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Flow cytometry analysis of germinating *Bacillus* spores, using membrane potential dye

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Abstract Germination of *Bacillus anthracis* spores is necessary for the transcription of plasmidic genes essential to the infection. Assessing germination potential is crucial to predict the risk associated with pathogenic *Bacillus* exposure. The aim of this study was to set up a viability assay based on membrane potential in order to predict the earliest germination event of spores. *B. cereus* and two strains of *B. subtilis* were used. The spores were isolated with a sodium bromide gradient. Approximately 10^7 spores were incubated at 37°C in tryptic soy broth (TSB). Aliquots were harvested at predetermined times and stained with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] or with bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$]. Fluorescence characteristics were obtained using flow cytometry. The earliest detectable activation of membrane potential occurred after 15 min of incubation in TSB using $DiOC_6(3)$. Using $DiBAC_4(3)$, the earliest detectable signal was after 4 h of incubation. Control experiments using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)-treated spores did not show any change in the fluorescence intensity over time. Since no membrane potential and no germination were detected in CCCP-treated spores, the activation of membrane potential seems to be associated with germination. $DiOC_6(3)$ can be used as an early membrane potential indicator for spores. $DiBAC_4(3)$, by contrast, is not a early membrane potential marker.

Keywords Membrane potential · Germination · *Bacillus anthracis* · Spore

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Introduction

The use of weapons of mass destruction such as spore-forming *Bacillus anthracis* constitutes a world threat. Since such harmful microorganisms are often odourless, tasteless, and colourless, detecting them in the atmosphere is difficult. Real-time detection of pathogens is a challenge. Moreover, the evaluation of the viability status of the detected microorganisms is crucial if sound decisions are to be made.

Culturability assay (plate count) is the standard method for evaluating viability (Barer and Harwood 1999). However, plate count does not take into account the following: (1) colony-forming units may be formed by single cells or clumps, (2) there can be a non-culturable portion of the microorganism populations (Edwards 2000), and (3) selectivity is inherent to the choice of any culture condition. In addition to these limitations, several days of incubation are often needed for bacteria to form countable colonies. Recently, new viability assays were developed for bacteria (Keer and Birch 2003). These tests were essentially based on respiratory activity (Bhupathiraju et al. 1999; Rodriguez et al. 1992), external membrane integrity (Nebe-von-Caron et al. 2000), intracellular pH (Chitara et al. 2000), and membrane potential (Deere et al. 1995; Manson et al. 1993, 1994, 1995). The major advantage of these new technologies is the rapidity of response and the multi-parametric nature of the collected information.

Some of these assays have been validated directly on *Bacillus* spores (Comas-Riu and Vives-Rego 2002; Laflamme et al. 2004). Although those tests gave new information about spores, they did not provide rapid information on the germination potential of the

organism. Evaluation of germination and growth potential, using rapid methods, would give information more rapidly than plate count.

The measurement of membrane potential has been used as an indicator of viability in numerous studies to test the susceptibility to antimicrobial agents (Kaprelyants and Kell 1992; Manson et al. 1994, 1995; Shapiro 1995). Cells with high membrane potential are often referred as energized or hyperpolarized cells. In contrast, de-energized or depolarized cells exhibit low or zero membrane potential. The measurement of membrane potential by flow cytometry was first described by Shapiro and collaborators (1979). In bacteria, all of the potential is developed across the cytoplasmic membrane. Membrane potential plays a critical role in bacterial proton-motrice force involved, especially in the generation of ATP (Dimroth 2000), but also in chemotaxis (Charon et al. 1992), glucose transport (Russel 1990), and survival at low pH (Scott et al. 2000). The membrane potential is generally dissipated by treatment with proton ionophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). It is speculated that establishment of membrane potential is likely to take place when dormant spores start to germinate in order to increase exchange with their environment.

Two major classes of membrane potential dyes are frequently referred to in the literature: carbocyanines and oxonols. Carbocyanine dyes accumulate on hyperpolarized membranes and are translocated into the lipid bilayer (Cabrini and Verkman 1986). 3,3′-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] has been the most widely used carbocyanine (Rottenberg and Wu 1998). One of the oxonol salts used most frequently to monitor membrane potential is bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (often cited as bis-oxonol). The dyes enter depolarized cells, where they bind to intracellular proteins or membranes and exhibit enhanced fluorescence. Thus, depolarization results in a greater influx of the anionic dye, which in turn leads to an increase in fluorescence (Suller and Lloyd 1999). Hyperpolarization, by contrast, results in a decrease of fluorescence.

We hypothesize that one of the earliest events in the germination of *Bacillus* spores is the activation of membrane potential (Keynan 1978). In fact, as reviewed recently by Moir et al. (2002) and Setlow (2003), the activation of membrane potential were in the cascade of reactions that encompass spore germination. The objective of this study is to set up a viability assay, based on membrane potential, that can rapidly predict the germination of purified *Bacillus* spores.

Materials and methods

Bacteria and culture conditions

Three strains of *Bacillus* were used: one strain of *B. cereus* (clinical strain), and two strains of *B. subtilis*. Of the latter, *B. subtilis* var. *niger*, the simulant strain for *B. anthracis*, was provided by National Defence of Canada, the other came from ATCC (9373). The strains' identities were confirmed to species level by analyzing cellular fatty acids (Microbial ID, Newark, Del., USA) and the first 500 base pairs of the 16S ribosomal DNA gene (MIDI Labs, Newark, Del., USA). The use of *B. anthracis* was not possible because of the bioconfinement facilities' requirements. Moreover, *B. cereus* and *B. anthracis* are considered as a single species (within a group called *B. cereus* group), phylogenetically and genetically, and can be used as surrogates at this step of the study (Helgason et al. 2000).

Production and purification of spores

One aliquot of each liquid culture was plated on a sporulation medium [nutrient agar (Difco) with 0.5% yeast extract, 7×10^{-4} M $CaCl_2$, 10^{-3} M $MgCl_2 \cdot 6\,H_2O$, and 5×10^{-5} M of $MnCl_2 \cdot 4\,H_2O$, final pH of 6.8] and incubated 72 h at 37°C. Colonies were harvested with a sterile cotton swab and transferred in 1 ml filtered (pore size, 0.22 µm) phosphate buffer saline (PBS). After two washing steps and following resuspension in PBS, the samples were gently put on top of a NaBr density gradient and centrifuged at 2,400 *g* (25°C) for 45 min. This gradient allows the formation of successive stages of NaBr density: 1.5, 1.4, 1.3, 1.2, 1.1 and 1.0 g ml^{-1}, according to Nicholson and Law (1999). Endospores were isolated at the 1.33 g ml^{-1} gradient level, whereas debris and vegetative cells are found at 1.1 and 1.2 levels, respectively. The isolated spores were washed twice in 1 ml PBS and kept in this buffer at 4°C to avoid germination.

Spore count, purity evaluation, and DNA staining

Spore preparation purity was tested by microscopy and heat resistance. A wet mount was used to evaluate the degree of purity of the spores isolated from the NaBr density gradient. In addition, to confirm the absence of debris and to quantify the spores, a fluorescent DNA marker, the 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI), was used to stain spores and vegetative cells, if present. Briefly, spore suspensions were washed twice with filtered PBS. The samples were pelleted and resuspended in 1 ml PBS with 10 nM DAPI. After a 10-min incubation in the dark, a wet mount was prepared directly from the reaction mixture and observed immediately using fluorescence microscopy. A minimum of five snapshots was taken. The spore counts were done directly on the photomicrographs. This procedure as well as plate counts were performed on each purified spores suspensions. Spore enrichment followed by NaBr gradient allowed the recovery of high-purity spore preparations (more than 95%). Almost no debris or vegetative cells were seen in the preparation. Moreover, no germinating spores were observed (phase-dark spores), i.e. all spores were phase-bright spores (Lafl-

amme et al. 2004). Obtaining a homogenous preparation is important, since this study emphasizes on the early detection of *Bacillus* spore germination.

Culture conditions

Samples consisting of 1×10^7 freshly purified (non-treated) spores were put in 25 ml tryptic soy broth (TSB) and incubated at 37°C in a shaking bath. Similar samples were prepared where CCCP was added at 100 μM (final concentration) in the culture media to serve as negative control for membrane potential activation as reported by van Schaik et al. (2004). Membrane potential analysis was done on aliquots at time 0, 15, 30, 45, 60, 90, and 120 min for $DiOC_6(3)$ analysis. The incubation time were extended to 240, 480, and 1,440 min for $DiBAC_4(3)$ analysis.

Membrane potential dyes

$DiOC_6(3)$ (excitation: 484 nm, emission: 500 nm) and $DiBAC_4(3)$ (excitation: 490 nm, emission: 520–550 nm) were obtained from Molecular Probes (Eugene, Or., USA). For $DiOC_6(3)$, a stock solution of 20 μM was dissolved in dimethyl sulfoxide and stored at −20°C. Aliquots of culture were incubated with 2.5 nM of $DiOC_6(3)$ for 2 min prior to flow cytometry analysis. A 1-mg ml^{-1} stock solution of $DiBAC_4(3)$ was prepared in 70% ethanol and stored at −20°C. $DiBAC_4(3)$ was used at a concentration of 2 μM in PBS (for 1×10^7 isolated spores) and incubated for 2 min in the dark at room temperature prior to analysis. The optimal dye concentration used in this study was established prior to experimentation. In most treatments, almost 100% of the cells were stained. Differences were seen in the intensity of the staining by the mean fluorescence intensity. The optimal concentration for *Bacillus* spore germination was determined at 2.5 nM for $DiOC_6(3)$ dye and 2 μM for $DiBAC_4(3)$. The latter concentration was reported by other authors (Jepras et al. 1997; Manson et al. 1995).

Flow cytometry

Flow cytometry analyses of the spores were performed using an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, Fla., USA) with acquisition software EXPO 32 (version 1.1c). The flow cytometer was equipped with an air-cooled, 15-mW argon laser as a light source. Fluorescence signals were collected through a 525-nm bandpass filter (FL1). Acquisition of fluorescence data was gated by forward-angle light scatter and side scatter, and the data rate was set at less than 500 events per second. Samples were allowed to run approximately 1 min before the acquisition of a minimum of 5,000 events.

Microscopic observation

To confirm that germination process had occurred in our samples, phase contrast microscopy (Leitz, Laborlux S) observation of the spores after 0, 1, 2, and 4 h of incubation in TSB was performed.

Statistical analyses

The statistical analyses were carried out with Statistical Analytical Software. Results were expressed as mean value ± SEM. Data were analyzed using the paired *t*-test. All reported *P*-values were declared significant at $P<0.05$.

Results

All three strains of *Bacillus* spores responded similarly to the various assays. When purified *Bacillus* spores were in contact with TSB, the membrane potential increased rapidly. In fact, a strong increase in the fluorescence signal of $DiOC_6(3)$ was observed after only 15 min of incubation for each strain tested (Fig. 1). During the first 15 min, signal increase was between 1.70- and 1.74-fold for *B. subtilis* var. *niger* (Fig. 1a, black line) and *B. subtilis* ATCC 9373 (Fig. 1b, black line) and 1.88 times for *B. cereus* when compared with the basal membrane potential at time zero (Fig. 1c, black line). In all cases, the increase from time zero to 15 min was highly significant ($P<0.0004$). After the first 15 min, the increase rate was more gradual. However, between 60 min and 90 min, the increase in fluorescence accelerated rapidly, and the peak of the intensity of fluorescence was observed at 120 min (2 h of incubation). After this time, the mean fluorescence of $DiOC_6(3)$ started to decrease until it returned to baseline (time zero) at 24 h (data not show). Generally, the measurement of fluorescence intensity of $DiOC_6(3)$ showed a rapid increase of membrane potential, followed by a short, stable phase. This phenomenon was followed by another increase to the maximum level. There was, for all strains tested, a diphasic signal of membrane potential activation. Subsequently, there was a decline phase ending back to the initial level. Further investigations are needed to explain the diphasic signal observed.

For each strain used, CCCP-exposed controls were performed. No significant change in the mean fluorescence intensity was observed over time (Fig. 1a–c, dark grey line). Moreover, *B. subtilis* var. *niger* spores, either heat-killed or ethanol-fixed, are commonly used as control (Novo et al. 1999). The heated-killed samples showed no change in $DiOC_6(3)$ fluorescence intensity over time (data not shown).

$DiBAC_4(3)$ was also evaluated in this study as a germination marker (Fig. 2, $n=3$ for each strain) for non-treated, freshly isolated spores. In contrast to $DiOC_6(3)$, the results was expressed in percentage of

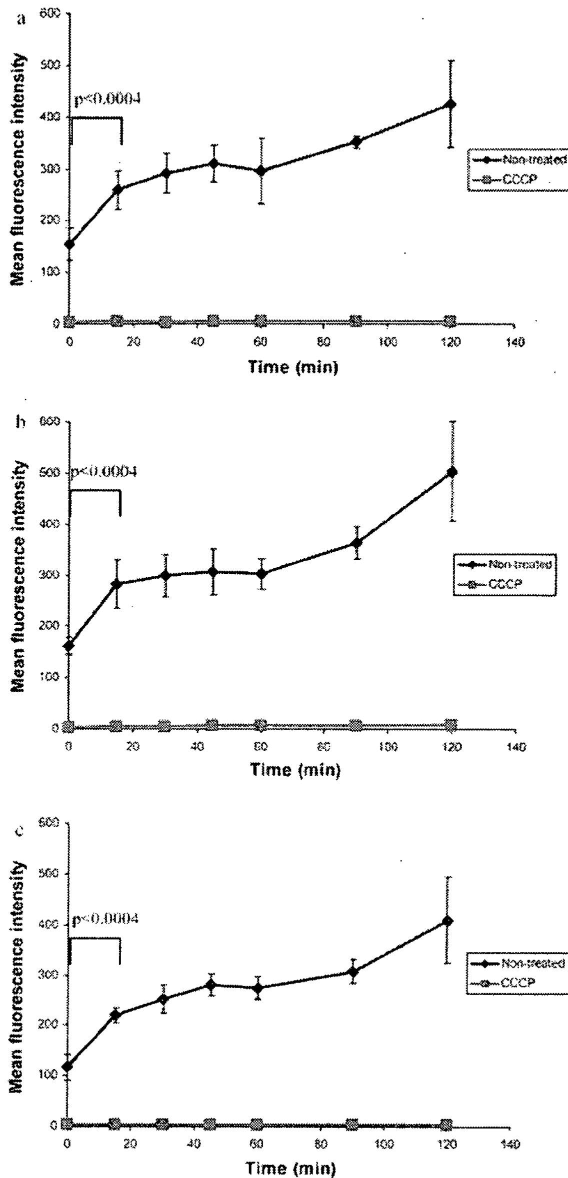

Fig. 1 Flow cytometry measurement of membrane potential over time, using 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] staining. *Bacillus cereus* (**a**), *B. subtilis* var. *niger* (**b**), and *B. subtilis* ATCC 9373 (**c**) purified spores were cultured in tryptic soy broth (TSB), with and without *m*-chlorophenylhydrazone (*CCCP*) (100 μM), harvested at different times (0, 15, 30, 45, 60, 90, 120, and 240 min), and stained with $DiOC_6(3)$. The mean fluorescence intensity was collected using a 525-nm bandpass filter (FL1 channel). A minimum of five readings (independent experiments) per harvesting time were done

positive cells. The pattern of reaction in time for the three strains used was relatively similar ($P=0.2$). $DiBAC_4(3)$ staining gave a positive signal of germination at 4 h of incubation in TSB; the percentage of positive cells started to decline indicating detection of active membrane potential. Unlike $DiOC_6(3)$, $DiBAC_4(3)$ is not a rapid germination marker, since it allowed the first detection of membrane potential within a minimum of 4 h.

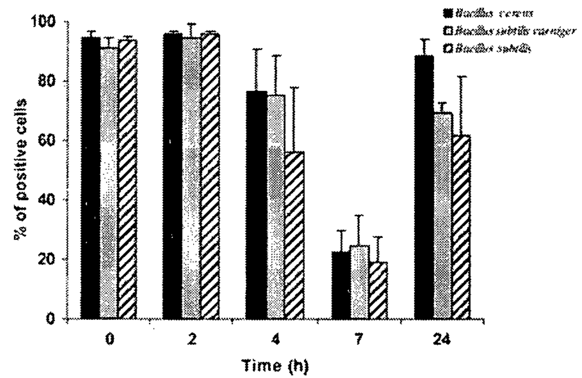

Fig. 2 Compilation of percentage of positive cells (non-treated) for bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] staining. *B. cereus*, *B. subtilis* var. *niger*, and *B. subtilis* ATCC 9373 were harvested at different times (0, 2, 4, 7, and 24 h) and stained with $DiBAC_4(3)$. The percentage of positive cells were collected. Three independent experiments were done for each strain

The phase contrast microscopic observation showed that roughly 100% of the spores had undergone germination process, since almost no spores were observable after 4 h of incubation (Fig. 3). No spores germinated when CCCP were used.

Discussion

The objective of this study was to evaluate the germination potential of *Bacillus* spores, using a test based on tracking membrane potential. This study shows that

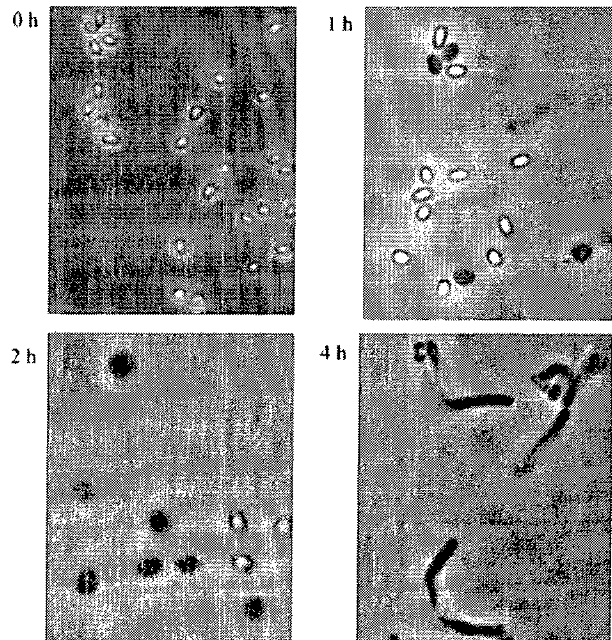

Fig. 3 Photography, using phase contrast microscopy, of *B. subtilis* var. *niger* purified spore after 0, 1, 2, and 4 h of incubation in TSB

$DiOC_6(3)$ is a very effective dye to reach that aim. In fact, the time needed to evaluate the germination status of a *Bacillus* spore samples is 15 min of incubation in TSB, 10 min of incubation with the dye, and a maximum of 5 min to analyze the samples [two tubes in this case: (1) non-incubated samples formerly named time zero and (2) the incubated samples] by flow cytometry. Thus, in approximately 30 min, it is possible to get relevant information about the spores' germination potential. $DiBAC_4(3)$ gives the same information but takes a minimum of 4 h of incubation, which is no more efficient than microscopic observation.

For all the strains tested, the results showed two points were an increase in the mean fluorescence intensity was substantial. One from time zero to time 15 min and a second from time 60 min to time 120 min. It is possible that the two rises in fluorescence are due to two separate germinating populations. Further experiments are required to explain this phenomenon. One possible approach is to heat-activate the spores in order to increase the synchronicity in the germination.

The mean fluorescence intensity of $DiOC_6(3)$ was used in this study, since, contrary to $DiBAC_4(3)$ staining, no difference can be observed when evaluating the percentage of positive cells. In fact, almost 100% of the cells were positive for $DiOC_6(3)$ staining for all times tested, but the intensity of this staining, measured by the mean fluorescence, varied with time. A possible explanation is that lipophylic probes tend to bind to the coat or in outer membrane. On the other hand, using CCCP-exposed spores, only few staining cells were detected.

Before germination, *Bacillus* spores exhibit low permeability to dyes. In the spore state, *Bacillus* are likely to have low or zero membrane potential. The results obtained with $DiOC_6(3)$ at time zero indicate low membrane potential, since low fluorescence intensity was detected. But the fluorescence detected at time zero, compared to the result obtain with CCCP, leads to the belief that minimal membrane potential exist on the dormant spores. This observation needs to be investigated.

The CCCP-treated spores were used in this study as depolarized control cells. Using these proton ionophores, membrane potential has been reduced to zero or near zero. This universally accepted control for membrane potential proved the efficiency of $DiOC_6(3)$ to finely detect membrane potential activation and exclude the hypothesis that the fluorescence emission seen for non-treated spore is not due to a phenomenon of permeation/adsorption, but instead was due to an uptake of the dye by the germinating spore. Heat-treated spores, using autoclaved spores, also showed inactivated membrane potential as detected by $DiOC_6(3)$.

The first membrane potential signal obtained in this study was at time 15 min of incubation using $DiOC_6(3)$ staining. This positive staining is observed at least 1 h before the phase-dark spore formation, which is the microscopic indication of germinating spores. A study showed that 3–4 min after the initiation of germination of *B. subtilis* 1,604 strain, production of ATP is detected (Venkatasubramanian and Johnstone 1989). Fluorescence monitoring of NADH and dosage of ATP in germinating spore of *B. megaterium* QM B1551 also support this conclusion (Sano et al. 1988). Although NADH and ATP measurements would have supported germination process, microscopic observation of spores disappearing is an irrefutable proof that the germination process had occurred. Thus, detection of membrane potential using $DiOC_6(3)$ coincides with these biochemical events. Additional studies are required to verify if membrane potential overlaps with other biochemical changes such as dipicolinic acid release that occur during spore germination (Moir et al. 2002).

This in vitro model used to monitor *Bacillus* spore germination is performed in very simple conditions when compared to conditions triggering germination of *B. anthracis* spore in vivo: the anthrax germination is highly specific and involves combinations of amino acids and purines/ribosides (Weiner et al. 2003). An in vitro model inducing *B. anthracis* spore germination have to be developed with $DiOC_6(3)$.

Conclusion

The membrane potential dye $DiOC_6(3)$ is a promising tool for the rapid evaluation of *Bacillus* spore germination.

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