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Terbium chloride influences *Clostridium difficile* spore germination

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Abstract

The germination of *Clostridium difficile* spores is an important stage of the *C. difficile* life cycle. In other endospore-forming bacteria, the composition of the medium in which the spores are generated influences the abundance of germination-specific proteins, thereby influencing the sensitivity of the spores towards germinants. In *C. difficile* media composition on the spores has only been reported to influence the number of spores produced. One of the measures of spore germination is the analysis of the release of DPA from the spore core. To detect DPA release in real time, terbium chloride is often added to the germination conditions because Tb^{3+} complexes with the released DPA and this can be detected using fluorescence measurements. Although *C. difficile* spores germinate in response to TA and glycine, recently calcium was identified as an enhancer for spore germination. Here, we find that germination by spores prepared in peptone rich media, such as 70:30, is positively influenced by terbium. We hypothesize that, in these assays, Tb^{3+} functions similarly to calcium. Although the mechanism(s) causing increased sensitivity of the *C. difficile* spores that are prepared in peptone rich media to terbium is still unknown, we suggest that the $TbCl_3$ concentration used in the analysis of *C. difficile* DPA release be carefully titrated so as not to misinterpret future findings.

Introduction

Patients treated with broad-spectrum antibiotics have a disrupted gut microbiome and, as a result, are vulnerable to *C. difficile* infection (CDI) due to the loss of the colonization resistance that is provided by the microbiota [1]. Upon colonization of the intestinal tract, *C. difficile* vegetative cells elicit the symptoms of disease through the secretion of two toxins, TcdA (an enterotoxin) and TcdB (a cytotoxin) and their eventual endocytosis by the colonic epithelial cells [2]. *C. difficile*-infected patients are commonly treated with other antibiotics, such as vancomycin or fidaxomicin, which relieve the primary symptoms of CDI by targeting the actively-growing, toxin-producing, vegetative forms [3]. However, and importantly, these antibiotics also disrupt the gut microbiome and may result in recurring episodes of disease[4].

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Though the *C. difficile* vegetative cells are strictly anaerobic, they form metabolically dormant spores that are resistant to toxic chemicals, high temperature, radiation and oxygen [5–7]. As a result of this dormancy, the spores can survive outside the host in the aerobic environmental setting where the strictly anaerobic, vegetative form cannot. Structurally, endospores are composed of several, well-defined layers [8]. The endospore core contains DNA, RNA, ribosomes and a large amount of dipicolinic acid (pyridine-2-, 6-dicarboxylic acid; DPA), chelated with calcium (CaDPA) that provides heat resistance [6, 9]. The core is surrounded by an inner spore membrane composed of phospholipids, followed by a layer of germ-cell wall (which later becomes the cell wall of the vegetative cell during outgrowth). A thick layer of specialized peptidoglycan, cortex, surrounds the germ-cell wall and protects the spore core against osmolysis. The cortex is surrounded by the outer membrane derived from the mother cell, coat proteins and an exosporium layer [6–8, 10]. When the environmental conditions become favorable, spores germinate and outgrow to the vegetative form [7, 11, 12].

C. difficile spore germination is initiated in response to the combinatorial actions of certain bile acids [*e.g.*, taurocholic acid (TA)] and amino acids (*e.g.*, glycine) [13–17]. In prior work, the bile acid germinant receptor was identified as the germination-specific, subtilisin-like, pseudoprotease, CspC [18]. More recently, we found that a small mutation in the *cspA* coding region of *cspBA* resulted in spores that germinate in response to TA alone (without the requirement for an amino acid), suggesting that the CspA protein is the amino acid germinant receptor (Shrestha and Sorg, submitted [19]).

In several endospore-forming bacteria, the abundance of the spore germinant receptors is significantly influenced by the medium used to generate the spores [20]. *C. difficile* spores are generally prepared in rich media at an optimal temperature and cell density for higher yields [21]. A study by Hornstra *et al.*, demonstrated that in *Bacillus cereus* ATCC 14579, the composition of the sporulation medium increased the transcription of the seven germinant receptors, and the nutrient-induced spore germination was significantly affected [22]. Similar results were also observed in other spore-forming bacteria (e.g., *B. subtilis*) [20], however, no studies have been performed in *C. difficile* on how the medium used to generate spores influences the properties of spore germination; although there are studies that show that high spore yield can be obtained when *C. difficile* spores are produced in peptone rich medium, such as 70:30 or SMC [21, 23–27]. Here, we report that media composition influences the abundance of the *C. difficile* spore germinant receptors and find that spores prepared on peptone-rich medium can germinate in response to TA and terbium ions (which may function as a calcium substitute). Because Tb^{3+} ions are often used to detect the presence of the dipicolinic acid that is released during the early events of spore germination [16, 17, 28–33], these findings could be useful for future analysis of *C. difficile* spore germination or for the germination of other bacteria that are influenced by calcium ions.

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MATERIALS AND METHODS

Growth conditions

C. difficile UK1 (ribotype 027) [18, 34, 35] and M68 (ribotype 078) [17, 35, 36] strains were grown on either BHIS agar medium [Brain heart infusion (Bacto BHI supplemented with 5 g / L yeast extract] or 70:30 agar medium [63 g / L Bacto peptone, 3.5 g / L protease peptone, 11.1 g / L BHI, 1.5 g/ L yeast extract, 1.06 g / L tris base and 0.7 g / L ammonium sulfate (NH_4SO_4)] or SMC agar medium (90 g / L Bacto peptone, 5 g / L protease peptone, 1 g / L NH_4SO_4 and 1.5 g / L tris base) [37] or TYG agar medium [(30 g / L Bacto typtone, 20 g / L yeast extract and 10 g / L glucose)] in an anaerobic environment (85% N_2, 10% H_2, and 5% CO_2) (Model B, Coy Laboratories, Grass Lake, MI) at 37 °C. The BHIS, 70:30 and SMC agar media were supplemented with 1 g / L of L-cysteine while TYG agar medium was supplemented with 1 g / L thioglycolate.

Spore purification

C. difficile spores were purified as described previously [16, 17]. Briefly, the *C. difficile* UK1 and *C. difficile* M68 strains were grown on either BHIS, 70:30, SMC or TYG agar medium as described above and allowed to grow for 4 days. Cells from each plate were scraped into 1 mL sterile water and incubated at 4 °C overnight. Next, the cells were washed five times with water to remove cell debris and combined into 2 mL total volume. The washed spores were layered on top of 8 mL of 50% sucrose and centrifuged at 4,000 X g for 20 minutes. The spore pellets were separated from the supernatant, and the spores were washed five times with water to remove any sucrose and incubated at 4 °C until use.

Germination of spores isolated from different media

The spores were characterized by measuring DPA release as well as changes to the optical density (OD_{600}) during germination, as described previously [17, 30]. Briefly, spores purified from various media were heat activated at 65 °C for 30 minutes and suspended in water at an OD_{600} = 50. In order to measure DPA release, the spores were added to final OD_{600} of 0.25 in 100 μL final volume of buffer (50 mM HEPES, 250 mM NaCl, pH 7.5) supplemented with 250 μM Tb^{3+} and containing either 10 mM TA or 30 mM glycine or both 10 mM TA and 30 mM glycine in a 96 well black plate. The DPA release was then measured for 2 hours at 37 °C using a SpectraMax M3 (Molecular Devices) plate reader with excitation at 270 nm and emission at 545 nm with a 420 nm cutoff.

In order to measure the germination by optical density, the spores were added to a final OD_{600} of 0.5 in HEPES buffer supplemented with 30 mM glycine alone or 10 mM TA alone or 10 mM TA and 30 mM glycine in 100 μL final volume with or without 250 μM Tb^{3+} in 96 well clear plates. OD_{600} was monitored at 37 °C for 2 hrs. using the plate reader. To determine the influence of metals on *C. difficile* spore germination, 70:30-prepared spores were incubated with either 10 mM TA or 10 mM TA supplemented with 10 mM or 250 μM of metal chlorides ($CaCl_2$, $MnCl_2$, $MgCl_2$, KCl, $LiCl_2$ or $TbCl_3$). Germination was monitored as above.

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Western blot

Samples for SleC activation were prepared by treating UK1 and M68 spores with either 10 mM TA or 30 mM glycine or both 10 mM TA and 30 mM glycine with or without 250 μM Tb^{3+} and incubated at 37 °C for 2 hours. Soluble protein samples were extracted by boiling the spores in NuPage buffer (Life Technologies) at 95 °C for 20 minutes and centrifuged at 20,000 x g for 10 minutes to separate supernatant from the spore pellet. Solubilized proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane for SleC detection by western blot. Similar technique was used to determine the influence of metals on *C. difficile* spore germination using SleC activation.

In order to quantify amounts of SleC, CspB, and CspC proteins in spores prepared from different media, solubilized proteins were extracted by boiling 2×10^9 spores per mL in NuPAGE (Life Technologies) sample buffer at 95 °C for 20 minutes. Equal volume of spore extracts and recombinant CspB, CspC or SleC standard proteins were separated by SDS-PAGE. Proteins were then transferred onto low-fluorescence polyvinylidene difluoride membrane (PVDF) at 30V for 16 hours. The membrane was then blocked in 10% skimmed milk in TBS (Tris-buffered saline) and washed thrice with TBS containing 0.1% (vol / vol) Tween-20 (TSBT) for 20 minutes each at room temperature. The membranes were then incubated with anti-CspB, anti-CspC or anti-SleC antibodies for 2 hours and washed thrice with TSBT. For the secondary antibody, AlexaFlour 555-labeled donkey anti-rabbit antibody was used to label the membranes for 2 hours in the dark. The membranes were washed again thrice with TBST in the dark, and scanned with a GE Typhoon Scanner using Cy3 setting, an appropriate wavelength for the Alexa Flour 555 fluorophore. The fluorescent bands were analyzed using ImageQuant TL 7.0 image analysis software. Intensity of the extracted protein in each blot was compared to the standard curve that was included on each blot.

Calcium measurement

Calcium concentrations in different media were measured using a Ca^{2+} detection assay kit (Abcam, ab102505). Standards for Ca^{2+} were prepared as advised in the kit. The Ca^{2+} concentration was then measured from BHI, BHIS, 70:30, SMC and TYG medium at OD_{575} in the plate reader. The OD_{575} values for the Ca^{2+} measurement were then converted into μg / L. For the control, 23 mg / L $CaCl_2$ suspended in water was used.

Statistical Analysis

All germination assays were performed in technical triplicate and data points represent the average of three independent experiments. Biological replicates showed the same findings but due to differences in time points for the OD assay and DPA content for the DPA release assays, the data could not be averaged. Calcium concentrations were measured in triplicate. Error bars represent the standard error of the mean. A one-way ANOVA with Tukey’s multiple comparisons test was used to compare the quantified protein amounts. Each blot was loaded with five standard proteins and three spore samples for quantification of proteins.

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RESULTS

Tb^{3+} enhances the germination of *C. difficile* spores with TA.

Recent work by Kochan *et al.*, on germination by *C. difficile* spores has shown that calcium plays a role as an enhancer of germination when added with TA [24]. In several studies, terbium has been used to replace calcium, mostly to observe the calcium binding because terbium fluoresces at certain wavelengths and calcium does not [38, 39]. One of the ways germination is observed is through the measurement of the DPA that is released from the spore core. Upon recognition of germinants by *C. difficile* spores, the cortex layer is degraded by the cortex lytic enzyme (SleC) and, subsequently, DPA is released from the core [40]. In the presence of terbium ions, the released DPA can associate with terbium and the terbium-DPA complex can be detected in a FRET-like assay.

During some of our experiments analyzing the requirements of *C. difficile* spore germination, we found that, at times, wild type *C. difficile* spores would seemingly release DPA in response to TA alone. To investigate this phenomenon further, we measured the DPA released from spores that were prepared on BHIS medium. We found that these spores started to release DPA in response to TA alone after 1 hr 30 min incubation in these conditions (Figure 1A). However, when germination was measured by OD at 600 nm, in absence of terbium, the OD did not change with TA alone (Figure 1B). Surprisingly, when the germination was measured by OD in presence of terbium, the OD changed in response to TA alone (Figure 1C) - the germination rate was much faster when glycine was added as a co-germinant. To confirm that our observations were due to the initiation of spore germination and not just an artifact of the assay conditions, we tested the activation of SleC after two hours of incubation in the indicated germination condition. We found that in presence of terbium, SleC is activated when the spores were treated with TA alone, similar to the activation of SleC in response to both TA and glycine (Figure 1D). These results suggest that terbium might be involved in germination of the spores, potentially similar to the response of *C. difficile* spores to calcium or other metals [23].

The TA-terbium response increased when spores were prepared in peptone rich medium.

Many *C. difficile* strains do not efficiently form spores in liquid BHIS medium. Therefore, most studies produce spores on agar medium (*e.g.*, BHIS, 70:30, or SMC) [21].To investigate if our observation that spores germinate in response to TA and Tb^{3+} was due to how spores were prepared (*i.e.*, BHIS agar medium, on which our laboratory normally produces spores), we prepared spores on BHIS, 70:30, SMC or TYG agar medium and tested how Tb^{3+} influences germination of these spores. Interestingly, we found that the TA-terbium phenotype increased when spores were prepared in the peptone rich media, when compared to BHIS agar medium. In figure 2A, 2D, and 2G we compared DPA release of wildtype *C. difficile* UK1 spores prepared from 70:30, TYG or SMC agar medium, respectively. We found that the influence of Tb^{3+} on *C. difficile* spore germination was greater in these media compared to BHIS-prepared spores (Figure 1A). Similar to what we observed for BHIS-prepared spores, none of these spores germinated, as measured by changes in OD_{600}, in the absence of Tb^{3+} (Figure, 2B, 2E, and 2H). However, when terbium was added with TA, the OD of the spore suspension dropped in response to TA and Tb^{3+}

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(Figure 2C, 2F and 2I). We also confirmed that the activation of SleC occurred in presence of TA and Tb^{3+} (Figure S1). These results strongly suggest that the medium used to prepare spores plays a role in the TA-terbium phenotype; the spores become more responsive to terbium when produced on a more peptone rich media (*e.g.*, 70:30, SMC and TYG).

TA-terbium phenotype is found in a different *C. difficile* ribotype.

Next, we wanted to test if the TA-terbium phenotype is only true for the *C. difficile* UK1 strain or if it is observed in another ribotype. In our prior work on *C. difficile* spore germination, we used the *C. difficile* M68 strain (ribotype 078) to test the impact of muricholic acids and the effect of different amino acids on *C. difficile* spore germination [17, 36]. Thus, we used this strain to investigate the impact of Tb^{3+} on germination by *C. difficile* spores. As for *C. difficile* UK1, we prepared *C. difficile* M68 spores in BHIS, 70:30 or SMC agar medium (M68 strains did not form spores on TYG medium for unknown reasons) and measured DPA release (Figure 3A, 3D and 3G). The M68 strain also germinated in response to TA and Tb^{3+} and, similar to *C. difficile* UK1 spores, this phenotype was stronger for spores prepared from 70:30 and SMC compared to spores prepared from BHIS agar medium. Similarly, when we tested germination by OD, in the absence of terbium, spores did not germinate in response to TA alone (Figure 3B, 3E and 3H). However, the addition of Tb^{3+} to the germination solution resulted in germination (Figure 3F and 3I); BHIS prepared spores (Figure 3C) had a much milder response to $TbCl_3$ addition than did 70:30- or SMC-prepared spores (Figure 3F and 3I). These observations were also confirmed, as for *C. difficile* UK1, by analyzing SleC activation (Figure S2).

The TA-terbium phenotype extends to other metals.

Recent work by Kochan et al. [23] has shown that apart from calcium, germination by *C. difficile* spores can also be stimulated by other metal ions such as $MgCl_2$ but not with $ZnCl_2$, $LiCl_2$, and KCl. Here, we also tested germination by *C. difficile* UK1 spores prepared from 70:30 medium, which are sensitive to terbium, with these metal chlorides as enhancers or cogerminants. Similar to the results by Kochan and colleagues [23], we also found that the spores germinated with $MgCl_2$ or $CaCl_2$, but not with $LiCl_2$ nor KCl, at either 10 mM (Figure 4A) or 250 μM (Figure 4B). We also tested $MnCl_2$ and found that at 10 mM $MnCl_2$, the spores germinated similar to $CaCl_2$ or $TbCl_3$ (Figure 4A) and germinated similar to $CaCl_2$ at 250 μM $MnCl_2$ (Figure 4B). The germination was confirmed by analyzing SleC activation. At 10 mM TA and 10 mM $CaCl_2$ or $MnCl_2$ or $MgCl_2$ or glycine, *C. difficile* spores activated SleC (Figure 4C). A 10 mM TA and 250 μM $CaCl_2$ or $MnCl_2$ or $MgCl_2$ or glycine, *C. difficile* also activated SleC indicating that these co-germinants are effective at low concentration (Figure 4D). Unfortunately, we could not test $ZnCl_2$. When dissolved, $ZnCl_2$ changed the pH of the solution to $pH < 4$ and any effort to neutralize the pH resulted in precipitation. Moreover, the effect of pH on *C. difficile* spore germination has been documented in prior work [23, 41].

Medium composition influences the abundance of *C. difficile* spore germinant receptors but does not explain TA-Tb^{3+} phenotype.

In *B. subtilis*, it was shown that spores prepared on different media resulted in changes to the amount of Ger proteins found within the spores [20]. We hypothesized that spores prepared

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in peptone rich media may have increased amounts of the germinosome proteins (*i.e.*, CspB, CspC or SleC) which could influence the sensitivity of spores to terbium. To test this hypothesis, we quantified the amount of CspB, CspC and SleC proteins purified from the different media mentioned above using a previously described technique (due to the quality of our CspA antibody, we could not quantify the abundance of CspA in the spores). We found that there was not a significant increase in SleC levels for *C. difficile* UK1 or *C. difficile* M68 spores that were purified from different media (Figure 5A and 5B, respectively). Though the differences in SleC abundance appear striking, they did not meet statistical significance. We observed an increase in CspB protein purified from 70:30, TYG or SMC media compared to BHIS-prepared medium for spores derived from the *C. difficile* UK1 strain (Figure 5C). However, for *C. difficile* M68, spores prepared from BHIS contained a larger amount of CspB than did spores prepared on 70:30 or SMC media (Figure 5D). We also observed small differences in abundance of CspC in both the strains purified from different media (Figure 5E, 5F). But, because CspC abundance in *C. difficile* UK1 SMC-prepared spores was lower than in TYG and *C. difficile* M68 SMC-prepared spores was more abundant the abundance of CspC is not likely to contribute to the observed phenotypes. These results suggest that producing *C. difficile* spores on different media can influence the abundance of some spore proteins. However, we did not observe a correlation between medium and the Tb^{3+} phenotype observed in Figures 1, 2 and 3.

The calcium concentrations are similar in different media.

In several studies, terbium has been used to substitute calcium. Previous studies by Kochan *et al.* have also shown that calcium is involved in enhancing germination by *C. difficile* spores in the presence of germinants (TA and glycine) [23, 24]. We hypothesized that TA-terbium is functioning similar to TA-calcium/other metals and that a difference in the calcium concentration in the media used to prepare spores might contribute to spores being sensitive to calcium or terbium in these germination assays. In order to test this hypothesis, we used a colorimetric assay to detect calcium concentration in various media. However, we observed no significant difference in the calcium concentration in BHIS, 70:30, SMC or TYG media (Figure 6). These results suggest that an unidentified factor contributes to the sensitivity to terbium during *C. difficile* spore germination and that to detect the release of DPA from germination of *C. difficile* spores, the concentration of terbium should be optimized so as not to influence germination during the assay conditions.

DISCUSSION

Growth media plays a significant role in formation of spores [20–22]. A common medium used for the growth of *C. difficile* vegetative cells is BHIS agar medium; however, *C. difficile* spores are frequently produced in / on 70:30 or SMC medium because of the improved spore yield in these media [21, 23–27]. Importantly, the different composition of sporulation media can impact the germination phenotype of the spores. For example, Ramirez-Peralta *et al.*, showed for *B. subtilis* spores the medium composition had a significant effect on the rate of spore germination [20]. The rate of germination of *B. subtilis* spores prepared from nutrient-rich, liquid medium was significantly higher compared to nutrient-poor, liquid medium. Similarly, we found that germination of spores prepared under

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different sporulation conditions (media) resulted in significant differences in germination. Specifically, when we prepared spores from the BHIS medium, at the end of 2 hours germination we observed slight increase in DPA release in presence of TA alone (Figure 1A); the release of DPA corresponds to germination. Oddly, when the OD assay was used to measure the germination, there was no germination in TA alone (Figure 1B). The difference between the assays is the addition of terbium to monitor DPA release. When terbium was added in the OD assay, we observed that, similar to DPA increase in TA alone, germination was observed with OD within 2 hours suggesting that it was terbium, along with TA, that induced the germination of the spores. Although *C. difficile* is known to germinate in the presence of bile acids (*e.g.*, TA) and amino acids (*e.g.*, glycine), calcium has been identified as an important contributor to spore germination, though its role remains undefined [23, 24, 42]. It is not clear whether calcium functions as a bona fide spore cogerminant, a co-factor for a process essential for spore germination and / or as an enhancer for amino acids. But, removal of calcium by chelation prevents spore germination, indicating that calcium is essential for *C. difficile* spore germination, and addition of calcium increases the sensitivity of spores to germinants [24].

We found that the TA-terbium germination phenotype increased when spores were prepared from peptone rich medium, such as 70:30, SMC or TYG, and compared to BHIS-prepared spores. As shown in Figure 2, when spores were produced from 70:30, TYG or SMC medium, the spores germinated in response to TA-terbium within 1 hour. The TA-terbium phenotype was stronger in spores prepared from SMC medium followed by TYG, 70:30 then BHIS medium. These results were also true for the *C. difficile* M68 strain (Figure 3). We found that in wildtype *C. difficile* UK1 spores, CspB abundance was significantly increased in SMC, 70:30 or TYG medium compared to BHIS medium, but this was not true for *C. difficile* M68 spores (Figure 5). Unfortunately, we could not analyze the abundance of CspA in this study. It is possible that CspA abundance correlates with the observed phenotype, but we cannot test this directly due to the quality of the CspA antibody.

During this study, we also found that $MnCl_2$ could function as an enhancer or cogerminant to a similar effect as $CaCl_2$ (Figure 4). Moreover, even though $MgCl_2$ was not as efficient as $CaCl_2$ or $MnCl_2$ at 250 μM (Figure 4B), when tested at 10 mM, it was equally effective. These observations for these three metals are similar to prior work on amino acids as co-germinants [15, 17]. In this prior work, there is a hierarchy of recognition of these amino acids and this hierarchy is largely conserved in another strain. Here, it appears that $CaCl_2$ and $MnCl_2$ are efficiently recognized as enhancer / co-germinants and $MgCl_2$ is weaker. Obviously, there is a growing need to determine the biochemistry of the germinant receptors to determine how they interact with germinants and how these metal ions influences these interactions.

We hypothesized that the calcium concentration in BHIS medium was greater, thus reducing the effect of Tb^{3+} on germination (*i.e.*, the spore would already be saturated with Ca^{2+} and be less influenced by Tb^{3+}). However, there were no significant differences in the calcium concentration in different media (Figure 6). Although the mechanism for the increase in terbium sensitivity in spores prepared in different media is not known, there could be various other media components that might relate to the terbium sensitivity. However, we feel that it

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is important to report the impact of Tb^{3+} on *C. difficile* spore germination because analysis of DPA release is a common screening technique in spore germination studies. An increase in sensitivity to terbium may be confused with a *bona fide* germination phenotype and it is important to confirm the germination phenotype with different assays, such as the OD assay or SleC activation during germination. Finally, we recommend that the Tb^{3+} concentration used for each *C. difficile* strain be titered accordingly so as not to influence the germination process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DPA release by germinating endospores is often detected using Tb^{3+} fluorescence when Tb^{3+} complexes with the released DPA.
- Tb^{3+} influences *C. difficile* spore germination, likely functioning as a Ca^{2+} substitute.
- For future analyses, the concentration of $TbCl_3$ used to detect DPA release by germinating *C. difficile* spore should be titered so as not to influence germination.

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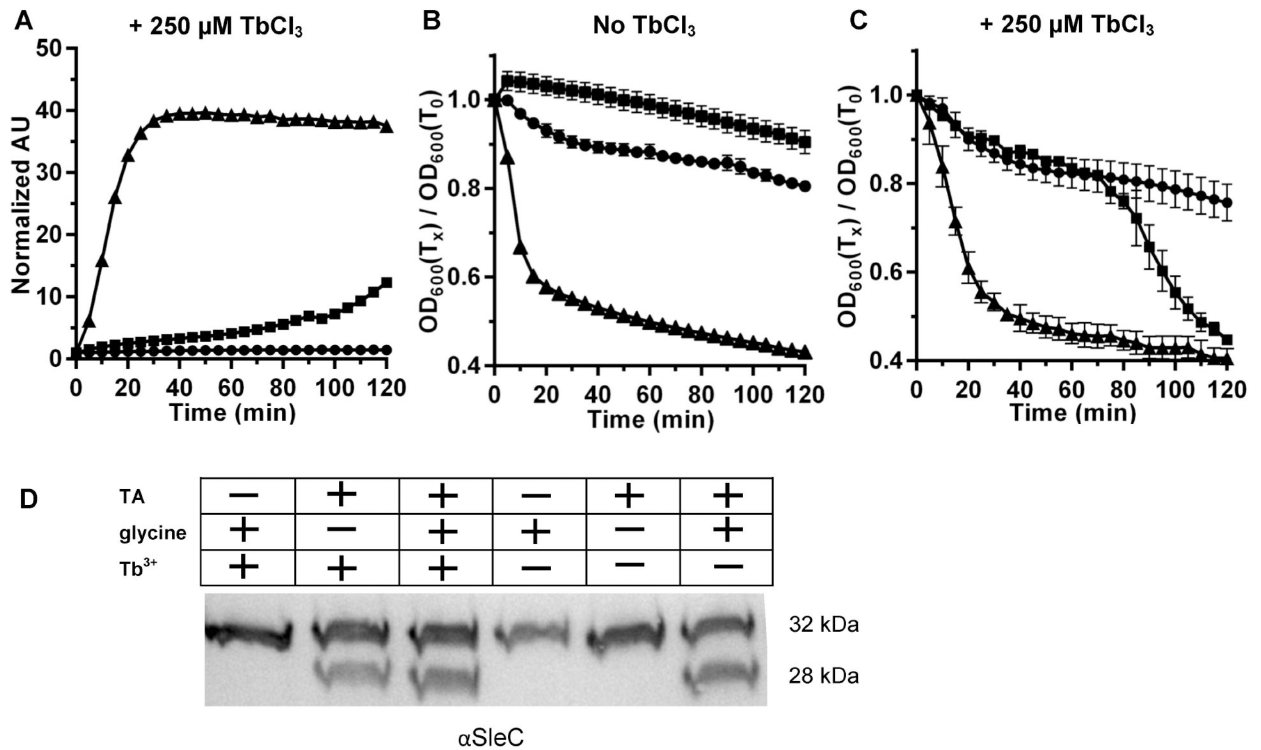

Figure 1. Comparison of germination of *C. difficile* UK1 spores in presence/absence of terbium.
C. difficile UK1 spores were prepared from BHIS medium as described in the materials and methods. (A) CaDPA release from the purified spores was analyzed by suspending the spores in buffer supplemented with 250 μM $TbCl_3$ and (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. The extent of germination was also analyzed by determining OD_{600} over time. Germination by *C. difficile* spores was analyzed in the absence of $TbCl_3$ (B) or in the presence of 250 μM $TbCl_3$ (C). Data points represent the averages from three independent experiments and error bars represent the standard error of the mean. (D) Equal numbers of spores were incubated with or without 250 μM $TbCl_3$ in 30 mM glycine or 10 mM TA or 10 mM TA and 30 mM glycine for 2 hours and soluble proteins were extracted with NuPAGE buffer and separated by SDS-PAGE followed by immunoblotting with SleC- specific antisera.

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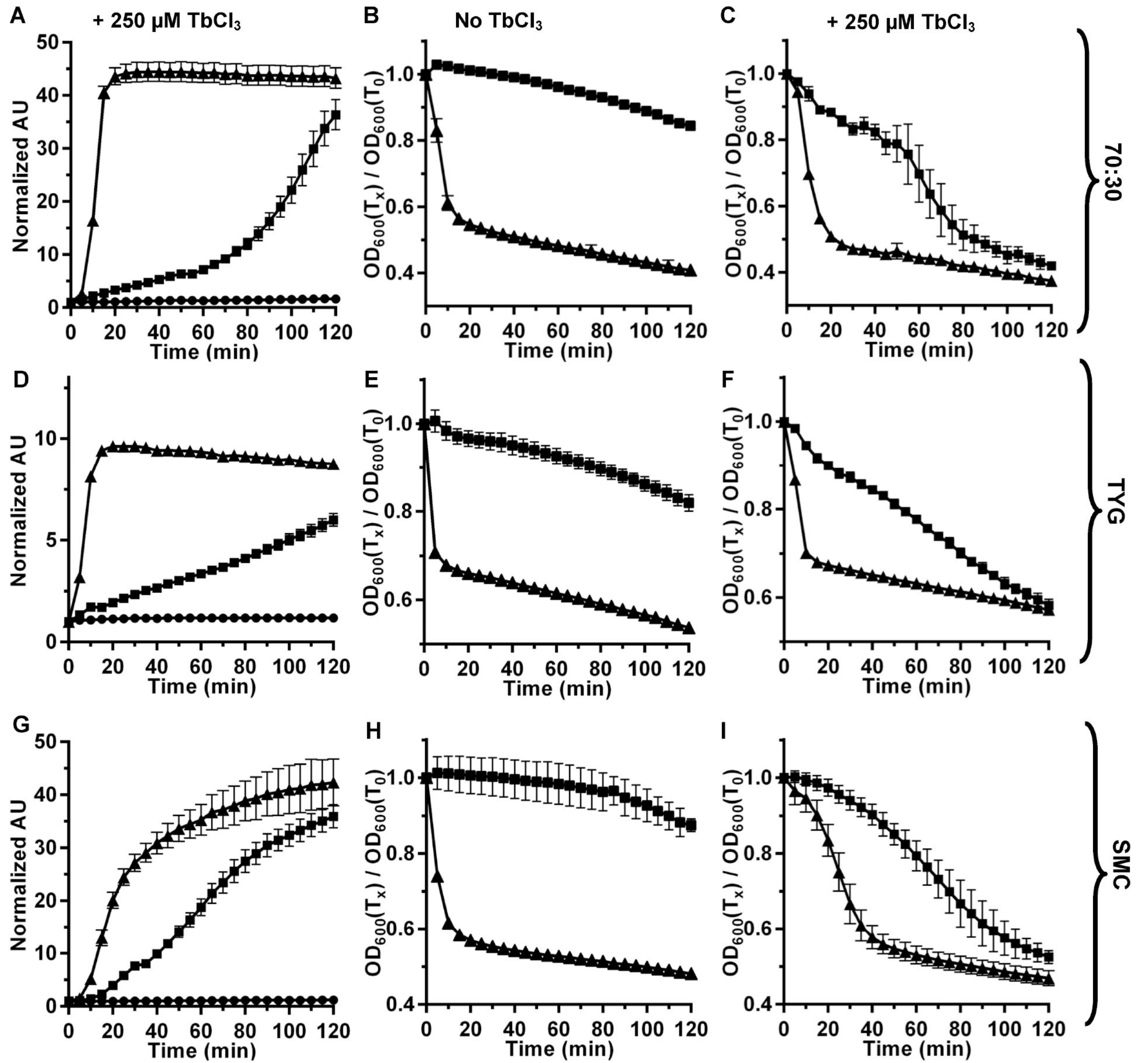

Figure 2. Comparison of germination of *C. difficile* UK1 spores prepared in different media.
The germination phenotype of wild type *C. difficile* UK1 spores prepared from (A, B, C) 70:30 medium, (D, E, F) TYG medium or (G, H, I) SMC medium were analyzed as described in Figure 1 (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean.

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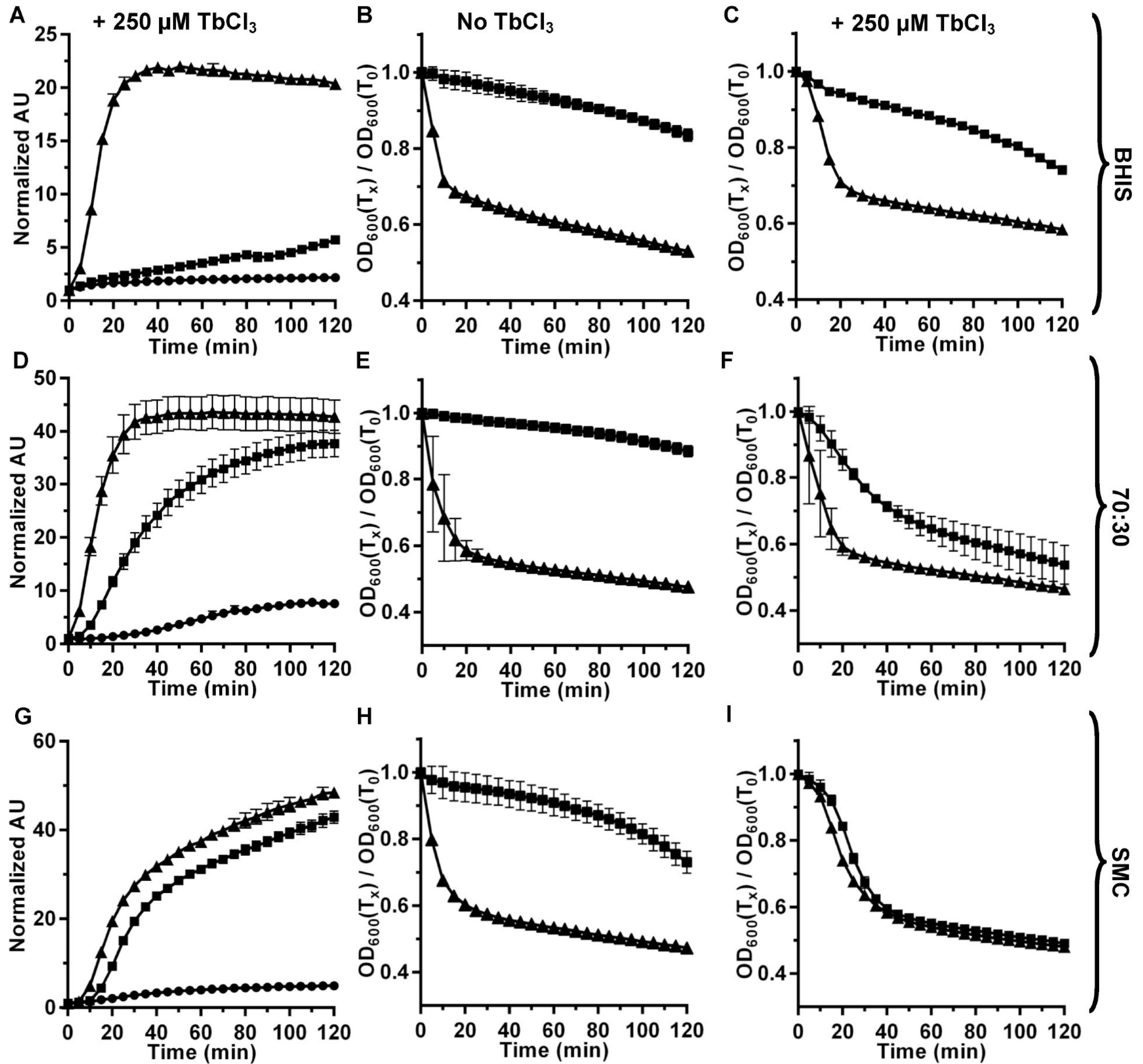

Figure 3. Comparison of germination of *C. difficile* M68 spores prepared in different media.

The germination phenotype of wild type *C. difficile* M68 spores prepared from (A, B, C) BHIS medium, (D, E, F) 70:30 medium and (G, H, I) SMC medium were analyzed as described in Figure 1 (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean.

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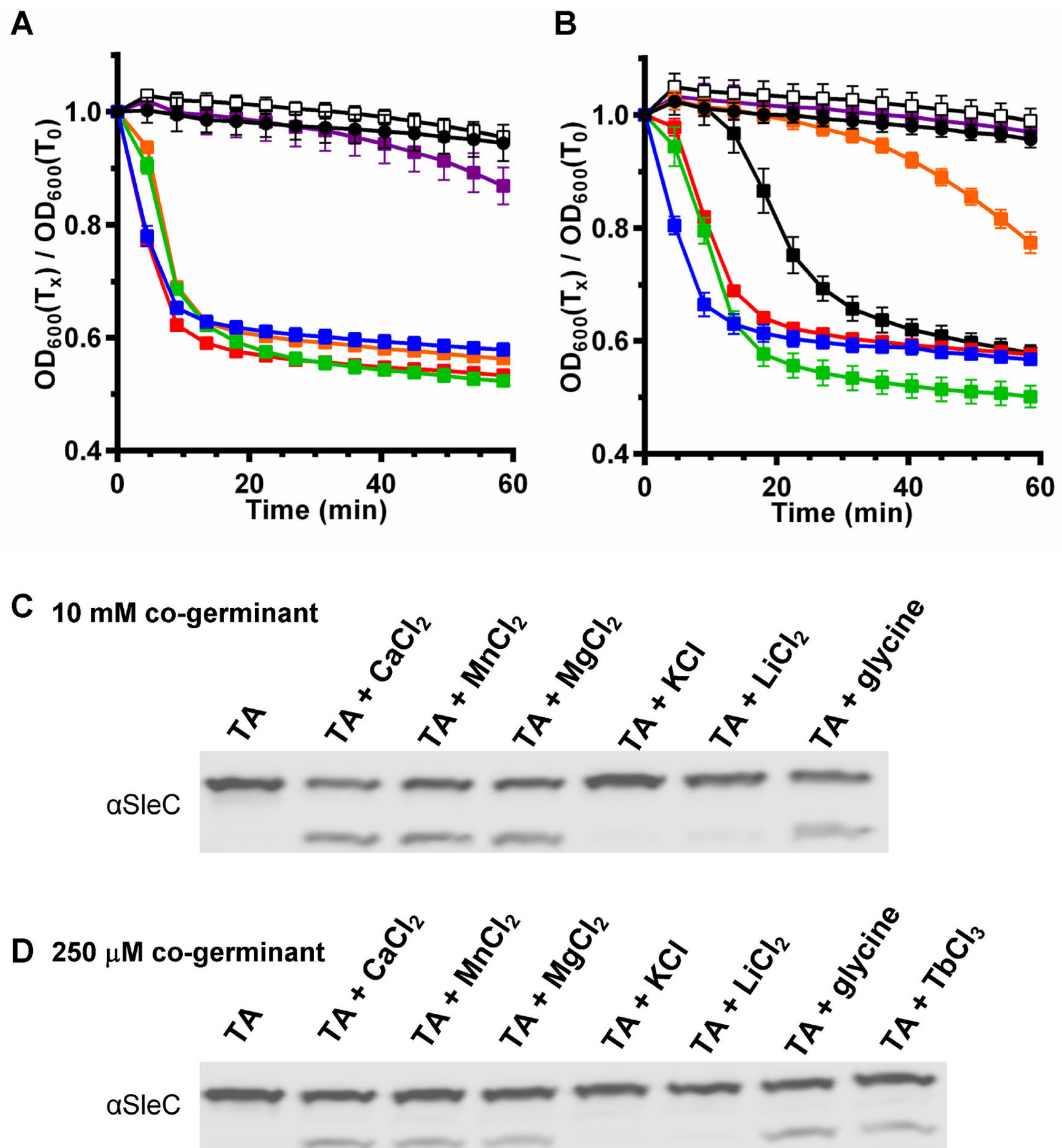

Figure 4. *C. difficile* spores are sensitive to other metals.

C. difficile UK1 spores were prepared from 70:30 medium and purified as described in the materials and methods. Purified spores were incubated with 10 mM TA and the indicated concentration of metals or co-germinant. Germination was assayed by OD for 1 hr at 37 °C in the presence 10 mM TA alone (black circles) or with 10 mM TA and (A) 10 mM or (B) 250 μM metals or co-germinants. $CaCl_2$ (blue), $MnCl_2$ (green), $MgCl_2$ (orange), KCl (open), $LiCl_2$ (purple), glycine (red), $TbCl_3$ (black squares). Respective SleC activation is shown in (C) and (D).

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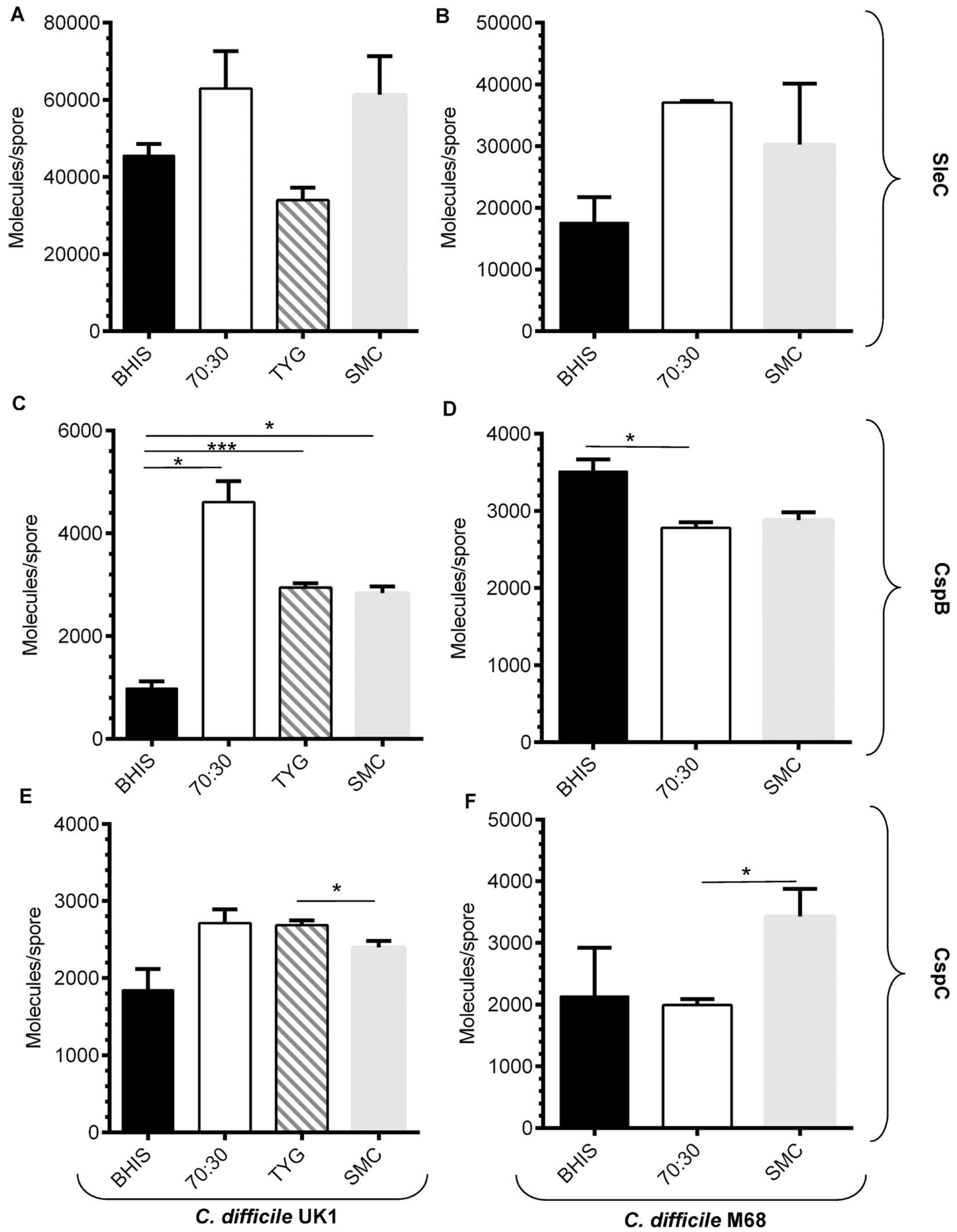

Figure 5. Quantifying the abundance of CspB, CspC and SleC in *C. difficile* spores.

2×10^9 spores purified from *C. difficile* UK1 strain (A, C, E) or the *C. difficile* M68 strain (B, D, F) that were generated in BHIS medium, 70:30 medium, TYG medium or SMC medium. The proteins were extracted with NuPAGE buffer and samples were separated by SDS-PAGE, transferred to low fluorescence PVDF membranes and blotted with antisera specific to the indicated proteins. Quantified proteins are expressed as molecules / spore and are represented in a bar graph form (A, B) SleC, (C, D) CspB and (E, F) CspC. The data presented represent the averages from three independent experiments and error bars

represent the standard error of the mean. Statistical significance was determined using a one-way ANOVA with Tukey's multiple comparisons test (* $p < 0.05$; *** $p < 0.001$).

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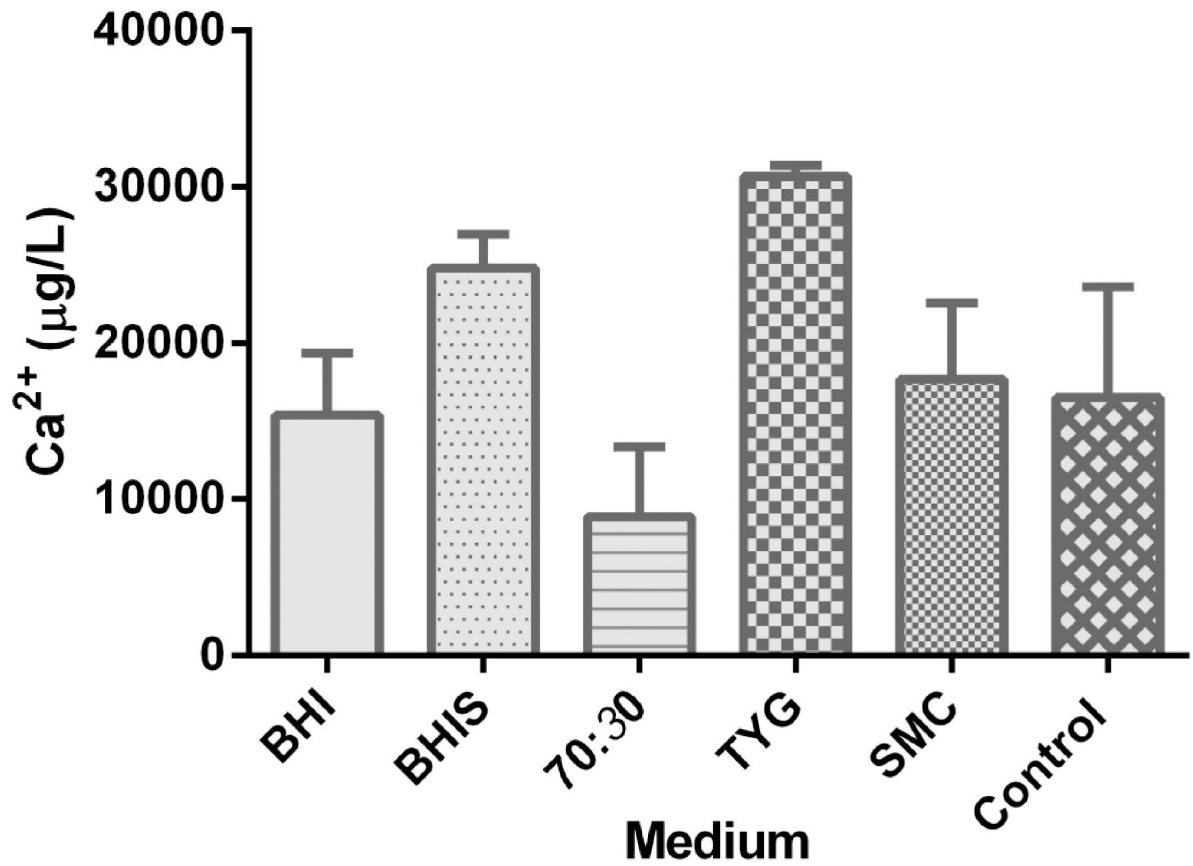

Figure 6. Detection of calcium concentration in different media types.

Bar graph representing the calcium concentration in μg / L from BHI, BHIS, 70:30, TYG and SMC media. 23 mg/mL of calcium chloride dissolved in water is used as a control.