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Dynamic Staining of *Bacillus* Endospores with Thioflavin T

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***Abstract*— Rapid detection and identification of endospores presents a range of complex challenges. Dynamic staining approach, developed in our lab, utilizes the time-course fluorescence enhancement of an amyloid-staining dye, Thioflavin T (ThT), after mixing with intact endospores. We examined the kinetics of staining *Bacillus atrophaeus* and *Bacillus thuringiensis* endospores, and the rates of staining were different for the two bacilli when intact endospores were treated with ThT. This finding demonstrates an avenue for attaining information about the sporulated bacterial species without lysing, germinating or other pretreatment steps.**

I. Introduction

This report demonstrates the use of thioflavin T (ThT) for dynamic staining of intact endospores of *Bacillus atrophaeus* (*B. atrophaeus*) and *Bacillus thuringiensis* (*B. thuringiensis*). While the fluorescence images of the ThT-stained endospores of the two species had similar appearances (Fig. 1a, b), the kinetics of the staining process were different for the two *Bacilli* (Fig. 1c).

Endospores are packed and resilient entities, and spore-forming bacteria are responsible for the production of some of the most puissant toxins. Despite numerous advances in biomedical technology, the rapid detection and identification of bacterial endospores remains challenging. Assays for bacterial endospores require a range of sample pretreatment steps, such as lysing to extract their content for analysis or allowing them to germinate and analyzing the resulting cultures of vegetative bacteria [1-4].

The endospore coats contain a wealth of information about the bacterial species [5-9]. Therefore, analytical techniques, targeting components of exosporium, provide the means for rapid endospore biosensing with certain species-specificity.

We previously demonstrated the use of ThT, an amyloid fluorescent stain, for the rapid visualization of endospores [10]. ThT binds with a high affinity to proteins with amyloid-like β-sheet motifs—like those that constitute the exosporium of the endospores. Furthermore, upon binding to endospores, the fluorescence quantum yield of ThT increases, resulting in the ability to image endospores in the presence of the leftover unbound dye. This emission enhancement effectively eliminates the necessity for pre or post-treatment steps for the visualization of endospores [10]. Furthermore, we previously reported no observable change on the endospores' ability to germinate after exposure to ThT [10].

Recently, we demonstrated that the kinetics of fluorescence enhancement of cyanine stains, when applied to different vegetative bacteria, are characteristic of the species, regardless of whether the bacteria are Gram positive or Gram negative [11]. The timed progression of staining, monitored via the enhancement of the fluorescence of the staining dye, differed for different species, which, in turn, provided venues for statistically significant discernibility among microbial organisms [11]. Subsequently, we previously demonstrated the completion of mixing for this experimental setup in less than 1-2 seconds [11].

Herein, we proceed to demonstrate the application of this dynamic approach to intact bacterial endospores by utilizing the emission enhancement of ThT. We observed that the rates of fluorescence staining of *B. thuringiensis* were faster than the rates of staining of *B. atrophaeus*, which can be representative of the composition of the endospore coats of the two species.

II. Methods

Following the methods we previously reported [10, 12], we inoculated the *Bacilli*, sporulated the cells, and purified the endospores. Bright field and epifluorescence microscopy allowed us to examine the samples (Fig. 1a, b) [10, 12]. We recorded the kinetics of fluorescence staining with ThT using protocols developed in our lab [11].

The experimental data was fit to a monoexponential rise function showing the increase in the emission intensity with time, $F(t)$. This analysis allowed us to determine the characteristic time constants, τ, for the quantification of the dynamics of the fluorescence staining (Fig. 1c) [11]:

$$F(t)_{t\geq t_0} = F(\infty) - \Delta F \exp\left(-\frac{t-t_0}{\tau}\right) \quad (1)$$

where $F(\infty)$ is the fluorescence intensity upon completion of the staining, ΔF is the maximum change in the fluorescence intensity, and t_0 is the starting time of endospore-stain interaction initiated by rapid mixing of the sample with the dye.

III. Results & Discussion

The intensity change, ΔF, varies with the dye concentration, C_{ThT}, and with the endospore density,

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represented as spore count, SC (Figure 2a, Table 1). Doubling the density from 7.5×10^4 to 15×10^4 spores ml^{-1} caused an increase in ΔF that exceeded twofold (Table 1).

TABLE 1. TIME CONSTANTS, τ / S, AND THE MAXIMUM CHANGE IN FLUORESCENCE INTENSITY, ΔF, FOR THE EMISSION ENHANCEMENT OF THT INDUCED BY *BACILLUS* ENDOSPORES.[a]

C_{ThT} [b] / μM	*B. atrophaeus* [c,d] (SC / spores ml^{-1}) [e]				*B. thuringiensis* [c,d] (SC / spores ml^{-1}) [e]			
	(7.5×10^4)		(1.5×10^5)		(7.5×10^4)		(1.5×10^5)	
	τ / s	$\Delta F \times10^{-3}$ / CPS	τ / s	$\Delta F \times10^{-3}$ / CPS	τ / s	$\Delta F \times10^{-3}$ / CPS	τ / s	$\Delta F \times10^{-3}$ / CPS
1	6.6 ± 1.2	27 ± 6	7.2 ± 1.5	59 ± 8	3.0 ± 0.5	20 ± 8	3.3 ± 0.3	43 ± 9
5	7.7 ± 0.8	42 ± 5	6.5 ± 1.7	97 ± 8	2.4 ± 0.6	40 ± 7	3.2 ± 0.8	97 ± 9
10	6.4 ± 2.4	37 ± 3	7.7 ± 2.5	87 ± 6	2.6 ± 1.2	41 ± 2	2.6 ± 0.7	120 ± 14

[a] The values of τ were extracted from monoexponential data fits of emission kinetic traces (λ_{ex} 430 nm, and λ_{em} 480 nm), recorded upon mixing of dye solutions and endospore suspensions (all in aqueous media: 2 mM Tris buffer at pH 8.5 with 1 mM TWEEN® 40). The average values and standard deviations of τ were obtained from four repeats. ΔF values represent the maximum difference between the average final emission intensity (~120s after the addition of endospores) and the average initial emission intensity (before the addition of endospores).

[b] Dye concentration, C_{ThT}.

[c] For two-factor analysis of variance (ANOVA2), the two null hypotheses were: $H_{0,1}$: the values of τ do not depend on C_{ThT}; and $H_{0,2}$: the values of τ do not depend on the spore count, SC. For *B. atrophaeus*, ANOVA2 produced $p_{SC} = 0.97$, $p_{ThT} = 0.75$, and $p_{SC,ThT} = 0.38$, i.e., neither $H_{0,1}$, nor $H_{0,2}$, can be rejected. For *B. thuringiensis,* ANOVA2 produced $p_{SC} = 0.36$, $p_{ThT} = 0.29$, and $p_{SC,ThT} = 0.53$, i.e., neither of the null hypotheses can be rejected.

[d] Comparing all values of τ measured for *B. atrophaeus* with all values of τ for *B. thuringiensis*, using one-factor ANOVA (H_0: the values of τ do not depend on the endospore species), produced $p = 1.8\times10^{-14}$. Therefore, H_0 can be rejected at any reasonable level of confidence.

[e] The spore counts, SC, after mixing the dye solutions with the endospore suspensions.

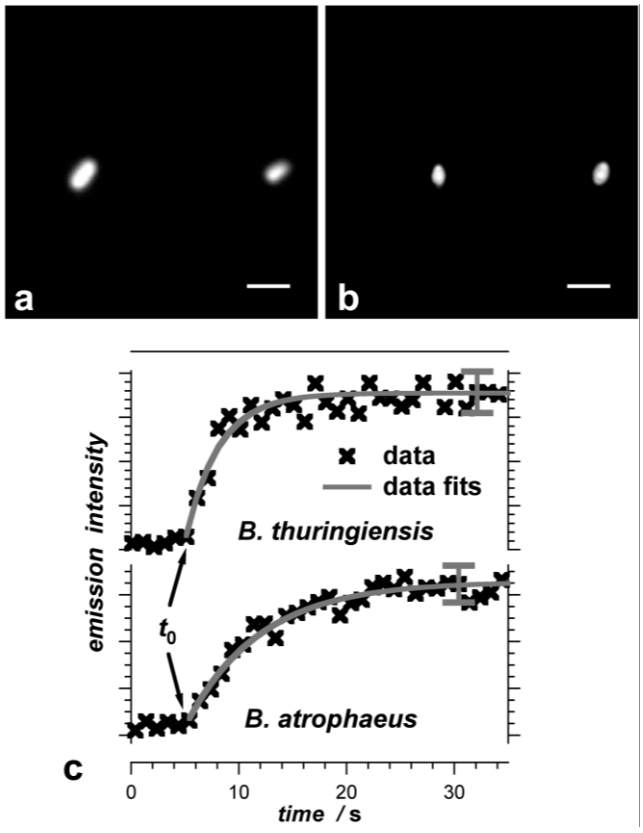

Figure 1. Fluorescence staining of endospores of *B. atrophaeus* and *B. thuringiensis* with ThT. (a, b) Epifluorescence microscope images of endospores of (a) *B. atrophaeus* and (b) *B. thuringiensis* stained by mixing with a ThT aqueous solution (C_{ThT} = 1 μM; 2 mM Tris buffer, pH 8.5, with 1 mM TWEEN® 40; λ_{ex} 482 nm, λ_{em} 536 nm, the band widths of the imaging filters were 25 nm; scale bar corresponds to 3 μm). (c) Kinetics of the enhancement of the emission of 1 μM ThT upon mixing with endospores, 1.5×10^5 spores ml^{-1}, at time t_0, with the monoexponential data fits (2 mM Tris buffer, pH 8.5, with 1 mM TWEEN® 40; λ_{ex} 430 nm, λ_{em} 480 nm).

Concurrently, a fivefold increase in C_{ThT} from 1 to 5 μM caused an increase in ΔF that was less than a factor of two. A further increase in C_{ThT} from 5 to 10 μM marginally affected ΔF (Figure 2a). These trends were similar for the endospores of both species (Figure 2a), indicating that static measurements of fluorescence intensity of the stained samples (i.e., $F(\infty)$ or ΔF) could not provide sufficient information for discerning between the two *Bacilli*. Conversely, the dynamic characteristic of the straining processes, τ, manifested distinctly different trends in comparison with the static characteristic, ΔF (Figure 2b). For endospore densities varying around 10^5 spores ml^{-1}, the time constants for each of the two *Bacilli* did not manifest a statistically significant dependence on the dye concentration in the range between 1 and 10 μM (Table 1, footnote c). Such a lack of statistically significant concentration dependence of the time constants agreed with the kinetic trends we have observed for vegetative bacterial cells [11].

For *B. atrophaeus* endospores, the τ values for staining with ThT clustered around 7 s, while *B. thuringiensis* τ values clustered around 3 s for the same dye. This difference constitutes a statistically significant discernibility in the respective τ values of the *Bacilli* (Table 1, footnote d). The faster rate of staining of *B. thuringiensis*, in comparison with *B. atrophaeus*, was attributed to the reduced amount of proteins on *B. thuringiensis'* surface to be stained, as reflected by its genotype [5].

The absolute values of fluorescence intensities, representing static characteristics such as $F(\infty)$, which is the sum of the initial fluorescence intensity and ΔF, depend on: (1) sample properties, such as the partition coefficient of ThT between the aqueous media and the endospores, the fluorescence quantum yield of the free and the bound dye, and the scattering properties of the endospore suspension;

and (2) instrumental settings, such as the manners of sample photoexcitation and emission measurement. Therefore, ΔF manifests dependence on SC and C_{ThT}, which is not necessarily linear (Figure 2a).

Conversely, the dynamic characteristics of staining, such as the time constants, τ, depend solely on the internal sample properties. Because τ characterizes the rates of fluorescence changes, the emission intensities prior to staining (unbound ThT) and after the completion of the staining process served as an internal standard, allowing for compensating for potential changes in the instrumental settings.

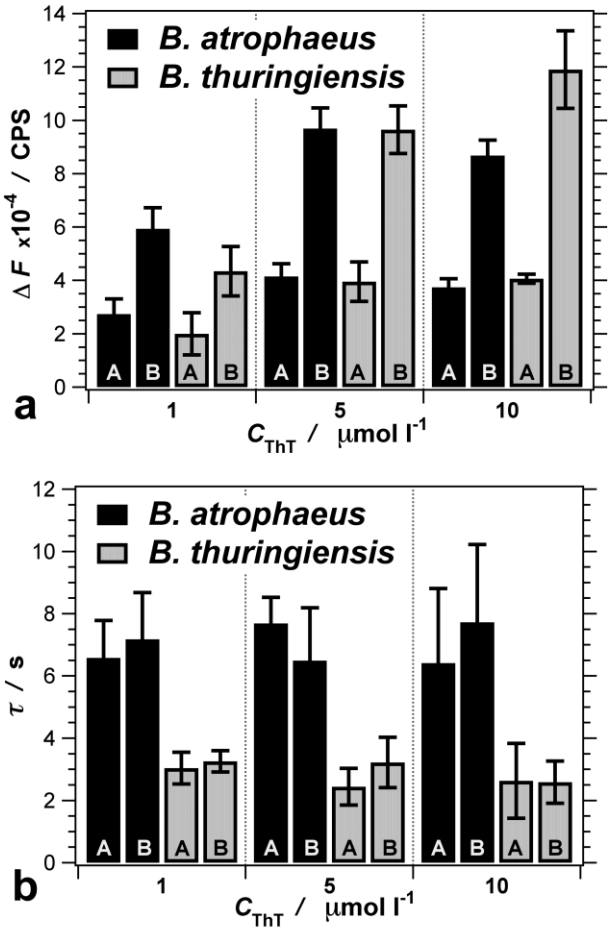

Figure 2. Category plots of: (a) static, ΔF, and (b) dynamic, τ, characteristics of staining of *B. atrophaeus* and *B. thuringiensis* with ThT. The time constants, τ, and the change in the fluorescence intensity, ΔF, were extracted from least-square data fits of kinetic emission data (Figure 1c) using equation 1 for different dye contraptions, C_{ThT}, and endospore densities of: A = 7.5×10^4 spores ml^{-1}, and B = 15×10^4 spores ml^{-1}.

Based on a diffusion model of staining, τ should exhibit negligible concentration dependence and, concurrently, a strong dependence on the cell or endospore media that takes up the staining dye [11, 13]. This simplified view appears to be in accord with the staining dynamic of species and stains that we currently report and previously investigated [11].

These findings show the species discernibility between intact bacterial endospores produced by the dynamic staining approach. Moreover, the observed trends demonstrate the advantages of applying dynamic, instead of static, approaches to bioanalysis. We believe that dynamic staining provides an avenue of unprecedented simplicity and facility for endospore assays.

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