2017-12

The potentiating effect of mandelate and lactate on chemically-induced germination in members of Bacillus cereus sensu lato.

Bishop, Alistair

10.1128/AEM.01722-17
Applied and Environmental Microbiology
American Society for Microbiology

The potentiating effect of mandelate and lactate on chemically-induced germination in members of *Bacillus cereus sensu lato.*

Running title: Effect of mandelate and lactate on spore germination

Alistair H. Bishop

School of Biological and Marine Sciences, University of Plymouth, Devon, U.K. PL4 8AA

Author for correspondence: alistair.bishop@plymouth.ac.uk

ABSTRACT

Endospores of the genus *Bacillus* can be triggered to germinate by a limited number of chemicals. Mandelate had a powerful, additive effect on the level and rate of germination produced in non-heat shocked spores of *Bacillus anthracis* Sterne, *Bacillus cereus* and *Bacillus thuringiensis* when combined with L-alanine and inosine. Mandelate had no germinant effect on its own but was active with these germinants in a dose-dependent manner at concentrations above 0.5 mM. The maximum rate and extent of germination was produced in *B. anthracis* by 100 mM L-alanine with 10 mM inosine: this was equalled by just 25% of these germinants when supplemented with 10 mM mandelate. Half the maximal germination rate was produced by 40% of the optimum germinant concentrations or 15% of them when supplemented with 0.8 mM mandelate. Germination rates in *B. thuringiensis* were highest around neutrality but the potentiating effect of mandelate was maintained over a wider pH range than was germination with L-alanine and inosine alone. For all species, lactate also promoted germination in the presence of L-alanine and inosine, this was further increased by mandelate. Ammonium ions also enhanced L-alanine and inosine-induced germination but only when mandelate was present. In spite of the structural similarities, mandelate did not compete with phenylalanine as a germinant. Mandelate appeared to bind to spores while enhancing germination. There was no effect when mandelate was used in conjunction with non-nutrient germinants. No effect was produced with spores of *Bacillus subtilis, Clostridium sporogenes* or *C. difficile*.

IMPORTANCE

The number of chemicals that can induce germination in the species related to *B. cereus* has been defined for many years and they conform to specific chemical types. Although not a germinant itself, mandelate has a different structure from these germination-active compounds and its addition to this list represents a significant discovery in the fundamental biology of spore germination. This novel activity may also have important applied relevance given the impact of spores of *B. cereus* in food-borne disease and *B. anthracis* as a threat agent. The destruction of spores of *B. anthracis*, for example, particularly over large outdoor areas, poses significant scientific and logistical problems. The addition of mandelate and lactate to the established mixtures of L-alanine and inosine would decrease the amount of the established germinants required and increase the speed and level of germination achieved. The large-scale application of 'germinate to decontaminate' strategy may, thus, become more practicable.

KEYWORDS Spore germination, *Bacillus anthracis*, mandelate, lactate, *Bacillus cereus*.

INTRODUCTION

Bacterial endospores are a highly resistant form into which some bacterial species are able to differentiate (1), typically in response to nutrient limitation. A number of triggers are capable of causing these spores to begin the process of returning to the vegetative form. These include heat shock, high pressure and also a number of chemicals that are somewhat specific to particular species (2). Spore germination is a relatively simple model of cellular differentiation and it is also of applied interest. Among the spore-forming species are those capable of causing human infection such as *Bacillus cereus* and *Clostridium difficile* and perhaps the most important agent of concern, *Bacillus anthracis.* Due to the high chemical resistance of spores, they are difficult to inactivate by disinfectants and only a few such chemicals are truly sporicidal. For wide-spread decontamination of hospital wards the concept of 'germinate to decontaminate' has been raised (3). This has also been applied as a suggested means to remove spores of *B. anthracis* after a malicious release (4, 5). A further advantage, given the relative ineffectiveness of chemical sporicides in soil, is to exploit the poor persistence of *B. anthracis* in soil once germinated (6).

The limited number of chemicals that are capable of triggering germination are well established. The mostly widely used experimental model, *Bacillus subtilis* will, for example, germinate when exposed to a mixture of L-asparagine, D-glucose, D-fructose and potassium ions (7). A prior heat shock is required to activate these spores maximally and make them receptive to the chemical germinants.

The less well-studied *Clostridium difficile* may also require a heat shock but the chemical germinants appear to be less well defined; glycine and bile salts have been shown to act as co-germinants and were maximally activated at 80^{o}C for 10 min. (8). For some clinical isolates, however, amino acids were insufficient and rich nutrient media were required for germination to occur; bile salts, however, were not required (9).

Members of *B. cereus sensu lato* (10) are interesting in that they show a high level of germination just with the appropriate chemical; germination is enhanced by a prior heat shock. For *B. anthracis* and *B. cereus*/ *B. thuringiensis* a powerful combination of chemical nutrients

is L-alanine and inosine (11). The stereo-specificity for the amino acid is crucial, with D-alanine acting as an inhibitor (12). Germinant receptors (GRs) have been identified on the inner membrane of the spore for different, specific chemical germinants that are active in *Bacillus* species (13, 14, 15). Each has specificity for one or more compounds, as has been hypothesized for *B. anthracis,* for example (16). This complexity is increased by positive and negative interactions between some of the chemical germinants (11). Other 'non-nutrient' chemicals (2) such as calcium dipicolinate and dodecylamine can trigger germination in both *Bacillus* and *Clostridium* species. In the former group this has been shown to be independent of binding to any GRs (17) but such germinant pathways may be involved for *Clostridium* species (18). Spores of *B. cereus sensu lato* will not develop into vegetative cells in the presence of just the nutrient germinants: the process halts with the loss of calcium dipicolinate, phase brightness and enhanced resistance to heat and anti-microbial compounds. The possession of these features typify the sporulated state. This is an ideal termination stage from an applied point of view because the germinated spores are now much more susceptible to decontamination measures but are not able to replicate and, potentially, worsen the contamination problem.

The limited number of specific chemical germinants, active on bacterial spores has remained unchanged for decades. Woese *et al.* (19) examined the effect on a number of amino acids, focusing also on analogs of L-alanine. More recently, a number of chemicals have been screened for activity as inhibitors of germination (20, 21). In connection with the work presented here a number of chemicals were screened for activity on spores of *B. cereus sensu lato.* The activity of three chemicals, mandelate, lactate and ammonium ions on spore germination is reported here.

RESULTS

Potentiating effect of mandelate on spore responsiveness to alanine and inosine. The maximal level of germination of *B.* anthracis Sterne spores was found at concentrations of about

100 mM L- alanine and 10 mM inosine. Using 15% of these concentrations produced the lowest level of germination shown in Table 1. This equated to 50% of the spore population becoming phase dark. The addition of 0.1 mM mandelate to 15 mM L-alanine and 1.5 mM inosine (15% of the optimal concentration) had no effect but progressively increasing this concentration to 1, 5, and 10 mM mandelate dramatically potentiated the germination response (Table 1). This resulted in increasing proportions of the spore population becoming phase dark (70, 80 and 90%, respectively). The minimal concentration of mandelate required to produce a detectable increase in germination under these conditions was 0.5 mM. Mandelate on its own at any concentration had no germinating effect. The increase in germination was proportional to the amount of mandelate added; it had no potentiating effect on the germination induced by L-alanine or inosine separately at any concentration of any of the chemicals.

Both (*R*)-(−) and (*S*)-(+) enantiomers of mandelic acid and mixtures thereof produced identical results. There was no toxic effect on the germinated spores at pH 7.2, even up to mandelate concentrations of 100 mM.

Effect of heat shock. Activating spores by heat shock mimicked and over-shadowed the increase in germination produced by mandelate. This was true of *B. anthracis* and, as illustrated in Table 2, of *B. thuringiensis* strain '*Btcry*' (22). The results obtained with the latter organism were representative throughout this work to those obtained with the *B. cereus* strains and 1230-88 (23) and ATCC 10876. Under microscopic examination, the final levels of phase dark spores were, for non-heat shocked spores: L-alanine and inosine alone, 10%; germinants with 5 mM mandelate, 40% and germinants with 10 mM mandelate, 70%. After heat shock, all of the spore preparations were over 95% phase dark.

Interactions with lactate and ammonium ions. Mandelate was not the only compound found to potentiate the triggering effect of L-alanine and inosine. Lactate, while ineffective on its own, was also able to increase the germinating effect of the germinants (Table 3). Its effect

was not as marked as mandelate, however: for a given concentration of L-alanine and inosine, the addition of 10 mM mandelate produced a greater level of germination than the addition of 25 mM lactate (Table 3). When mandelate and lactate were added together to the germinants they produced an additive effect, resulting in the greatest rate and extent of germination (Table 3). Surprisingly, D-and L- forms of lactate had identical effects and combinations of the two were additive.

Ammonium ions were found to promote the stimulation by mandelate of the germination induced by alanine plus inosine. It is noteworthy that they did not increase spore germination in the absence of mandelate (Table 3). The inclusion of all three stimulants had a small but reproducible promotion of germination beyond the combination of mandelate and ammonium ions or mandelate and lactate. The same stimulation of germination induced by L-alanine and inosine with mandelate, ammonium ions and lactate was observed in the *B. cereus* strains and *Btcry*$^-$; as with *B. anthracis*, lactate had no effect alone with the germinants but had an additive effect in the presence of mandelate.

Kinetics of the mandelate and lactate effects. A double reciprocal plot of the rate of germination of *Btcry*$^-$ spores over 10 min in varying concentrations of mandelate and of L-alanine and inosine was constructed (data not shown). The lines do not intersect at a single point, indicating that mandelate does not have to be at its putative receptor at the same time as L-alanine and inosine are at theirs (20). There is an almost doubling of affinity of the spores for mandelate over a four-fold range in L-alanine and inosine concentration, indicating a degree of co-operativity between the germinants and the adjuvant. The apparent germination V_{max} increases with increasing concentration of L-alanine plus inosine and indicates that mandelate binds at a different site. The apparent V_{max} and K_m values are shown in Table 4.

The interactions of lactate with L-alanine and inosine were much more complex. Linear relationship between the rate of germination and the concentration of lactate for given

concentrations of L-alanine and inosine were not observed. No deductions about the interactions between these germinant chemicals were, therefore, possible.

The maximum rate of decrease in optical density produced in *B. anthracis* Sterne spores with L-alanine (100 mM) and inosine (10 mM) was 0.0054 OD units/min (data not shown). The maximal rate of germination was reproduced by 15% of the optimal germinant concentration by the addition of 10 mM mandelate. Half of the maximum rate, termed C50, was produced by 15% of this concentration of both germinants when supplemented with 0.8 mM mandelate. Equally, if only 2.5% of both germinants were used, the C50 value was restored by the addition of 100 mM mandelate. If L-alanine and inosine were used alone, 40% of the optimal concentration of germinants was required to achieve C50.

Spore binding is implicated in mandelate activity. Two approaches were taken to demonstrate that mandelate binds to spores in order to stimulate L-alanine/inosine-induced germination. Pre-incubation of spores of either *B. anthracis* Sterne or *Btcry*$^-$ in mandelate (2 mM) followed by centrifugation and resuspension in the germinants produced the same rate and extent of germination in L-alanine/inosine as spores incubated throughout in 2 mM mandelate with these germinants. Similarly, spores that had been pre-incubated as above but where the mandelate was then diluted to a concentration of 0.02 mM (a non-active concentration, Table 1) with a solution of L-alanine and inosine again produced an identical germination response to those where the concentration of mandelate was 2 mM throughout the assay (data not shown). This was true even when the spores had been exposed to mandelate up to 4 h before incubation with L-alanine and inosine. There was no enhancement of germination when spores were pre-incubated with concentrations of lactate that, when added simultaneously, would have increased germination with L-alanine and inosine.

Interaction with other amino acids. Phenylalanine, in combination with inosine, has a powerful effect on germination in *B. anthracis* and *B. cereus*. Given the structural similarity between this amino acid and mandelate it was considered possible that the same germination

receptor was used. When added separately and in combination with sub-maximal levels of L-alanine plus inosine it was evident that there was no competition in the germination of *Btcry*$^-$ spores but rather an additive effect of mandelate and phenylalanine occurred (Table 5). This was true when saturating levels (100 mM) of both were used; the response was additive: adding just 200 mM mandelate was less effective. This was also true for *B. anthracis* (data not shown).

Positive and negative interactions have been identified between some of the amino acids that can contribute to spore germination (11, 16). Mandelate had an additive effect with for all of the combinations of amino acids tested with *B. anthracis* (Table 6). Surprisingly, the negative interaction between methionine and valine (11) appeared to be relieved when mandelate was added (Table 6). An alternative interpretation is that it simply exerted an additive effect with one or both of the amino acids present.

Dependence on pH value. The optimum pH value for germination with L-alanine and inosine using non-heat shocked spores of *Btcry*$^-$ spores was around pH 7.0 (Table 7). The same was true when mandelate (25 mM) was added but higher rates of germination were evident and were also maintained over a broad range of pH values. When heat shocked spores were used, this difference disappeared, as shown in Table 2, and near complete germination across the pH range was observed in all cases.

Effect of mandelate on *B. subtilis*, *B. atrophaeus and Clostridium*, spp. With all of the strains used there was no difference in the rate or extent of germination in the presence of mandelate. There was no effect on heat-shocked spores.

DISCUSSION

Other screening programs may have been under-taken but, particularly if they were unsuccessful, have not been reported. The discovery of mandelate as a compound active in the germination of some *Bacillus* species opens up a new line of investigation in the

222 fundamental biology of spore germination. It is not a 'nutrient germinant' such as the purines
223 and amino acids, nor is it a spore constituent like calcium dipicolinate. Unlike this and the
224 other well-known non-nutrient germinant, dodecylamine, mandelate does seem to bind to the
225 spore and also interacts with the germinants that are required for its activity to be apparent.
226 The GR used by aromatic acids in the *B. cereus* group does not, however, seem to be used
227 by mandelate: there is a lack of competition with phenylalanine, which argues against its
228 involvement. Having a different chemical structure to the other known germination-active
229 chemicals increases the possibility that other such chemicals may exist.

230 The mechanism of action of mandelate is unknown. The identity and specificity of any
231 putative receptor for mandelate has not yet been investigated. Mandelate had a potentiating
232 effect on spore germination with all of the amino acids tested when inosine was present
233 (Table 6). This, perhaps, argues against it operating through the GRs used by these amino
234 acids. There is a precise requirement for the L- isomer of amino acids (16, 19). It was
235 surprising that the R- and S- stereoisomers of mandelate were equally active. Given the
236 stimulatory effect on mixtures of amino acids and inosine it might be that mandelate somehow
237 operates as a general sensitizer to germinating chemicals as does a heat shock. No direct
238 evidence is presented here that mandelate actually binds to the spores but it remains a
239 possibility. Unlike with mandelate, pre-incubation of spores with lactate, produced no
240 enhancement of germination when subsequently exposed to L-alanine and mandelate.

241 Related chemicals like mandelonitrile, phenylpyruvic acid and methylbenzoyl formate were
242 shown in the screening program to have no activity. Moreover, methyl anthranilate was found
243 to have an inhibitory effect on L-alanine-induced germination of *B. subtilis* (24).

244 As found by Woese *et al.* (19), lactate alone was ineffective at triggering spore
245 germination. When combined with L-alanine and inosine there was a potentiating effect on
246 germination (Table 3) although it required a higher concentration than mandelate to achieve
247 the same effect. Lactate has a similar molecular structure to alanine and, conceivably,
248 operates by interaction with the GR that recognises the amino acid. It is important to note,
249 however, that lactate has no activity in the absence L-alanine and/or inosine. Pyruvate also

has a similar structure but was found to be ineffective, indicating a degree of molecular selectivity by the GR if, indeed, that is the mechanism. It is surprisingly that both stereo-isomers of mandelate and lactate had equal effect on promoting germination. Given the structural similarity it might be surmised that lactate causes its effect through interaction with the alanine GR. If this were so the acute specificity that is shown for the D- and L- forms of the amino acid is completely absent with respect to lactate. Similar to the findings here it has been shown that L-lactate, while not capable of inducing germination on its own, increased the rate and extent of germination in *C. botulinum* in the presence of L-alanine and also some other amino acids (25). It is of interest that this effect was, as shown here, irrespective of the stereoisomer used while there was an absolute requirement for the L-form of alanine. Lactate has, however, been shown to have an inhibitory effect on the germination of spores of *C. perfringens* (26).

Ammonium ions have previously been reported to have a stimulatory effect on *B. cereus* germination using 1 mM L-alanine (27). This finding was not reproduced here and even the presence of much higher concentrations of L-alanine and inosine (25 mM and 2.5 mM, respectively) did not benefit from the addition of ammonium ions (25 mM). This combination, when supplemented with mandelate, however, produced a much higher level of germination in *B. anthracis* spores than with L-alanine, inosine and mandelate alone (Table 3). Ammonium ions were found to stimulate the germinating effect of L-alanine and inosine (7) in one strain of *B. cereus* but it was reported to be inhibitory in another (28). The mechanism for this is unknown and has not yet been explored further.

Mandelate has never previously been associated with bacterial spore germination. Mandelic acid is known for its antibacterial effects at acidic pH values (29, 30) and as a mild exfoliant cosmetic (31) and in the treatment of certain dermatological conditions such as inflammation. The (R)-form is a key intermediate in the production of semi-synthetic penicillins and cephalosporins (32). It also has a long history of usage by oral dosage as a derivative of methenamine (33) for persistent urinary tract infections. It is, therefore, conceivable that mandelate could be used in the food industry to increase the germination of *B. cereus* spores

prior to inactivation. The two strains of *B. cereus* used in this study behaved in all respects very similarly to *Btcry*$^-$.

Another area of applied relevance for this work is in the decontamination of *B. anthracis.* To achieve a 'germinate to decontaminate' regime for *B. anthracis* over a wide area would require large amounts of L-alanine and inosine. Furthermore, the outdoor application of these nutrients might be hampered by their being readily metabolised by soil micro-organisms. The application of concentrated solutions of L-alanine and inosine was successful in the laboratory at promoting the 'self-decontamination' by microcosms of *B. anthracis* spores (6). The logistics and effectiveness of transferring this to the field have yet to be demonstrated. *Btcry*$^-$ was used in this study because it has been used as a simulant *B. anthracis* (34, 35). The data presented here show that the addition of mandelate to L-alanine and inosine would greatly decrease the requirement for these chemicals to achieve the same level of germination. This could either mean that less of the latter chemicals would be needed in the germinant cocktail or that the efficacy of the cocktail could be maintained as they were utilised by soil micro-organisms. Although subject to degradation by certain micro-organisms (36, 37), mandelate is not a conventional nutrient of micro-organisms. Given the restricted presence of the mandelate racemase degradation pathway it would be assumed that mandelate would have a greater persistence in the environment than the nutrient germinants. Work is currently underway to study the germination of spores in soil with and without the presence of mandelate.

MATERIALS AND METHODS

Spore production. Spores of *B. anthracis Sterne*, *B. thuringiensis* subsp. *kurstaki* HD-1 *cry*$^-$ ('*Btcry*$^-$'), *B. cereus* 1230-88 (23) and ATCC 10876, *B. atrophaeus* NCTC 10073 and *B. subtilis* ATCC 55405 and 133 were produced and washed as previously described (34). Spore purity was greater than 95%, as judged by phase contrast microscopy. *C. difficile* strains 1634 and 1813 were a gift from Prof. Les Baillie (University of Cardiff, U.K.) and strain 13566 was purchased from NCTC (Salisbury, U.K.) and were grown and purified according to the methods of Edwards and McBride (38). *Clostridium sporogenes* strain 701792 was purchased

306 from NCIMB (Aberdeen, U.K.) and was grown in anaerobic jars on reinforced *Clostridium*
307 medium (Oxoid, Basingstoke, U.K.). Vegetative cells were scraped from the plates and used
308 to inoculate the sporulation medium of Yang *et al.* (39). The harvesting and washing of spores
309 was as described above (38). All spores were stored in sterile distilled water at 4^{o}C for up to
310 two months. The heat shock treatments used were 70^{o}C for 30 min for *B.* anthracis, *Btcry*$^{-}$, *B.*
311 *cereus* and *B. subtilis* while 80^{o}C for 10 min was used for *C. sporogenes* and *C. difficile*. All
312 heat-shocked spores were stored on ice and used within 8 h.
313
314 **Germinants.** The standard germinant mix used for *B. anthracis* and *Btcry*$^{-}$ was inosine (10
315 mM) and L-alanine (100 mM) in phosphate buffer, pH 7.2 (50 mM). Other pH values were
316 obtained using acetate buffer (pH 5.0); phosphate buffer (pH 6-8) and CHES (pH 9.0), all at
317 50 mM final concentration. The germinants used for *B. subtilis* were D-glucose (10 mM), D-
318 fructose (10 mM) and potassium chloride (10 mM), with and without supplementation with L-
319 valine (2 mM) and L-asparagine (2 mM). Stock solution of mandelic acid (0.5 M and 0.1 M)
320 were adjusted to pH 7.2 with sodium hydroxide solution. For *C. sporogenes* the germinants
321 used were L-alanine (50 mM), L-lactate (25 mM) and sodium bicarbonate (25 mM) in 25 mM
322 Tris, pH 7.4. Spores of *C. difficile* were germinated in sodium taurocholate (10 mM) and L-
323 glycine (50 mM) in 25 mM Tris, pH 7.4 or Brain Heart Infusion broth (Oxoid, Basingstoke,
324 U.K.) with and without sodium taurocholate (10 mM). Dodecylamine was used at
325 concentrations between 1 and 10 mM. Calcium dipicolinate was used at a concentration of 60
326 mM. To study the interactions with amino acids, the concentration of L-histidine, L-methionine,
327 L-alanine, L-serine, L-valine and L-phenylalanine used was 5 mM, unless stated otherwise.
328 The concentration of inosine was 2.5 mM and that of mandelate, 20 mM. All chemicals were
329 obtained from Sigma Aldrich (Gillingham, U.K.). Mandelic acid was also purchased from
330 Fisher Scientific (Loughborough, U.K.) and Organics Merck Millipore (Watford, UK).
331
332 **Germination assays.** At least two separate preparations of spores were used to derive the
333 data presented. Experiments were repeated three times and triplicate readings were taken for

each data point. Germination assays were assessed in 96-well microtitre plates and the decrease in absorbance at 595 nm measured in a plate reader (Tecan, Männedorf, Switzerland). For members of the *B. cereus* group all assays were carried out at 25°C. For other bacteria the germination temperature was 37°C. Released spore DPA was measured by measuring its fluorescence with Tb^{3+} as previously described (40). The extent of germination was also monitored at the end of all experiments by the examination of over 200 spores by phase-contrast microscopy. Maximum rates were measured over the linear portion of the germination response (16). D-cycloserine (1 mg/ml) was incorporated as an inhibitor of alanine racemase (5) but similar results were obtained with all strains when it was omitted.

Spore binding. To demonstrate whether binding of mandelate to spores is involved in its stimulatory effect of L-alanine plus inosine-induced germination two approaches were used. First, spores were incubated in mandelate (2 mM) in 50 mM phosphate buffer, pH 7.2 for 10 min at 25°C and then centrifuged (13,000 x *g* for 5 min). The supernatant was removed and the spores were re-suspended in phosphate buffer. They were then added to a germination mixture to give a final concentration of L-alanine (20 mM) and inosine (2 mM). The rate and extent of germination was then compared to spores that had not been pre-incubated in mandelate but were in L-alanine (20mM) and inosine (2 mM) in phosphate buffer, with and without mandelate (2 mM). Alternatively, spores were incubated for 10 min at 25°C in mandelate (2 mM) in 50 mM phosphate buffer, pH 7.2. This suspension was then diluted 1:100 with L-alanine (20 mM) and inosine (2 mM) in phosphate buffer. Germination was then monitored in comparison with the positive and negative controls used above. These procedures were repeated with intervals of 4, 3, 2 and 1 h before the mandelate-treated spores were exposed to L-alanine and inosine.

The same procedures were carried out using lactate instead of mandelate but the initial concentrations were 25 mM.

ACKNOWLEDGEMENTS

The author is very grateful to Matt Emery for excellent technical assistance and to Prof. Anne Moir and Dr Natasha Sharp for helpful advice.

REFERENCES

1. McKenney, PT, Driks, A, Eichenberger, P. 2013. The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. Nature Rev Microbiol 11:33-44.
2. Setlow P. 2014. Germination of spores of *Bacillus* species: what we know and do not know. J Bacteriol 196:1297-305.
3. Nerandzic, MM, Donskey, CJ. 2016. A quaternary ammonium disinfectant containing germinants reduces *Clostridium difficile* spores on surfaces by inducing susceptibility to environmental stressors. Open Forum Infect Dis 3:ofw196.
4. Szabo JG, Muhammad N, Heckman L, Rice EW, Hall J. 2012. Germinant-enhanced decontamination of *Bacillus* spores adhered to iron and cement-mortar drinking water infrastructures. Appl Environ Microbiol 78:2449-2451.
5. Omotade, TO, Bernhards, RC, Klimko, P, Matthews, ME, Hill, AJ, Hunter, MS, Webster, WM, Bozue, JA, Welkos, SL, Cote, CK. 2014. The impact of inducing germination of *Bacillus anthracis* and *Bacillus thuringiensis* spores on potential secondary decontamination strategies. J Appl Microbiol 117:1614–1633.
6. Bishop, AH. 2014. Germination and persistence of Bac*illus anthracis* and *Bacillus thuringiensis* in soil microcosms. J Appl Microbiol 117:1274-1282.
7. Clements, MO, Moir, A. 1998. Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. J Bacteriol 184:1296-1303.
8. Sorg, JA, Sonenshein, AL. 2008. Bile salts and glycine as cogerminants for *Clostridium difficile spores.* J Bacteriol 190:2505-2512.
9. Paredes-Sabja, D, Bond, C, Carman, RJ, Setlow, P, Sarker, MR. 2008. Germination of spores of *Clostridium difficile* strains, including isolates from a hospital outbreak of *Clostridium difficile*-associated disease (CDAD) Microbiol 154:2241-2250.

10. Okinaka RT, Keim P. 2016. The phylogeny of *Bacillus cereus* sensu lato. Microbiol Spectr doi: 10.1128/microbiolspec.TBS-0012-2012.

11. Luu H, Akoachere M, Patra M, Abel-Santos E. 2011. Cooperativity and interference of germination pathways in *Bacillus anthracis* spores. J Bacteriol 193:4192-4198.

12. Stewart, BT, Halvorson, HO. 1953. Studies on the spores of aerobic bacteria I. The occurrence of alanine racemase. J Bacteriol 65:160-165.

13. Hudson, KD, Corfe, BM, Kemp, EH, Feavers, IM, Coote, PJ Moir, A. 2001. Localisation of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. J Bacteriol 183:4317-4322.

14. Paidhungat, M, Setlow, P. 2001. Localization of a germinant receptor protein (GerBA)to the inner membrane of *Bacillus subtilis* spores. J Bacteriol 193:3982-3990.

15. Borsch-Pedersen, K, Lindback, I, Madslien, H, Kidd, SW, O'Sullivan, K, Granum, PE, Aspholm, M. 2016. The cooperative and interdependent roles of *GerA, GerK* and Ynd in germination of *Bacillus licheniformis*. Appl Environ Microbiol 82:4279-4287.

16. Ireland, JAW, Hanna, PC. 2002. Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* ΔSterne endospores: *gerS* mediates responses to aromatic ring structures. J Bacteriol 184:1296–1303.

17. Paidhungat M, Setlow P. 2000. Role of Ger proteins in nutrient and non-nutrient triggering of spore germination in *Bacillus subtilis*. J Bacteriol 182:2513-2519.

18. Bhattacharjee, D, McAllister, KN, Sorg, JA. 2017. Germinants and their receptors in Clostridia. J Bacteriol 198:2767-277.

19. Woese, CR, Morowitz, HJ, Hutchison, CA. 1958. Analysis of action of L-alanine analogues in spore germination. J Bacteriol 76:578-88.

20. Akoachere, M, Squires, RC, Nour, AM, Angelov, L, Brojatsch, J, Abel-Santos, E. 2007. Identification of an *in vivo* inhibitor of *Bacillus anthracis* spore germination. J Biol Chem 282:12112-12118.

21. Alvarez, Z, Lee, K, Abel-Santos, E. 2010. Testing nucleosides analogues as inhibitors of *Bacillus anthracis* spore germination *in vivo* and macrophage cell culture. Antimicrob Agents Chemother 54:5329-5336.

22. Bishop AH, Robinson CV. 2014. *Bacillus thuringiensis* HD-1 Cry$^-$: development of a safe, non-insecticidal simulant for *Bacillus anthracis*. J Appl Microbiol 117:654–662.

23. Bizzarri MF, Prabhakar A, Bishop AH. (2008) Multiple-locus sequence typing analysis of *Bacillus thuringiensis* recovered from the phylloplane of clover (*Trifolium hybridum*) in vegetative form. Microb Ecol 55:619–625

24. Prasad, C, Srinivasan, VR. 1969. Methyl anthranilate, an inhibitor for the germination of spores of aerobic bacilli. Appl Microbiol 18:131-132.

25. Plowman, J, Peck, MW, 2002. Use of a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide range of germinants and conditions. J Appl Microbiol 92: 681-694.

26. Kennedy, KM, Milkowski, AL, Glass, KA. 2013. Inhibition of *Clostridium perfringens* growth by potassium lactate during an extended cooling of cooked uncured ground turkey breasts. J. Food Prot 76:1972-1976.

27. Preston, RA, Douthit, HA. 1984. Germination of *Bacillus cereus* spores: critical control by DL- alanine racemase. J Gen Microbiol 130: 3123-3133.

28. Shibata H, Takamatsu H, Tani, I. 1978. Inhibition by ammonium ion of germination of unactivated spores of *Bacillus cereus* T induced by L-alanine and inosine. Microbiol Immunol 22:123-31.

29. Hamilton-Miller, JM, Brumfitt, W. 1977. Methenamine and its salts as urinary tract antiseptics: variables affecting the antibacterial activity of formaldehyde, mandelic acid, and hippuric acid *in vitro*. Invest Urol 14:287-291.

30. Jeon, JM, Lee, HI, Kim, SG, Han, SH, So, JS. 2010. Differential inactivation of food poisoning bacteria and lactobacillus sp by mandelic acid. Food Sci Biotech 19:183-587.

31. Wójcik A, Kubiak M, Rotsztejn H. 2013. Influence of azelaic and mandelic acid peels on sebum secretion in ageing women. Adv Dermatol Allergol 30:140-145.

32. Yadav, GD, Sajgure, AD, Dhoot, SB. 2008. Insight into microwave irradiation and enzyme catalysis in enantioselective resolution of RS-(±)-methyl mandelate. J Chem Technol Biotechnol 83:1145-1153.

33. Lo, TS, Hammer, KDP, Zegarra, M, Cho, WCS. 2014. Methenamine: a forgotten drug for preventing recurrent urinary tract infection in a multidrug resistance era. Expert Rev Anti Infect Ther 12:549-554.

34. Bishop AH, Stapleton HL. 2016. Aerosol and surface deposition characteristics of two surrogates for *Bacillus anthracis* spores. Appl Environ Microbiol 82:6682–6690.

35. Bishop, AH. 2014. Germination and persistence of *Bacillus anthracis* and *Bacillus thuringiensis* in soil microcosms. J Appl Microbiol 117:1274–1282.

36. Chen, YP, Dilworth, MJ, Glenn, AR. 1989. Degradation of mandelate and 4-hydroxymandelate by *Rhizobium leguminosarum* biovar *trifolii* TA1. Arch Microbiol. 151:520-523.

37. Schafer, SL, Barrett, WC, Kallarakal, AT, Mitra, B, Kozarich, JW, Gerlt, JA, Clifton, JG, Petsko, GA, Kenyon, GL. 1996. Mechanism of the reaction catalyzed by mandelate racemase: structure and mechanistic properties of the D270N mutant. Biochemistry 35:5662-5669.

38. Edwards, AN, McBride, SN. 2016. Isolating and purifying *Clostridium difficile* spores. Meth Mol Biol. 1476:117–128.

39. Yang, W-W, Crow-Willard, EN, Ponce, A. 2008. Production and characterization of pure *Clostridium* spore suspensions. J Appl Microbiol 106:27–33.

40. Yi, X, Setlow P. 2010. Studies of the commitment step in the germination of spores of *Bacillus* species. J Bacteriol 192:3424–3433.

468

469

470

471

472

473

474

475

476 **TABLES**

477

Germination mixture	Maximum germination rate. Change in RFU/ min.	Final RFU
L-alanine (100 mM), inosine, (10 mM)	196.8 (9.2)	6712 (332.7)
L-alanine (15 mM), inosine, (1.5 mM), mandelate (25 mM)	123.7 (3.6)	5304 (274.9)
L-alanine (15 mM), inosine (1.5 mM), mandelate (10 mM)	101.4 (5.1)	4725 (180.7)
L-alanine (15 mM), inosine (1.5 mM), mandelate (5 mM)	94.6 (6.1)	4523 (195.3)
L-alanine (15 mM), inosine (1.5 mM), mandelate (1 mM)	88.3 (3.7)	4116 (216.2)
L-alanine (15 mM), inosine	77.7 (4.4)	2903 (195.7)

(1.5 mM), mandelate (0.5 mM)		
L-alanine (15 mM), inosine (1.5 mM), mandelate (0.1 mM)	59.2 (3.3)	2748 (118.9)
L-alanine (15 mM), inosine (1.5 mM)	58. 1 (3.7)	2741 (214.7)

478

479 **TABLE 1** Enhancement by mandelate of germination induced by L-alanine and inosine in *B.*
480 *anthracis* Sterne spores. This was monitored by Tb-DPA fluorescence and measured in
481 relative fluorescence units (RFU). The final RFU was measured after 60 min of incubation at
482 25^{o}C.

483

484

	No heat shock			**Heat shock**		
	L-alanine/ inosine alone	**L-alanine/ inosine/ mandelate (5 mM)**	**L-alanine/ inosine/ mandelate (50 mM)**	**L-alanine/ inosine alone**	**L-alanine/ inosine/ mandelate (5 mM)**	**L-alanine/ inosine/ mandelate (50 mM)**
Percentage change in optical density	3.39 (0.61)	15.37 (0.43)	23.40 (2.65)	41.71 (5.66)	42.59 (3.64)	42.63 (4.21)

485

486

487 **TABLE 2** Response of heat shocked and non-heat shocked *B. thuringiensis Btcry*$^{-}$ spores to
488 L-alanine (10 mM) plus inosine (1mM), with and without varying concentrations of
489 mandelate. The changes in optical density had ceased after 20 min when the final readings
490 were taken. The percentages were measured as the decrease from the initial optical density.
491 Standard deviation is shown in parentheses.

492

Germinant combinations	**Maximum rate of germination (% germination per minute)**	**Final percentage germination after 40 min.**
Germinants with NH_4Cl (25mM)	2.1 (0.5)	45.9 (2.4)
L-alanine (10 mM) and inosine (1 mM) alone	2.2 (0.7)	46.8 (2.4)
Germinants with lactate (25mM)	2.8 (0.4)	64.8 (3.0)
Germinants with mandelate (5 mM)	3.1 (0.5)	68.1 (3.1)
Germinants with mandelate (5 mM) and NH_4Cl (25mM)	4.7 (0.6)	83.7 (2.5)
Germinants with mandelate (5 mM) and lactate (25 mM)	5.0 (0.4)	87.3 (2.1)
Germinants with mandelate (5 mM), lactate (25 mM) and NH_4Cl (25mM)	5.2 (0.3)	90.0 (2.0)

493

494 **TABLE 3** Germination of spores of *B. anthracis* Sterne in the presence of L-alanine (10 mM)
495 and inosine (1 mM) with and without supplementation by mandelate, lactate and ammonium
496 ions. The levels of germination were assessed using optical density changes. Percentages
497 were calculated by comparison to the data using 100 mM L-alanine and 10 mM inosine: this
498 was taken to produce the maximum change in optical density and resulted in complete
499 conversion to phase dark spores.

500

Germinant concentrations	V_{max} (OD units/min)	K_m (µM)
80 mM L-alanine + 8 mM inosine	0.0091 OD units/ min (0.0093 - 0.0083)	3.25 µM (3.13-3.38)
60 mM L-alanine + 6 mM inosine	0.0086 OD units/ min (0.0129 - 0.0065)	4.19 µM (2.32-7.83)
40 mM L-alanine + 4 mM inosine	0.0076 OD units/ min (0.0090 - 0.0060)	3.86 µM (2.86-5.52)
20 mM L-alanine + 2 mM inosine	0.0071 OD units/ min (0.0087 - 0.0061)	6.12 µM (4.71-8.14)

501

502 **TABLE 4** V_{max} and K_m values for mandelate (20 mM) with *Btcry*$^-$ spores in varying
503 concentrations of L-alanine and inosine. Data shown in parentheses represents 95%
504 confidence intervals.

Treatment	Percentage decrease in optical density after 20 min	Maximum rate of optical density decrease (OD unit/ min)
L-alanine + inosine	0.74 (0.16)	0.0034 (0)
L-alanine + inosine + phenylalanine (10 mM)	9.91 (1.1)	0.0109 (0.002)
L-alanine + inosine + mandelate (10 mM)	11.89 (1.1)	0.0122 (0.002)
L-alanine + inosine + phenylalanine (100 mM)	19.48 (1.49)	0.0131 (0.003)
L-alanine + inosine + mandelate (10 mM) + phenylalanine (100 mM)	19.71 (2.4)	0.016 (0.002)
L-alanine + inosine + mandelate (100 mM)	21.80 (1.34)	0.021 (0.002)
L-alanine + inosine + mandelate (100 mM) + phenylalanine (100 mM)	31.47 (1.19)	0.023 (0.002)

505

506 **TABLE 5** Interactions of mandelate and phenylalanine in combination with the germinants L-
507 alanine (15 mM) and inosine (1.5 mM). Non-heat shocked *B. thuringiensis Btcry*$^-$ spores were

508 incubated at 25°C. The percentages were measured as the decrease from the initial optical
509 density.

Germinant combination	Percent germination
Inosine	3.1 (0.9)
Inosine + histidine	30.4 (3.6)
Inosine + histidine + mandelate	94.8 (3.4)
Inosine + methionine + valine	46.8 (3.3)
Inosine + methionine + valine + mandelate	76.4 (4.1)
Inosine + methionine	35.8 (2.8)
inosine + alanine	41.3 (3.2)
Inosine+ alanine + mandelate	98.2 (3.1)
Inosine + serine	35.8 (2.7)
Inosine + serine + mandelate	94.8 (3.0)
Inosine + valine	76.4 (4.4)
Inosine + valine + mandelate	94.8 (3.1)
Inosine + phenylalanine	76.4 (4.6)
Inosine + phenylalanine + mandelate	94.8 (2.9)

510

511 **TABLE 6** The additive effect of mandelate in combination with inosine and germinant-active
512 amino acids. Mean percentage germination in *B. anthracis* Sterne as judged by microscopic
513 evaluation of phase dark spores after 20 min at 25°C. All of the amino acids were used at 5
514 mM concentration with inosine and mandelate being used at 2.5 mM and 20 mM, respectively.
515 Standard deviation is shown in parentheses.

516

517

Treatment	Percentage germination
	pH value

	5.0	6.0	7.0	8.0	9.0
Germinants alone	10.1 (2.4)	38.3 (2.9)	51.9 (2.7)	44.2 (2.8)	39.0 (1.6)
Germinants with mandelate	75.9 (1.9)	91.2 (2.3)	90.4 (2.9)	84.6 (2.6)	76.0 (2.9)

518 **TABLE 7** Dependence on pH value of germination of non-heat-shocked *B. thuringiensis Btcry*$^-$
519 spores at 25^{o}C after 20 min. Germinants for each treatment were L-alanine (10 mM) plus
520 inosine (1 mM) alone or the same germinants with mandelate (25mM). Germination was
521 assessed by microscopic enumeration of phase dark spores. Standard deviation is shown in
522 parentheses.

523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540

541

542