A rapid staining technique for the detection of the initiation of germination of bacterial spores

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Aims: We propose to apply the Wirtz–Conklin staining technique to evaluate spore germination.

Methods and Results: Spores at different stages of germination were stained with modified spore stain (Wirtz–Conklin) and evaluated for staining properties. *Bacillus* spores suspended in deionized water, which does not support germination, stained greenish-blue. Spores suspended in germination enhancers that did not form bacilli stained pink, indicating the initiation of germination. Spores suspended in culture media, which promotes bacterial outgrowth, formed bacilli and were also stained pink.

Conclusions: Modified spore stain (Wirtz–Conklin) was found to be useful to detect the initiation of spore germination as early as 30 min following incubation in a germination environment.

Significance and Impact of the Study: This simple staining procedure is useful in detecting the initiation of germination of bacterial spores.

INTRODUCTION

Spore-forming bacteria are highly resistant to adverse environmental conditions including dryness, heat and poor nutrient supply. They are also highly resistant to chemical disinfectants, desiccation, and extremes in pH, temperature, pressure, and ultraviolet and ionizing radiation (Gould 1977). Spores are highly refractile bodies, consisting of a central core surrounded by five layers; plasma membrane, germ cell wall, cortex, coats, and exosporium. The core, plasma membrane and germ cell wall constitute a condensed cell which is self-contained and is protected by the outer integument. The complexity of the coat varies between different species (Warth 1978a).

Detection of spore germination is a challenging task. It is vital to detect germination of spores for the study of germination events and to evaluate the effect of sporicidal agents. Spore germination is accompanied by the loss of refractility, which can be observed visually using phase contrast microscopy (Vary 1965; Parker 1969; Power *et al.*

1989). Determination of the reduction in spore optical density has also been used to assess spore germination. The percent germination can be calculated from the absorbance decrease at 625 nm, a 60% decrease of initial absorbance for intact spores represents 100% germination of the spore population (Vary 1965; Parker 1969; Boschwitz *et al.* 1983; Power *et al.* 1989; Senesi *et al.* 1992; Makino *et al.* 1994). Other methods used to study spore germination include determining the loss of heat resistance during germination (Warth 1978b), or the amount of dipicolinic acid (DPA) released by germinating spores (Janssen *et al.* 1958; Warth 1980). These methods are primarily useful for studying germination kinetics in populations of spores. The use of tetrazolium overlay technique can be useful to distinguish germination mutants (Lafferty and Moir 1977). Recently, Bruno and Mayo suggested the use of colour fluorescence image analysis of acridine orange stained spores for the assessment of germination (Bruno and Mayo 1995).

In this study we evaluated the usefulness of modifying a differential spore stain (Wirtz–Conklin) as a direct and straightforward method for detecting the initiation of bacterial spore germination.

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MATERIALS AND METHODS

Spore preparation

For induction of spore formation, *B. subtilis* (ATCC 51189) were grown for a week at 37°C on NAYEMn agar (Nutrient Agar with 0·1% (w/v) Yeast Extract and 5 mg l^{-1} MnSO$_4$). The plates were scraped and the bacteria/spores suspended in sterile 50% ethanol and incubated at 22°C overnight with agitation in order to lyse the remaining vegetative bacteria. The suspension was centrifuged at 2500 *g* for 10 min and the pellet washed three times in cold deionized water. The spores were stored at 4°C (Hamouda *et al.* 1999). The spores were counted using serial dilution and plating on TSA overnight.

Spore treatment

Bacillus spores, 5×10^7, were resuspended in 1 ml of deionized H$_2$O, germination enhancers (GE) (Shibata *et al.* 1976), or in Trypticase soy broth (TSB) (BBL–Becton Dickinson, Sparks, MD, USA). The spore suspensions were incubated on a rotator for various intervals and then a sample of each was used for staining.

Modified Spore (Wirtz-Conklin) stain (Schaeffer and MacDonald 1933)

The spore suspension was smeared on a glass slide and fixed with a Bunsen flame. Slides were flooded with five percent aqueous malachite green (Fisher Scientific Co. Fair Lawn, NJ, USA). Slides were intermittently heated with a Bunsen flame for approximately five min, to ensure that the dye remained hot but not boiling. Slides were rinsed with tap water, then counterstained with 0·5% Safranin-O (Sigma Chemical Co., St. Louis. MO, USA) for 1 min. After drying, the slides were examined using the oil immersion power of a light microscope (Olympus BH-2, Tokyo, Japan).

Susceptibility of *Bacillus* spores to antimicrobial nanoemulsions

To confirm the microscopic findings, the susceptibly of spores, under the different treatments, to antimicrobial nanoemulsion was tested. NanoSafe1™ (NanoBio Corporation, Ann Arbor, MI, USA), an oil–in–water nanoemulsion that has been investigated in our lab for its antimicrobial activity (Hamouda *et al.* 1999; Hamouda and Baker 2000; Hamouda *et al.* 2001) was used. *Bacillus* spores, 5×10^7, were suspended in deionized H$_2$O, or GE in PBS, or in TSB in an Eppendorf tube. Different concentrations of Nano–Safe1™ (NanoBio Corp., Ann Arbor, MI, USA) were added to each of the tubes and incubated at room temperature for up to 4 h on a rotator. After treatment, suspensions were serially diluted in deionized H$_2$O, plated on TSB agar, and incubated overnight to determine the viable count. Log reduction in spore count was determined by the following equation: Log reduction = Log$_{10}$ (cfu pre-treatment/cfu post-treatment).

RESULTS

Microscopic examination of *Bacillus* spores stained with Wirtz-Conklin modified spore stain, showed that *Bacillus* spores resuspended in water for up to 24 h, appeared as greenish-blue spheres (spores) (Figs 1a and 2a). In GE, some spores were stained pink (Fig. 1b) as early as 30 min after suspension. The percentage of pink spores increased over time, with the majority of the spores staining pink at 4 h. Even after prolonged incubation, up to 24 h, in GE, the spores stained pink without loss of morphology and no bacilli had been formed (Fig. 2b). In TSB, *Bacillus* spores acquired pink stain in approximately 30 min. At 3 h, most of these spores had germinated and had become vegetative bacilli (pink rods) (Figs 1c and 2c). Other *Bacillus* species (*Bacillus subtilis* var. *niger* ATCC 9372 and *Bacillus subtilis* (*Bacillus niger* Migula) ATCC 6537) were also tested and showed similar ratios between greenish-blue *vs.* pink spores, and pink bacilli, depending on their germination conditions (data not shown).

To synchronize the staining affinity with the microbicidal susceptibility, the spores were exposed to antimicrobial nanoemulsions under the different nutritional conditions. *B. subtilis* suspended in deionized H$_2$O was resistant to the nanoemulsion for 4 h at room temperature (Fig. 3). Incubation of the spores with the nanoemulsion with either GE or TSB resulted in approximately a 3 log reduction in spore count in 4 h (Fig. 3).

DISCUSSION

Modified Wirtz-Conklin stain showed a clear distinction between different treatments of spores which indicate different stages of spore germination. In environments that do not promote spore germinations such as deionized H$_2$O, *Bacillus* spores appeared as greenish-blue spheres (spores). In the presence of GE, which rapidly initiates germination without complete transition to bacilli, spores were stained as pink spheres. In contrast, environments that promote complete germination and outgrowth of the spores to the vegetative bacilli, the germinating spores acquired the pink stain whether they morphologically appear as spheres or rods. The variability of spore germination appeared to be due to the asynchrony in spore population germination following exposure to spore germinants (Moir *et al.* 1979). To confirm the association between the germination events and the alteration in staining, the spores were exposed to bactericidal nanoemulsion under the different incubation conditions. These nanoemulsions have been developed in
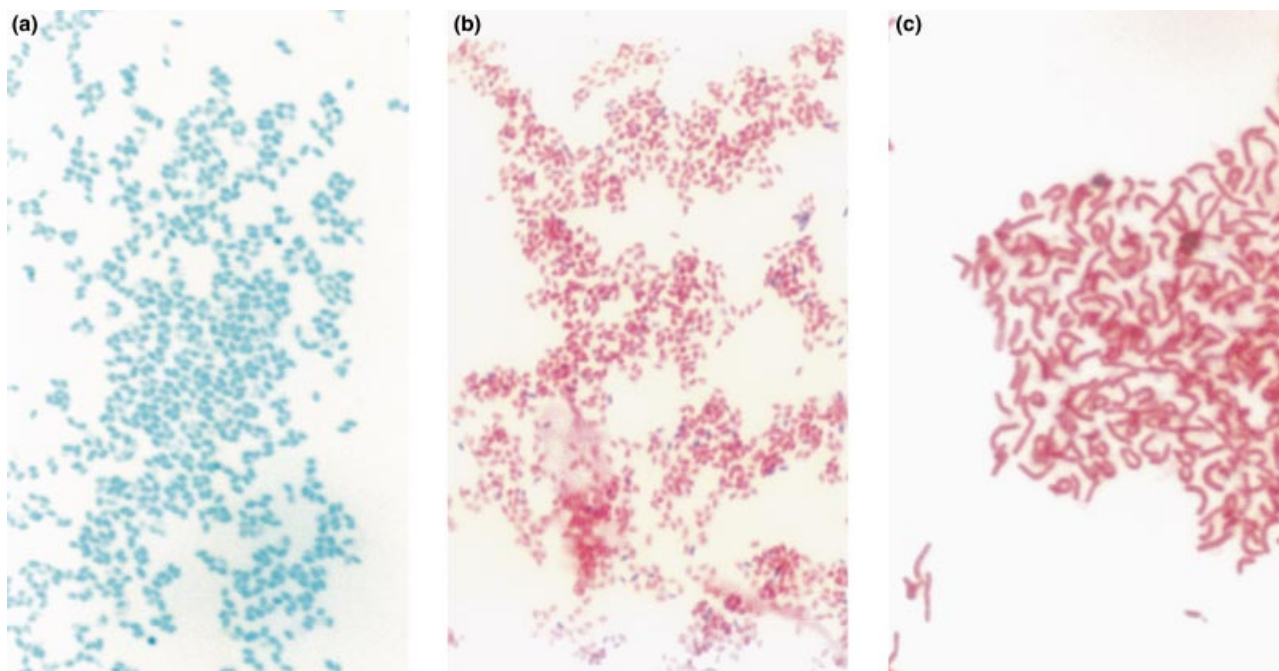
Fig. 1 Micrographs of *B. subtilis* stained with a modified Wirtz-Conklin stain. Spores were suspended in various solutions for 4 h at room temperature on a rotator, then stained with Wirtz-Conklin stain. (a) In deionized H_2O, spores stained greenish-blue, with no initiation of germination or outgrowth (blue spheres); (b) In GE, spores were stained pink and the initiation of germination took place without outgrowth to bacilli (red spheres); (c) In TSB, pink bacilli are observed due to complete outgrowth to vegetative bacilli (pink rods)

our lab in the past four years, and have been studied extensively for their antimicrobial activity (Hamouda *et al.* 1999; Donovan *et al.* 2000; Hamouda and Baker 2000; Hamouda *et al.* 2001). These agents kill spores that have initiated germination but not quiescent spores. *B. subtilis* spores suspended in deionized H_2O, were resistant to the nanoemulsion at room temperature (Fig. 3), and these spores were stained greenish-blue with Wirtz-Conklin stain. Incubation of the spores with the nanoemulsion with either GE or TSB resulted in approximately a 3 log reduction in spore count in 4 h (Fig. 3). Microscopic examination of stained spores, showed that spores incubated with GE (Fig. 2b) did not develop into bacilli for up to 24 h, due to the absence of essential nutrients required for outgrowth. However, they changed in their staining affinity, acquiring a pink colour, instead of a greenish-blue colour following Wirtz-Conklin staining. This change in their staining affinity is associated with initiation of germination in the presence of GE without complete outgrowth to the vegetative bacilli. Germination makes them susceptible to the antimicrobial activity of the nanoemulsions. Spores incubated with complete media will germinate into vegetative bacilli in 3 h. These bacilli will acquire a pink stain, similar to germinating spores, and are also vulnerable to antimicrobial nanoemulsion.

As described in previous reviews, spores lose many of their characteristic properties during germination including refractility, resistance, and dormancy. Early germination events include commitment, which cannot be detected, and loss of heat resistance, and DPA release both of which can be detected minutes after the addition of germinants. Late germination events include, selective cortex hydrolysis, loss of soluble hexosamine, decrease in A600, net ATP synthesis, and the onset of general spore metabolism (Foster and Johnstone 1990). The use of any of these methods for detection of spore germination is complicated and time consuming, and can not identify these events in individual spores. Differentiation of germination using phase contrast microscopy is inaccurate and requires a trained eye since it depends on distinguishing the ratio of phase bright to phase dark spores. Decrease in A600 and phase contrast refractility are only accurate when germination is less than 98%; if germination exceeds this, the estimation of the surviving heat-resistant spores by enumeration would be a more accurate method (Gould and Sale 1970).

Lafferty and Moir used tetrazolium overlay technique to distinguish between (Ger⁻) mutants from the wild type (Ger⁺). The response to tetrazolium was dependent on the efficiency of sporulation in the colony, and this was affected by some exotrophic markers (Moir *et al.* 1979). Powell used hot carbol fuchsin, nigrosin followed by
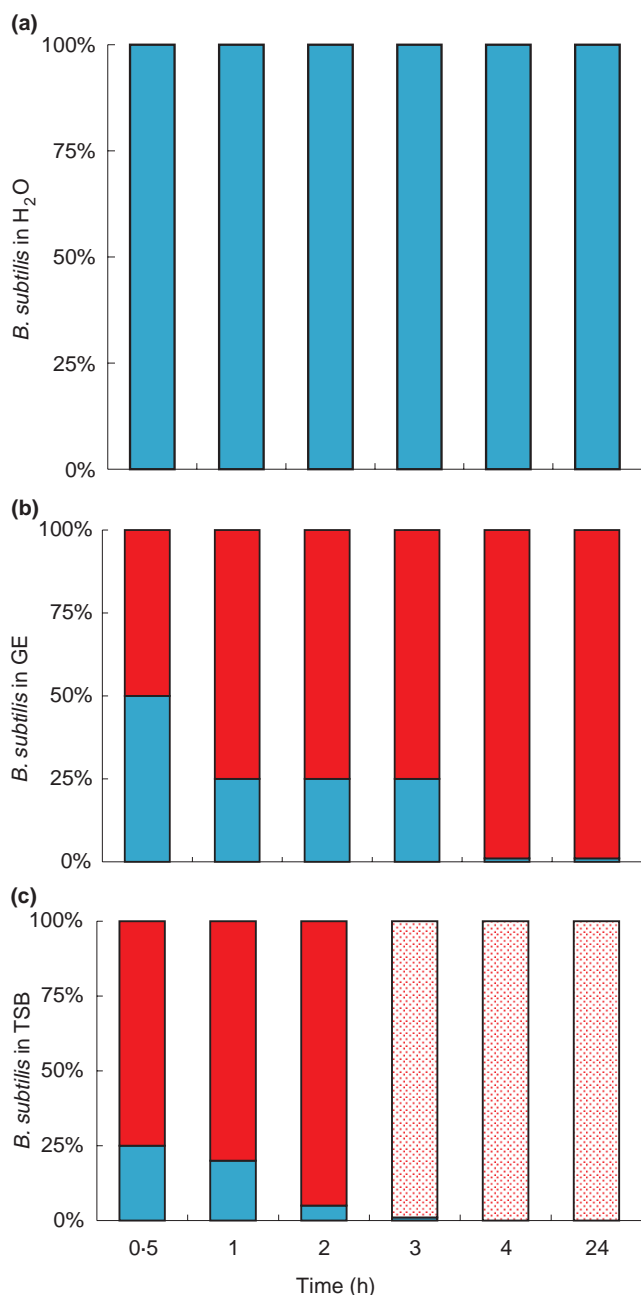
(a)

(b)

(c)

Fig. 2 The graph demonstrates the ratio of greenish-blue spores to red spores to pink bacilli in each of the different spore treatment (a) deionized H_2O (b) GE (c) TSB, in relation to time

methylene blue to identify germinating spores. However, over-staining with carbol fuchsin made differentiation between the two forms very difficult (Powell 1950). Sharma and Prasad proposed the use of aqueous acridine orange to differentiate non-viable spores which fluoresce lemon-green, and viable spores which fluoresce orange red. This technique requires 4 h, and the use of different
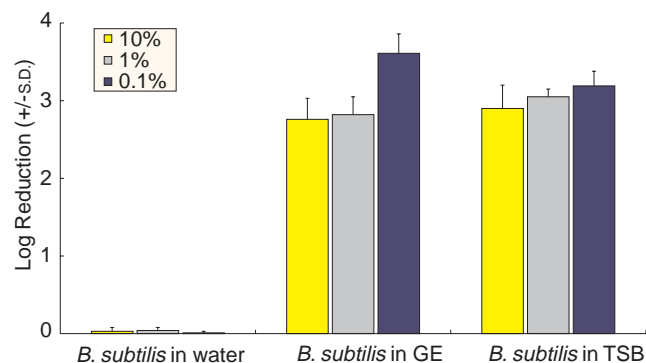

Fig. 3 Log reduction of *B. subtilis* suspended in various solutions (deionized H_2O, GE or TSB) and treated with antimicrobial nanoemulsion (NanoSafe1™) for 4 h at room temperature

microscope (Sharma and Prasad 1992). Bruno and Mayo used colour fluorescence image analysis of acridine orange stained germinating bacilli where stained spores produced a green fluorescence. Following incubation with glucose solution, due to spore germination, larger reddish or metachromatic cells were detected (Bruno and Mayo 1995). However, this technique lacked reproducibility in determining germination, required intensive scanning of the slide and the use of a fluorescent microscope. Ease of performance and quantification, make the use of modified Wirtz-Conklin stain is a valuable technique for the detection of initiation of spore germination.

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