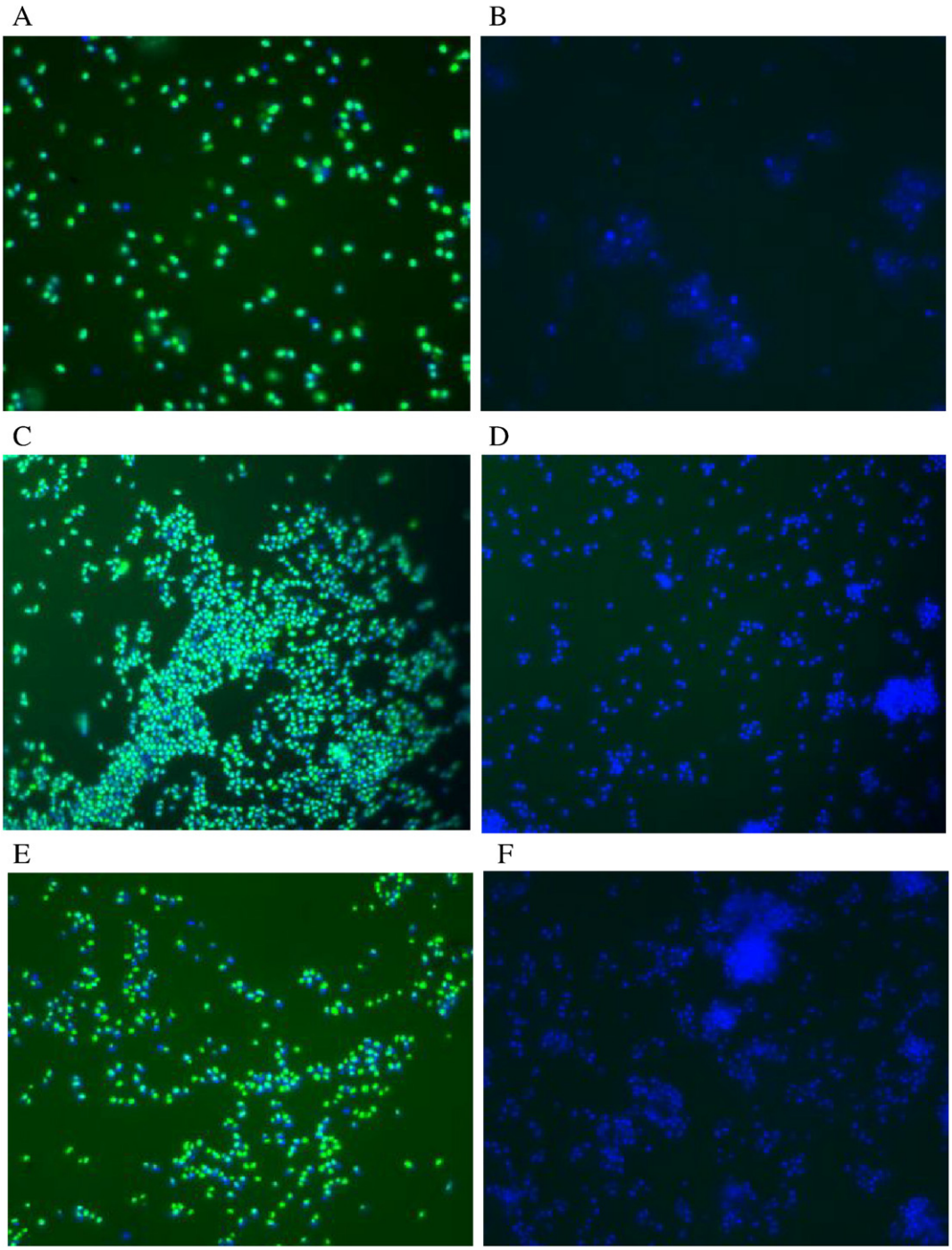


**Fig. 1.** FISH staining on different species of *Bacillus* spores permeabilized and hybridized with the rapid protocol. The spores were hybridized with a universal probe EUB338 (left) and with a control probe *Not*EUB338 (right). All the spores were counterstained with DAPI (blue) and hybridized with EUB-FITC (green). A and B) *B. megaterium*, C and D) *B. atrophaeus*, E and F) *B. cereus*. Magnification: 1000×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hybridization was performed in buffer 2 for 15 min at 46 °C. The reactions were stopped in 300 μL of ice-cold PBS.

### 2.5. Fluorescence microscopy

After the hybridization, the spores were counterstained with 0.1 μM of 4′6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and then filtered under 10 kPa of pressure onto polycarbonate filters 0.22 μm (Millipore, Bedford, MA, USA). The samples were washed twice with PBS. A drop of mowiol (Calbiochem, San Diego, CA, USA) was put between the filter and the microscopic slide. The spores were observed using a fluorescent microscopy (Nikon Eclipse 6600 connected with a system for picture capture SPOT RT slider version 3.1, (Diagnostic instrument, INC).

### 2.6. Specific detection of B. cereus in mixed cultures

*B. cereus* spores were mixed with equal quantity of *L. lactis*, *E. coli* and *B. cereus* vegetative cells cultures. The permeabilization treatment described above for *B. cereus* spores was used. The hybridization was optimized to obtain maximum fluorescence signal-to-noise characteristics. It was performed with two probes simultaneously: a

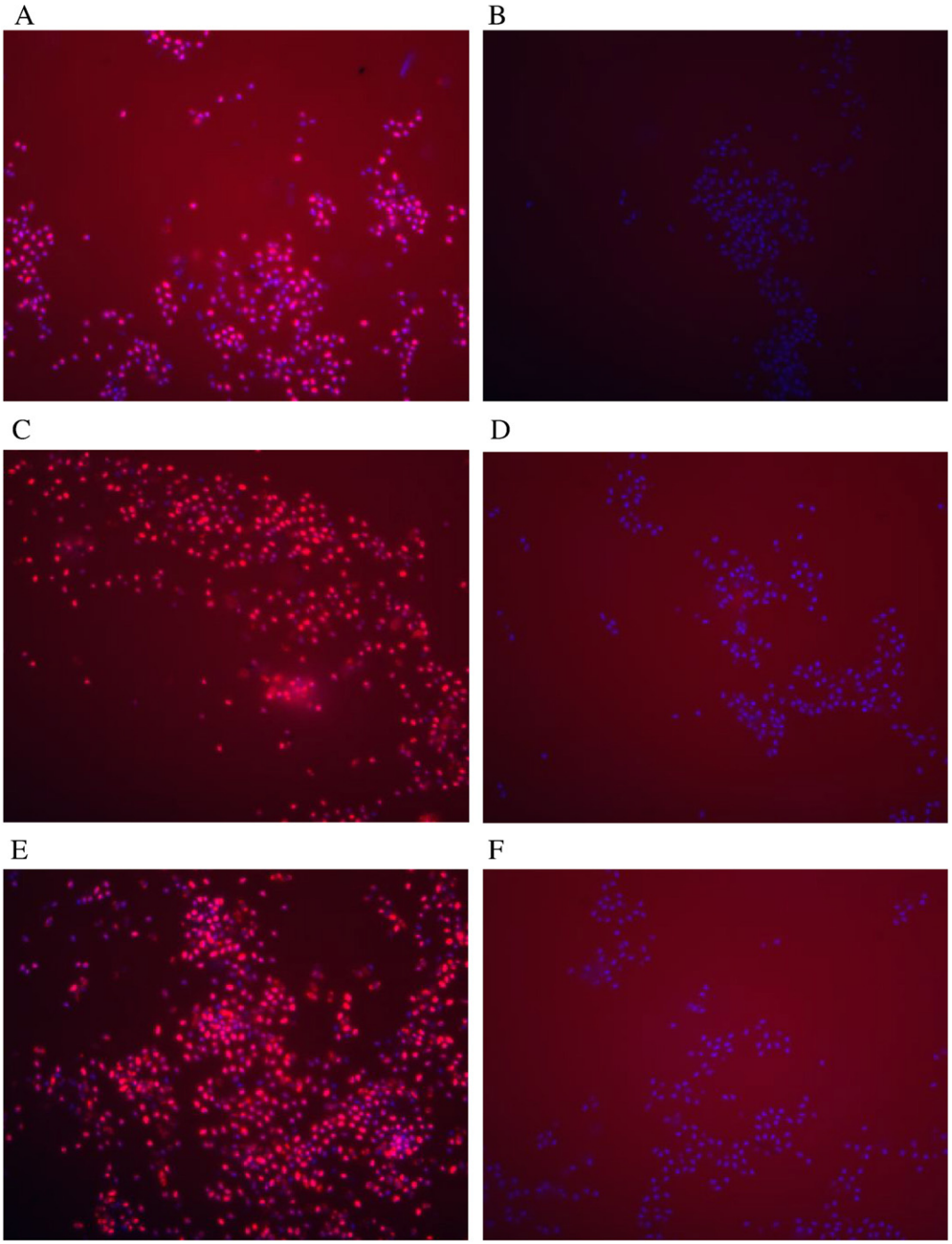


**Fig. 2.** FISH staining with the universal protocol on *Bacillus* spores. They were hybridized with a universal probe (left) and with a control probe (right). All the spores were counterstained with DAPI (blue) and hybridized with Alexa fluor 546 probe (red). A and B) B. *megaterium*, C and D) *B. atrophaeus*, E and F) B. *cereus*. Magnification: 1000×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

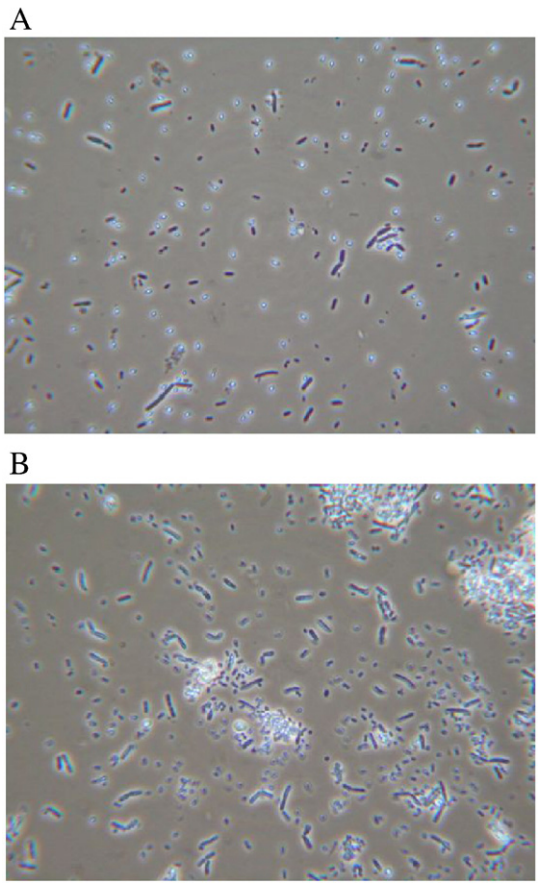


**Fig. 3.** Phase contrast microscopy of the mixed culture *L. lactis*, *E. coli* and *B. cereus* (vegetative cells and spores) A) before treatment and B) after treatment. Magnification: 1000×.

universal probe (EUB338 or *not*EUB338) coupled with Alexa Fluor 546 dye (2.46 ng/μL) and a specific probe for *B. cereus* (pB394 or *not*pB394) coupled with Alexa Fluor 488 dye (6.67 ng/μL) as shown in Table 1. The hybridization was performed in buffer 2 for 15 min at 46 °C then filtered and analyzed by fluorescent microscopy. Furthermore, each bacterial species was permeabilized and hybridized individually with the same protocol.

### 2.7. Scanning electron microscopy

The control and treated spore preparations were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.3 (Canemco, Lakefield, Qc, Canada) for 24 h at 4 °C. The spores were washed three times in cacodylate buffer for 10 min. Post-fixation was done using osmium tetroxide 1% (Canemco) in the buffer for 90 min. The spores were washed three times for 10 min with the buffer. Ethanol dehydrations were performed in successive concentrations (50, 70, 95 and 100%) for 10 min each. The cultures were put in two successive steps of 100% ethanol for 40 and 10 min and further treated twice in hexamethyldisilazane (Sigma-Aldrich) for 30 min to facilitate the drying. Finally the samples were air dried under a hood overnight and placed on an observation support. The samples were gilding and observed under scanning electron microscopy JEOL JSM-6360LV. Photographs were taken directly with the integrated camera.

### 2.8. Control experiments

For each FISH protocol, control experiments were performed using antisense probes (named *not*-). The interpretation of the FISH results (fluorescence) was always compared with the appropriate control experiment, performed in the same conditions and using the control probe. Positive FISH results without a control that was completely negative were not retained as appropriate protocol. The only exception was in the universal protocol where a few positive FISH cells were obtained in the control and this is mention in the appropriate Results section.

## 3. Results

The results presented show typical results from over ten replicate experiments.

### 3.1. FISH protocols

Permeabilization and hybridization protocols for FISH technique were developed for strains of *B. megaterium*, *B. atrophaeus* and *B. cereus* spores (Table 2). The protocols were optimized for each strain to allow the best detection possible e.g. intensity of fluorescence and proportion of positive spores in each *Bacillus*. The samples exhibited significant proportion of positively stained individuals with bright appearance (Fig. 1). The proportion of positively stained spores can be observed by the fraction of green (positive) versus blue (negative). As expected, the respective controls with *not*EUB338 probe gave negative results (no green fluorescence was observed).

A universal protocol was also created. It was developed to facilitate the detection of unknown *Bacillus* spores in environments. The protocol allows the detection of the three strains of *Bacillus* spores (Fig. 2). The intensity of fluorescence and the proportion of positive spores were lower than the optimized protocols, but high enough to be easily detected by fluorescent microscopy. Controls (*not*EUB338) were negative, even if some individual stained spores occasionally appeared positive.

### 3.2. Detection of B. cereus spores in mixed sample

*B. cereus* spores were detected in a mixed sample including *E. coli* DH5α, *L. lactis* MG1363 and *B. cereus* (both spores and vegetative forms). The mixed cultures were permeabilized with *B. cereus* optimized protocol. All the bacteria in the mixed culture were visible by optical microscopy before and after the permeabilization protocol (Fig. 3). However under fluorescence microscopy, only *L. lactis* and *B.*

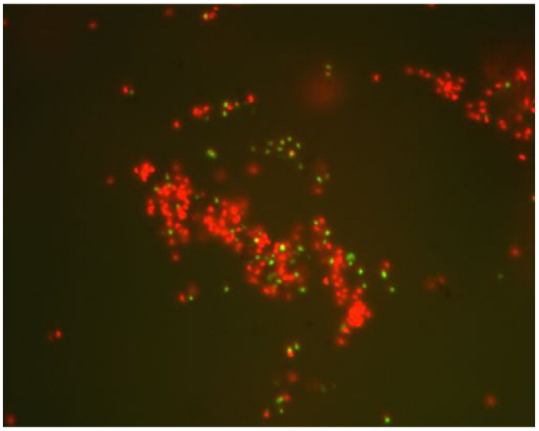

**Fig. 4.** Mixed cultures staining with universal EUB338 (Alexa-546, red) and *B. cereus* specific probes pB394 (Alexa-488, green). *L. lactis* and *B. cereus* (spores) were detected with EUB338 probes, but only *B. cereus* (spores) was detected with the specific probe. *E. coli* and *B. cereus* vegetative cells were not visible under fluorescence microscopy. Magnification: 1000×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

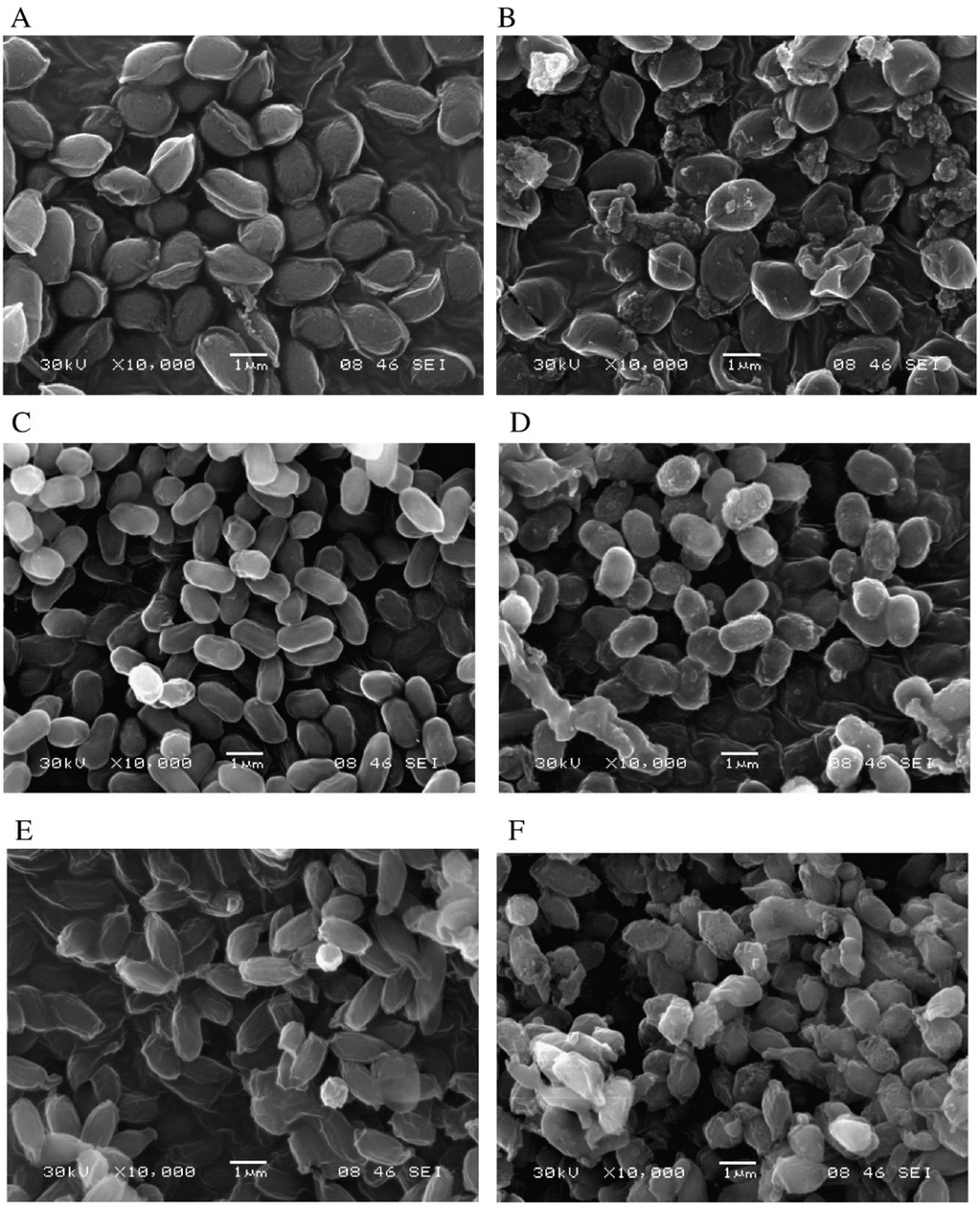


**Fig. 5.** Scanning electron microscopy with untreated (left) and treated (right) spores A and B) *B. megaterium*, C and D) *B. atrophaeus*, E and F) *B. cereus*. Magnification: 10 000×.

*cereus* spores were detected with the universal probe EUB338-Alexa 546 (red) (Fig. 4). The fluorescence of both species was as strong as in pure cultures and the proportion of positive bacteria was as high. Only *B. cereus* spores were detected with the specific probe pB394-Alexa 488 (green) (Fig. 4). The majority of *B. cereus* spores were positive and the fluorescence signal was strong enough to be easily detected by microscopy. The controls performed with the *not*EUB338-Alexa 546 and/or the *not*pB394-Alexa 488 were negative. In fluorescence microscopy, vegetative *B. cereus* and *E. coli* were not visible with DAPI counterstaining in the mixed cultures and individually after the permeabilization treatment (data not shown). Moreover, these species were not detectable with the FISH probes (pB394 and/or EUB338) in any situation (pure cultures or mixed cultures) with the *B. cereus* spores optimized protocol.

### 3.3. Scanning electron microscopy

Scanning electron microscopy was performed on *Bacillus* spores treated with their optimized protocol. Scanning electron microscopy revealed surface differences between treated and untreated spores (Fig. 5). The untreated *B. megaterium*, spores were rougher and bumpier than the treated spores that were smoother. Amongst the treated spores, more cell debris were visible but the spore membranes seemed intact. The opposite results were obtained when treated and the untreated *B. atrophaeus* spores were compared. The treated spores were bumpier and rougher than the untreated spores that seemed smoother. But, amongst the treated spores, more cell debris were visible than in the untreated spores such as *B. megaterium*. However, even when permeabilized, the spores conserved their original shape. Finally *B. cereus* showed similar results as *B. atrophaeus*.

## 4. Discussion

For the optimized protocol, permeabilization treatments, comprising various chemicals and enzymes, were developed in accordance with the composition of the different spore layers. The proteinase K was used to degrade the proteins that compose the majority of the molecules of the exosporium of *B. cereus* (Ebeling et al., 1974). This enzyme was not used with *B. atrophaeus* because of the absence of exosporium (Driks, 2007). Urea, SDS and DTT were used to denature the proteins present in the coat layer (Bennion and Daggett, 2003; Fischer et al., 1995; Jirgensons, 1981). Lysozyme and mutanolysin were used to degrade the peptidoglycans present in the cortex (Lichtman et al., 1992; Vollmer and Tomasz, 2002). *B. megaterium* spores were more easily permeabilized than the others since the proteinase K, urea and mutanolysin were not useful to obtain successful FISH labeling. Since no universal or standard spore permeabilization procedure was available in the literature, adapted protocols had to be developed for each spore type, according to their thickness, the lipid content of each layer, their protein content, peptidoglycans and glycoproteins structure, and the presence of exosporium. However, a universal protocol was still created from the optimized protocol. This protocol can be used for the detection of unknown *Bacillus* spores in environments.

Previously published FISH protocols (permeabilization and hybridization) usually take several hours up to a few days. When optimizing the FISH protocols for *Bacillus* spores, it was realized that a lot of the steps could be dramatically reduced or suppressed without altering the signal quality. The proposed FISH protocols reduced the time of execution and allowed the detection of the three *Bacillus* spore models in less than an hour using universal probes (permeabilization and hybridization included). Also, *B. cereus* spores were successfully detected using specific probes in a mixed sample in less than 1 h. This method is faster than the two previously published protocols for spores. *B. megaterium* spores protocol took about 3 days before to get the results (Fischer et al., 1995) and than an other derived protocol for *Bacillus* ribotype DA001 endospores about 2 days to get the results (Felske et al., 1998). Moreover, these previous protocols did not allow the staining and the detection of *B. atrophaeus* and *B. cereus* spores by FISH. Our protocols are also more rapid than other existing protocols such as protocols for vegetative cells (1–2 days to get the results) (Rusch and Amend, 2004; Samarbaf-Zadeh et al., 2006; Sekar et al., 2004; Sekar et al., 2003). Most published permeabilization and hybridization for FISH protocols were developed on microscope slides to demonstrate consortium structural relationships. In the present method, performing the reactions in suspension provided quicker results with better signal-to-noise characteristics.

In the mixed cultures, *E. coli* and *B. cereus* vegetative cells were not stained with DAPI. *B. cereus* vegetative cells and *E. coli* were neither observed with the universal probes. But using phase contrast optical microscopy, the bacteria were still visible amongst the mixed sample and seemed intact. This result suggests that the permeabilization treatments are too harsh for vegetative cells, leading to leakage of genetic material as evidenced by negative FISH and DAPI staining (Fazi et al., 2007; Schonhuber et al., 1997).

Foegeding and Busta (1983) employed scanning electron microscopy to show that *Clostridium botulinum* spores treated with hypochlorite appeared to have different surface characteristics (Foegeding and Busta, 1983). They explained that the appearance was probably due to extraction of protein material from the spore coat. In the present work, scanning electron microscopy results also suggest that the various chemical treatments had some effect on spore surface. This result was predictable since the bacteria, if it was completely damaged or destroyed, would have lost its cell content, thus would not be detectable by FISH (Jin and Lloyd, 1997). Also, the treatment performed is supposed to create very small pores that cannot be visible with the scanning microscopy.

In summary, we have reduced the complexity of FISH protocols to a level never published before. In addition, we developed a series of effective and rapid protocols optimized for FISH directly on *Bacillus* spores. These new protocols reduce time of execution down to about 1 h and retained effectiveness in mixed cell populations. This work could be applied to the rapid detection of *B. cereus* spores in food-borne diseases. Moreover, this protocol could be adapted to other kind of bacterial spore such as *Clostridium* with the similar layered degradation strategy.

## Acknowledgments

The authors are thankful to Richard Janvier for electron microscopy. This work was funded by the Defence Research and Development Suffield, Canada. GF is a CIHR Strategic Training Fellow in PHARE. CL acknowledges the IRSST and Engineering Research Council of Canada (NSERC) studentships. CD acknowledges the IRSST/Canadian Institutes of Health Research and FRSQ scholarships.

## References

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

Amann, R., Fuchs, B.M., Behrens, S., 2001. The identification of microorganisms by fluorescence in situ hybridisation. Curr. Opin. Biotechnol. 12, 231–236.

Bennion, B.J., Daggett, V., 2003. The molecular basis for the chemical denaturation of proteins by urea. Proc. Natl. Acad. Sci. U. S. A. 100, 5142–5147.

Bertaux, J., Gloger, U., Schmid, M., Hartmann, A., Scheu, S., 2007. Routine fluorescence in situ hybridization in soil. J. Microbiol. Methods 69, 451–460.

Bottari, B., Ercolini, D., Gatti, M., Neviani, E., 2006. Application of FISH technology for microbiological analysis: current state and prospects. Appl. Microbiol. Biotechnol. 73, 485–494.

Castanha, E.R., Fox, A., Fox, K.F., 2006. Rapid discrimination of Bacillus anthracis from other members of the *B. cereus* group by mass and sequence of "intact" small acid soluble proteins (SASPs) using mass spectrometry. J. Microbiol. Methods 67, 230–240.

Chambon, P., Deutscher, M.P., Kornberg, A., 1968. Biochemical studies of bacterial sporulation and germination. X. Ribosomes and nucleic acids of vegetative cells and spores of *Bacillus megaterium*. J. Biol. Chem. 243, 5110–5116.

de Vries, Y.P., Atmadja, R.D., Hornstra, L.M., de Vos, W.M., Abee, T., 2005. Influence of glutamate on growth, sporulation, and spore properties of *Bacillus cereus* ATCC 14579 in defined medium. Appl. Environ. Microbiol. 71, 3248–3254.

Driks, A., 2007. Surface appendages of bacterial spores. Mol. Microbiol. 63, 623–625.

Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H.D., Lang, H., 1974. Proteinase K from *Tritirachium album* Limber. Eur. J. Biochem. 47, 91–97.

Fazi, S., Amalfitano, S., Pizzetti, I., Pernthaler, J., 2007. Efficiency of fluorescence in situ hybridization for bacterial cell identification in temporary river sediments with contrasting water content. Syst. Appl. Microbiol. 30, 463–470.

Felske, A., Akkermans, A.D., De Vos, W.M., 1998. In situ detection of an uncultured predominant *Bacillus* in Dutch grassland soils. Appl. Environ. Microbiol. 64, 4588–4590.

Fischer, K., Hanh, D., Hönerlage, W., Schönholzer, F., 1995. In situ detection of spores and vegetative cells of *Bacillus megaterium* in soil by whole cell hybridization. Syst. Appl. Microbiol. 18, 265–273.

Foegeding, P.M., Busta, F.F., 1983. Proposed mechanism for sensitization by hypochlorite treatment of *Clostridium botulinum* spores. Appl. Environ. Microbiol. 45, 1374–1379.

Glockner, F.O., Fuchs, B.M., Amann, R., 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. 65, 3721–3726.

Jin, L., Lloyd, R.V., 1997. In situ hybridization: methods and applications. J. Clin. Lab. Anal. 11, 2–9.

Jirgensons, B., 1981. Circular dichroism study on reconstructive denaturation of proteins by sodium dodecyl sulfate in neutral and acid solutions. Makromol. Chem., Rapid Commun. 2, 213–217.

Laflamme, C., Ho, J., Veillette, M., de Latremoille, M.C., Verreault, D., Meriaux, A., et al., 2005. Flow cytometry analysis of germinating *Bacillus* spores using membrane potential dye. Arch. Microbiol. 183, 107–112.

Lange, J.L., Thorne, P.S., Lynch, N., 1997. Application of flow cytometry and fluorescent in situ hybridization for assessment of exposures to airborne bacteria. Appl. Environ. Microbiol. 63, 1557–1563.

Lenaerts, J., Lappin-Scott, H.M., Porter, J., 2007. Improved fluorescent in situ hybridization method for detection of bacteria from activated sludge and river water by using DNA molecular beacons and flow cytometry. Appl. Environ. Microbiol. 73, 2020–2023.

Lichtman, S.N., Okoruwa, E.E., Keku, J., Schwab, J.H., Sartor, R.B., 1992. Degradation of endogenous bacterial cell wall polymers by the muralytic enzyme mutanolysin prevents hepatobiliary injury in genetically susceptible rats with experimental intestinal bacterial overgrowth. J. Clin. Invest. 90, 1313–1322.

Liu, W.T., Mirzabekov, A.D., Stahl, D.A., 2001. Optimization of an oligonucleotide microchip for microbial identification studies: a non-equilibrium dissociation approach. Environ. Microbiol. 3, 619–629.
Oncu, S., Oncu, S., Sakarya, S., 2003. Anthrax—an overview. Med. Sci. Monit. 9, RA276–RA283.
Panessa-Warren, B.J., Tortora, G.T., Warren, J.B., 1997. Exosporial membrane plasticity of *Clostridium sporogenes* and *Clostridium difficile*. Tissue Cell 29, 449–461.
Pernthaler, A., Pernthaler, J., Amann, R., 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. 68, 3094–3101.
Rusch, A., Amend, J.P., 2004. Order-specific 16S rRNA-targeted oligonucleotide probes for (hyper)thermophilic archaea and bacteria. Extremophiles 8, 357–366.
Samarbaf-Zadeh, A.R., Tajbakhsh, S., Moosavian, S.M., Sadeghi-Zadeh, M., Azmi, M., Hashemi, J., et al., 2006. Application of fluorescent in situ hybridization (FISH) for the detection of *Helicobacter pylori*. Med. Sci. Monit. 12, CR426–CR430.
Schonhuber, W., Fuchs, B., Juretschko, S., Amann, R., 1997. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl. Environ. Microbiol. 63, 3268–3273.
Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T., Amann, R., 2003. An improved protocol for quantification of freshwater Actinobacteria by fluorescence in situ hybridization. Appl. Environ. Microbiol. 69, 2928–2935.
Sekar, R., Fuchs, B.M., Amann, R., Pernthaler, J., 2004. Flow sorting of marine bacterioplankton after fluorescence in situ hybridization. Appl. Environ. Microbiol. 70, 6210–6219.
Setlow, P., 2003. Spore germination. Curr. Opin. Microbiol. 6, 550–556.
Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101, 514–525.
Vollmer, W., Tomasz, A., 2002. Peptidoglycan *N*-acetylglucosamine deacetylase, a putative virulence factor in *Streptococcus pneumoniae*. Infect. Immun. 70, 7176–7178.
Yokogawa, K., Kawata, S., Nishimura, S., Ikeda, Y., Yoshimura, Y., 1974. Mutanolysin, bacteriolytic agent for cariogenic Streptococci: partial purification and properties. Antimicrob. Agents Chemother. 6, 156–165.