Efficient Inhibition of Germination of Coat-Deficient Bacterial Spores by Multivalent Metal Cations, Including Terbium (Tb^{3+})[▽]

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Release of dipicolinic acid (DPA) and its fluorescence with terbium (Tb^{3+}) allow rapid measurement of the germination and viability of spores of *Bacillus* and *Clostridium* species. However, germination of coat-deficient *Bacillus* spores was strongly inhibited by Tb^{3+} and some other multivalent cations. Tb^{3+} also inhibited germination of coat-deficient *Clostridium perfringens* spores.

Many *Bacillus* and *Clostridium* species can form dormant, resistant spores under conditions unfavorable for growth (24, 25). These spores can resist extreme conditions and survive for years in the absence of nutrients. However, when nutrients return, these spores can germinate and become growing cells (16, 23, 25). Some spore-forming bacteria can also cause food spoilage, food poisoning, and disease, and spores of *Bacillus anthracis* are a potential bioterrorism agent (9, 13, 25). Therefore, rapid, sensitive assays for bacterial spores have applied importance.

An assay has been developed to detect low spore concentrations, based on spores' high concentration (~10% [dry weight]) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (9). DPA forms a strong 1:1 highly fluorescent complex with terbium (Tb^{3+}) ions, and spore quantification by Tb^{3+} -DPA fluorescence takes only a few minutes (4, 12). DPA is also released in the first minutes of spore germination (23), and since germination is essential for spore viability, Tb^{3+} -DPA fluorescence has been used to detect spore germination, and there is a good correlation between DPA release and spore viability (4, 26, 28, 29, 31, 32). However, we report a flaw in the latter assay of spore viability, as Tb^{3+} and some other multivalent metal cations strongly inhibit germination of coat-deficient spores. The coats comprise the outermost layer of spores of many species, including *Bacillus subtilis*, and protect spores against many agents, including enzymes and reactive chemicals (8, 10, 11, 24).

The *B. subtilis* strains used are isogenic derivatives of a laboratory 168 strain and are (i) PS533 (wild type), carrying plasmid pUB110, encoding resistance to kanamycin (22); (ii) PS3328 (ΔcotE mutant), in which much of the *cotE* coding sequence was replaced with a tetracycline resistance (Tc^r) cassette (15); and (iii) PS3738 (ΔsafA mutant), which carries a Tc^r cassette replacing much of the *safA* coding sequence (10). These strains were sporulated at 37°C on 2× Schaeffer's-glucose (SG) agar plates without antibiotics, and spores were

harvested and cleaned as described previously (14). The *Bacillus cereus* strain used was strain T obtained from H. O. Halvorson, and its spores were prepared at 30°C in a defined liquid medium and were purified as described previously (3). The *Bacillus megaterium* strain used was strain ATCC 12782, and its spores were prepared at 30°C in liquid supplemented nutrient broth and were harvested and cleaned as described previously (5). The *Clostridium perfringens* strain used was MRS101 ($\Delta\text{cpe}::\text{catP}$ mutant) in which the enterotoxin gene (*cpe*) in the *C. perfringens* food poisoning isolate SM101 has been inactivated by insertion of a chloramphenicol resistance gene (18). The *C. perfringens* spores were prepared and purified as described previously (17, 18). All spores used in this work were free (>98%) of growing or sporulating cells, germinated spores, and cell debris, as determined by phase-contrast microscopy. For chemical de-coating, spores at an optical density at 600 nm (OD_{600}) of ~25 were incubated at 70°C for 2 h in 1% SDS-0.1 M NaOH-0.1 M NaCl-0.1 M dithiothreitol, and decoated spores were washed and stored as described previously (1).

Prior to germination, spores at an OD_{600} of ~10 were heat activated at 75°C for 30 min (*B. subtilis*), 70°C for 20 min (*B. cereus*), or 60°C for 15 min (*B. megaterium*); activated spores were cooled on ice before germination. Spores were routinely germinated at an OD_{600} of 0.5 at 37°C in a 96-well plate in 200 μl of 25 mM K-HEPES buffer (pH 7.4) (*B. subtilis* and *C. perfringens*), 25 mM Tris-HCl buffer (pH 8.8) (*B. cereus*), or 25 mM Tris-HCl buffer (pH 7.4) (*B. megaterium*), with or without 50 μM TbCl_3 (30). Spore germination was initiated by the addition of germinants to final concentrations of 2 mM L-valine (*B. subtilis*) (valine was used instead of L-alanine to preclude germination inhibition by D-alanine generated by spore-associated alanine racemase [23, 25]), 40 mM NH_4Cl plus 10 mM L-alanine (*B. cereus*), or 10 mM glucose (*B. megaterium*). Dodecylamine germination did not require heat activation and was carried out at 45°C in 40 mM dodecylamine with 25 mM K-HEPES buffer, pH 7.4 (19). For wild-type spores, germination was measured in the presence of 50 μM TbCl_3 by quantifying DPA release in a fluorescence plate reader by measuring the formation of Tb^{3+} -DPA (30). To measure germination of coat-deficient spores, 50 μM TbCl_3 was added to germination incubations at various times and the fluorescence intensity was recorded. However, to measure whether Tb^{3+} inhibited

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FIG. 1. Effect of Tb^{3+} on DPA release during spore germination. DPA release during germination of intact ($\circ$, $\bullet$) or chemically decoated (∇ , $\blacktriangledown$) spores in the presence ($\bullet$, $\blacktriangledown$) or absence ($\circ$, ∇) of TbCl_3 was measured as described in the text. In panels A, B, and C, the TbCl_3 concentration was either 50 μM ($\bullet$, $\blacktriangledown$) or 10 μM (∇ , $\blacktriangledown$), and in panel D TbCl_3 was at 50 μM ($\bullet$, $\blacktriangledown$). (A) *B. subtilis* PS533 (wild-type) spores germinating in 2 mM L-valine; (B) *B. subtilis* PS533 (wild-type) spores germinating in 40 mM dodecylamine; (C) *B. cereus* spores germinating in 40 mM NH_4Cl plus 10 mM L-alanine; and (D) *B. megaterium* spores germinating in 10 mM D-glucose. With intact spores germinating with nutrient germinants, the amounts of fluorescence in the TbCl_3 assay were similar with 10 μM and 50 μM TbCl_3 present throughout the assay (data not shown).

DPA release from coat-deficient spores, 10 μM TbCl_3 was added to germination incubations unless otherwise specified. The percentages of germinated spores at the end of incubations were checked by examination of ≥ 100 spores by phase-contrast microscopy. All germination experiments were repeated at least twice with similar results (less than or equal to $\pm 10\%$ of all values reported).

To evaluate the effects of Tb^{3+} on germination of coat-deficient spores, the valine germination of intact and chemically decoated wild-type *B. subtilis* spores was measured with or without Tb^{3+} . To measure germination plus Tb^{3+} , TbCl_3 was added at the beginning of germination, and DPA release was determined by continuously monitoring Tb^{3+} -DPA fluorescence. To measure spore germination without Tb^{3+} , TbCl_3 was added at various times during germination, and the fluorescence immediately after TbCl_3 addition was considered the DPA released by that time point. Intact wild-type *B. subtilis* spores released DPA similarly with or without 50 μM Tb^{3+} (Fig. 1A), and $\geq 95\%$ of these spores germinated after 2.5 h, as determined by phase-contrast microscopy. Without Tb^{3+} , decoated *B. subtilis* spores released DPA slower with L-valine than did intact spores (Fig. 1A), but 85% of the decoated spores germinated in 2.5 h, as determined by phase-contrast microscopy. However, with 10 μM Tb^{3+} present continuously, no DPA release was detected from decoated spores incubated with L-valine (Fig. 1A), and $\geq 99\%$ of these spores remained phase bright after 2.5 h (data not shown).

We also investigated if Tb^{3+} interfered with germination triggered by the cationic surfactant dodecylamine that triggers germination independent of spores' nutrient germinant receptors (GRs), in contrast to germination by L-valine that is GR dependent (19, 23). Intact wild-type *B. subtilis* spores germinated similarly with dodecylamine and with or without Tb^{3+} and released most of their DPA within 3 h (Fig. 1B). Without Tb^{3+} present continuously, dodecylamine germination of decoated wild-type *B. subtilis* spores was like that of intact spores (Fig. 1B), consistent with previous work (19). However, 10 μM

TABLE 1. Effect of various Tb^{3+} concentrations on the dodecylamine germination of intact and decoated *C. perfringens* spores^a

Tb^{3+} concn (μM)	% spore germination in 2.5 h	
	Intact spores	Decoated spores
0	100	100
50	100	56
500	92	32
2,500	83	21

^a Intact and chemically decoated spores of *C. perfringens* were germinated with dodecylamine plus various TbCl_3 concentrations, and the extent of germination was assessed by monitoring DPA release fluorometrically as described in the text. Use of 2.5 mM TbCl_3 in the fluorometric DPA assay did not alter the fluorescence compared to that with the standard 50 μM TbCl_3 normally present.

TABLE 2. Effect of various Tb^{3+} concentrations on the valine germination of intact and coat-deficient *B. subtilis* spores^a

Spore type used	% spore germination in 2.5 h at a Tb^{3+} concentration of:					
	0	500 nM	2 μ M	10 μ M	50 μ M	200 μ M
Intact wild type	96	92	96	94	96	95
Decoated wild type	80	80	84	0	0	0
$\Delta cotE$ mutant	76	68	78	0	0	0
$\Delta safA$ mutant	78	86	78	0	0	0

^a Spores of various *B. subtilis* strains, including intact and chemically decoated wild-type (PS533) spores, were germinated for 2.5 h at 37°C with 2 mM L-valine plus various $TbCl_3$ concentrations, and the degree of spore germination was determined by phase-contrast microscopy as described in the text.

Tb^{3+} present continuously inhibited the dodecylamine germination of decoated wild-type *B. subtilis* spores.

The effects of Tb^{3+} on the germination of spores of other species were also examined. Intact *B. cereus* and *B. megaterium* spores incubated with NH_4Cl plus L-alanine and glucose, respectively, released almost all DPA within 10 to 30 min with or without Tb^{3+} (Fig. 1C and D). Without Tb^{3+} , decoated *B. cereus* and *B. megaterium* spores germinated normally (*B. megaterium*) or germinated somewhat slower (*B. cereus*) than intact spores. However, 10 and 50 μ M Tb^{3+} completely inhibited DPA release from decoated *B. cereus* and *B. megaterium* spores, respectively (Fig. 1C and D); this inhibition of germination was confirmed by phase-contrast microscopy (data not shown). We also tested effects of Tb^{3+} on the dodecylamine germination of intact and decoated *C. perfringens* spores (Table 1). The effects of Tb^{3+} on germination by KCl, a particularly effective germinant for *C. perfringens* MRS101 spores (17), were not tested, because KCl germination of these decoated spores was extremely slow (data not shown); however, dodecylamine germination of these decoated spores was relatively normal. Tb^{3+} gave only minimal inhibition of the dodecylamine germination of intact *C. perfringens* spores but inhibited dodecylamine germination of the decoated spores, albeit not as strongly as decoated *B. subtilis* spores (Fig. 1B; Table 1).

Since Tb^{3+} strongly inhibited the germination of at least *Bacillus* spores that had been chemically decoated, we then asked if Tb^{3+} inhibited the germination of spores that were coat deficient due to specific mutations. Spores of *B. subtilis* were used that lacked either CotE or SafA, morphogenic proteins essential for the proper spore coat assembly and for coats' protection against enzymes and reactive chemicals (8, 10). As expected, there was no significant effect on valine germination

of intact wild-type *B. subtilis* spores with $\leq 200 \mu$ M Tb^{3+} (Table 2). However, valine germination of chemically decoated wild-type spores and of intact $\Delta cotE$ and $\Delta safA$ spores was completely inhibited by 10 μ M Tb^{3+} (Table 2).

Other multivalent metal cations were also examined for their ability to inhibit germination of coat-deficient spores (Table 3). With intact wild-type *B. subtilis* spores, all cations tested had no significant effect on germination except for 5 mM Zn^{2+} (Table 3). However, with decoated spores, two lanthanide cations completely inhibited germination at all concentrations tested, and Zn^{2+} had a similar inhibitory effect, although Mg^{2+} and Ca^{2+} were relatively ineffective.

This communication shows that low concentrations of Tb^{3+} and some other multivalent metal ions strongly inhibit the germination of coat-deficient spores. This finding is notable, as these same low Tb^{3+} concentrations are used to assay viable spores by measuring DPA release in spore germination (26, 28, 29, 31, 32). Since coat-deficient spores have viability comparable to that of intact spores (8, 15), Tb^{3+} -based assays of spore viability could yield false-negative results with coat-deficient spores. Consequently, the Tb^{3+} inhibition of spore germination may be significant when Tb^{3+} -based assays are used to quantitate viable spores in either old environmental samples or by the food industry.

While the Tb^{3+} inhibition of germination of coat-deficient spores is of applied importance, the mechanism of this inhibition is not clear. One possibility is that spore coat defects facilitate access of cations such as Tb^{3+} to inner spore layers not accessible in an intact spore, and cation binding to some now accessible target inhibits germination. One such target could be the GRs in spores' inner membranes, and GR inhibition could abolish nutrient germination (16, 23). However, while Tb^{3+} and similar cations might inhibit GR function, this cannot explain these cations' effects on spore germination, since Tb^{3+} also inhibits dodecylamine germination, and dodecylamine germination is GR independent (19). Other possible targets of Tb^{3+} are the cortex-lytic enzymes (CLEs) that hydrolyze spores' peptidoglycan cortexes, a process essential to complete spore germination. However, while Tb^{3+} and similar multivalent cations may inhibit CLEs, such inhibition again cannot explain such cations' inhibition of DPA release during germination, because spores of *Bacillus* species lacking CLEs release DPA completely during germination, albeit slower than wild-type spores, and dodecylamine germination is completely CLE independent (7, 19, 20, 21). Since effects of Tb^{3+} and similar cations on spores' GRs or CLEs cannot explain these

TABLE 3. Effects of various multivalent cations on the valine germination of intact and decoated wild-type *B. subtilis* spores^a

Cation	% spore germination in:									
	Intact spores at cation concn of:					Decoated spores at cation concn of:				
	0	50 μ M	500 μ M	2.5 mM	5 mM	0	50 μ M	500 μ M	2.5 mM	5 mM
Gd^{3+}	96	92	94	84		85	0	0	0	
Dy^{3+}	96	92	94	96		84	0	0	0	
Ca^{2+}	94	88	92		88	82	72	64		60
Mg^{2+}	94	88	90		82	82	76	64		48
Zn^{2+}	94	90	88		20	82	0	0		0

^a Intact and chemically decoated wild-type *B. subtilis* spores (PS533) were germinated for 2.5 h at 37°C with 2 mM L-valine plus various concentrations of divalent and trivalent cation chlorides, and the percentages of germinated spores were determined by phase-contrast microscopy as described in the text.

cations' inhibition of DPA release during spore germination, the only other likely target is the channel through which DPA is released during germination. This DPA channel has not been definitively identified but seems likely to contain proteins encoded by the *spoVA* operon, and if such channels were blocked, this would block not only DPA release during germination but also almost certainly subsequent germination events (16, 23, 27). How Tb^{3+} and similar cations might inhibit such DPA channels is not clear, but it is interesting that the efficacy of the various cations' inhibition of the germination of coat-deficient spores of *Bacillus* species parallels the values of the logarithms of the stability constants for $M^{2/3+}$ -DPA complexes (where M is any metal), and the stability constants for the formation of multi- $M^{2/3+}$ ion-DPA complexes also have this same relative order (2, 6) (data not shown). These observations suggest that DPA channels may be blocked by formation of $M^{2/3+}$ complexes that act like a cork to plug the DPA channel. While this mechanism is certainly speculative, and Tb^{3+} was not as effective in blocking *C. perfringens* spore germination, this suggestion seems worth further study.

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REFERENCES

1. Bagyan, I., M. Noback, S. Bron, M. Paidhungat, and P. Setlow. 1998. Characterization of *yhcN*, a new forespore-specific gene of *Bacillus subtilis*. *Gene* 212:179–188.
2. Chung, L., K. S. Rajan, E. Merdinger, and N. Grecz. 1971. Coordinative binding of divalent cations with ligands related to bacterial spores—equilibrium studies. *Biophys. J.* 11:469–582.
3. Clements, M. O., and A. Moir. 1998. Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. *J. Bacteriol.* 180:6729–6735.
4. Fichtel, J., J. Köster, J. Rullkötter, and H. Sass. 2007. Spore dipicolinic acid contents used for estimating the number of endospores in sediments. *FEMS Microbiol. Ecol.* 61:522–532.
5. Goldrick, S., and P. Setlow. 1983. Expression of a *Bacillus megaterium* sporulation-specific gene during sporulation of *Bacillus subtilis*. *J. Bacteriol.* 155:1459–1462.
6. Grenthe, I. 1961. Stability relationships among the rare earth dipicolinates. *J. Amer. Chem. Soc.* 83:360–364.
7. Heffron, J. D., E. A. Lambert, N. Sherry, and D. L. Popham. 2010. Contributions of four cortex lytic enzymes to germination of *Bacillus anthracis* spores. *J. Bacteriol.* 192:763–770.
8. Henriques, A. O., and C. P. Moran, Jr. 2007. Structure, assembly, and function of the spore surface layers. *Annu. Rev. Microbiol.* 61:555–588.
9. Hindle, A. A., and E. A. Hall. 1999. Dipicolinic acid (DPA) assay revisited and appraised for spore detection. *Analyst* 124:1599–1604.
10. Klobutcher, L. A., K. Ragkousi, and P. Setlow. 2006. The *Bacillus subtilis* spore coat provides “eat resistance” during phagosomal predation by the protozoan *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. U. S. A.* 103:165–170.
11. Laaberki, M. H., and J. Dworkin. 2008. Role of spore coat proteins in the resistance of *Bacillus subtilis* spores to *Caenorhabditis elegans* predation. *J. Bacteriol.* 190:6197–6203.
12. Li, Q., P. K. Dasgupta, and H. K. Temkins. 2008. Airborne bacterial spore counts by terbium-enhanced luminescence detection: pitfalls and real values. *Environ. Sci. Technol.* 42:2799–2804.
13. Mock, M., and A. Fouet. 2001. Anthrax. *Annu. Rev. Microbiol.* 55:647–671.
14. Nicholson, W. L., and P. Setlow. 1990. Sporulation, germination and outgrowth, p. 391–450. In C. R. Harwood and S. M. Cutting (ed.), *Molecular biological methods for Bacillus*. John Wiley and Sons, Chichester, United Kingdom.
15. Paidhungat, M., K. Ragkousi, and P. Setlow. 2001. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+} -dipicolinate. *J. Bacteriol.* 183:4886–4893.
16. Paredes-Sabja, D., P. Setlow, and M. R. Sarker. 2011. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. *Trends Microbiol.* 19:85–94.
17. Paredes-Sabja, D., J. A. Torres, P. Setlow, and M. R. Sarker. 2008. *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *J. Bacteriol.* 190:1190–1201.
18. Sarker, M. R., R. J. Carman, and B. A. McClane. 1999. Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* endotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Mol. Microbiol.* 33:946–958.
19. Setlow, B., A. E. Cowan, and P. Setlow. 2003. Germination of spores of *Bacillus subtilis* with dodecylamine. *J. Appl. Microbiol.* 95:637–648.
20. Setlow, B., E. Melly, and P. Setlow. 2001. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage of spore germination. *J. Bacteriol.* 183:4894–4899.
21. Setlow, B., et al. 2009. Characterization of the germination of *Bacillus megaterium* spores lacking enzymes that degrade the spore cortex. *J. Appl. Microbiol.* 107:318–328.
22. Setlow, B., and P. Setlow. 1996. Role of DNA repair in *Bacillus subtilis* spore resistance. *J. Bacteriol.* 178:3486–3495.
23. Setlow, P. 2003. Spore germination. *Curr. Opin. Microbiol.* 6:550–556.
24. Setlow, P. 2006. Spores of *Bacillus subtilis*: their resistance to radiation, heat and chemicals. *J. Appl. Microbiol.* 101:514–525.
25. Setlow, P., and E. A. Johnson. 2007. Spores and their significance, p. 35–67. In M. P. Doyle and L. R. Beuchat (ed.), *Food microbiology: fundamentals and frontiers*, 3rd edition. ASM Press, Washington, DC.
26. Shafaat, H. S., and A. Ponce. 2006. Applications of a rapid endospore viability assay for monitoring UV inactivation and characterizing arctic ice cores. *Appl. Environ. Microbiol.* 72:6808–6814.
27. Vepachedu, V. R., and P. Setlow. 2004. Analysis of the germination of spores of *Bacillus subtilis* with temperature sensitive *spo* mutations in the *spoVA* operon. *FEMS Microbiol. Lett.* 239:71–77.
28. Yang, W. W., and A. Ponce. 2009. Rapid endospore viability assay of *Clostridium sporogenes* spores. *Int. J. Food Microbiol.* 133:213–216.
29. Yang, W. W., and A. Ponce. 2011. Validation of a *Clostridium* endospore viability assay and analysis of Greenland ices and Atacama Desert soils. *Appl. Environ. Microbiol.* 77:2352–2358.
30. Yi, X., and P. Setlow. 2010. Studies of the commitment step in the germination of spores of *Bacillus* species. *J. Bacteriol.* 192:3424–3433.
31. Yung, P. T., and A. Ponce. 2008. Fast sterility assessment by germinable-endospore biodosimetry. *Appl. Environ. Microbiol.* 74:7669–7674.
32. Yung, P. T., H. S. Shafaat, S. A. Cannon, and A. Ponce. 2007. Quantification of viable endospores from a Greenland ice core. *FEMS Microbiol. Ecol.* 59:300–306.