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

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Running title: Effect of mandelate and lactate on spore germination

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17 **ABSTRACT**

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

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38

39 **IMPORTANCE**

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

52

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

54

55 **INTRODUCTION**

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

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

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

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

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

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

106

107 **RESULTS**

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

111 100 mM L- alanine and 10 mM inosine. Using 15% of these concentrations produced the
112 lowest
113 level of germination shown in Table 1. This equated to 50% of the spore population becoming
114 phase dark. The addition of 0.1 mM mandelate to 15 mM L-alanine and 1.5 mM inosine (15%
115 of the optimal concentration) had no effect but progressively increasing this concentration to 1,
116 5, and 10 mM mandelate dramatically potentiated the germination response (Table 1). This
117 resulted in increasing proportions of the spore population becoming phase dark (70, 80 and
118 90%, respectively). The minimal concentration of mandelate required to produce a detectable
119 increase in germination under these conditions was 0.5 mM. Mandelate on its own at any
120 concentration had no germinating effect. The increase in germination was proportional to the
121 amount of mandelate added; it had no potentiating effect on the germination induced by L-
122 alanine or inosine separately at any concentration of any of the chemicals.

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

126
127 **Effect of heat shock.** Activating spores by heat shock mimicked and over-shadowed the
128 increase in germination produced by mandelate. This was true of *B. anthracis* and, as
129 illustrated in Table 2, of *B. thuringiensis* strain '*Btcry*' (22). The results obtained with the latter
130 organism were representative throughout this work to those obtained with the *B. cereus*
131 strains and 1230-88 (23) and ATCC 10876. Under microscopic examination, the final levels of
132 phase dark spores were, for non-heat shocked spores: L-alanine and inosine alone, 10%;
133 germinants with 5 mM mandelate, 40% and germinants with 10 mM mandelate, 70%. After
134 heat shock, all of the spore preparations were over 95% phase dark.

135
136 **Interactions with lactate and ammonium ions.** Mandelate was not the only compound
137 found to potentiate the triggering effect of L-alanine and inosine. Lactate, while ineffective on
138 its own, was also able to increase the germinating effect of the germinants (Table 3). Its effect

139 was not as marked as mandelate, however: for a given concentration of L-alanine and inosine,
140 the addition of 10 mM mandelate produced a greater level of germination than the addition of
141 25 mM lactate (Table 3). When mandelate and lactate were added together to the germinants
142 they produced an additive effect, resulting in the greatest rate and extent of germination
143 (Table 3). Surprisingly, D- and L- forms of lactate had identical effects and combinations of the
144 two were additive.

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

153

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

163

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

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

168 The maximum rate of decrease in optical density produced in *B. anthracis* Sterne spores
169 with L-alanine (100 mM) and inosine (10 mM) was 0.0054 OD units/min (data not shown). The
170 maximal rate of germination was reproduced by 15% of the optimal germinant concentration
171 by the addition of 10 mM mandelate. Half of the maximum rate, termed C50, was produced by
172 15% of this concentration of both germinants when supplemented with 0.8 mM mandelate.
173 Equally, if only 2.5% of both germinants were used, the C50 value was restored by the
174 addition of 100 mM mandelate. If L-alanine and inosine were used alone, 40% of the optimal
175 concentration of germinants was required to achieve C50.

176

177 **Spore binding is implicated in mandelate activity.** Two approaches were taken to
178 demonstrate that mandelate binds to spores in order to stimulate L-alanine/inosine-induced
179 germination. Pre-incubation of spores of either *B. anthracis* Sterne or *Btcr7* in mandelate (2
180 mM) followed by centrifugation and resuspension in the germinants produced the same rate
181 and extent of germination in L-alanine/inosine as spores incubated throughout in 2 mM
182 mandelate with these germinants. Similarly, spores that had been pre-incubated as above but
183 where the mandelate was then diluted to a concentration of 0.02 mM (a non-active
184 concentration, Table 1) with a solution of L-alanine and inosine again produced an identical
185 germination response to those where the concentration of mandelate was 2 mM throughout
186 the assay (data not shown). This was true even when the spores had been exposed to
187 mandelate up to 4 h before incubation with L-alanine and inosine. There was no enhancement
188 of germination when spores were pre-incubated with concentrations of lactate that, when
189 added simultaneously, would have increased germination with L-alanine and inosine.

190

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

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

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

206

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

213

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

217

218 DISCUSSION

219 Other screening programs may have been under-taken but, particularly if they were
220 unsuccessful, have not been reported. The discovery of mandelate as a compound active in
221 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

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

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

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

278 prior to inactivation. The two strains of *B. cereus* used in this study behaved in all respects
279 very similarly to *Btcry*'.

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

297

298 MATERIALS AND METHODS

299 **Spore production.** Spores of *B. anthracis* Sterne, *B. thuringiensis* subsp. *kurstaki* HD-1 *cry*
300 ('*Btcry*'), *B. cereus* 1230-88 (23) and ATCC 10876, *B. atrophaeus* NCTC 10073 and *B.*
301 *subtilis* ATCC 55405 and 133 were produced and washed as previously described (34). Spore
302 purity was greater than 95%, as judged by phase contrast microscopy. *C. difficile* strains 1634
303 and 1813 were a gift from Prof. Les Baillie (University of Cardiff, U.K.) and strain 13566 was
304 purchased from NCTC (Salisbury, U.K.) and were grown and purified according to the
305 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°C for up to
310 two months. The heat shock treatments used were 70°C for 30 min for *B. anthracis*, *Btcr7*, *B.*
311 *cereus* and *B. subtilis* while 80°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 *Btcr7* 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

334 each data point. Germination assays were assessed in 96-well microtitre plates and the
335 decrease in absorbance at 595 nm measured in a plate reader (Tecan, Männedorf,
336 Switzerland). For members of the *B. cereus* group all assays were carried out at 25°C. For
337 other bacteria the germination temperature was 37°C. Released spore DPA was measured by
338 measuring its fluorescence with Tb³⁺ as previously described (40). The extent of germination
339 was also monitored at the end of all experiments by the examination of over 200 spores by
340 phase-contrast microscopy. Maximum rates were measured over the linear portion of the
341 germination response (16). D-cycloserine (1 mg/ml) was incorporated as an inhibitor of
342 alanine racemase (5) but similar results were obtained with all strains when it was omitted.

343

344 **Spore binding.** To demonstrate whether binding of mandelate to spores is involved in its
345 stimulatory effect of L-alanine plus inosine-induced germination two approaches were used.
346 First, spores were incubated in mandelate (2 mM) in 50 mM phosphate buffer, pH 7.2 for 10
347 min at 25°C and then centrifuged (13,000 x g for 5 min). The supernatant was removed and
348 the spores were re-suspended in phosphate buffer. They were then added to a germination
349 mixture to give a final concentration of L-alanine (20 mM) and inosine (2 mM). The rate and
350 extent of germination was then compared to spores that had not been pre-incubated in
351 mandelate but were in L-alanine (20mM) and inosine (2 mM) in phosphate buffer, with and
352 without mandelate (2 mM). Alternatively, spores were incubated for 10 min at 25°C in
353 mandelate (2 mM) in 50 mM phosphate buffer, pH 7.2. This suspension was then diluted
354 1:100 with L-alanine (20 mM) and inosine (2 mM) in phosphate buffer. Germination was then
355 monitored in comparison with the positive and negative controls used above. These
356 procedures were repeated with intervals of 4, 3, 2 and 1 h before the mandelate-treated
357 spores were exposed to L-alanine and inosine.

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

360

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364

365 REFERENCES

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

- 389 10. Okinaka RT, Keim P. 2016. The phylogeny of *Bacillus cereus* sensu lato. Microbiol
390 Spectr doi: 10.1128/microbiolspec.TBS-0012-2012.
- 391 11. Luu H, Akoachere M, Patra M, Abel-Santos E. 2011. Cooperativity and interference of
392 germination pathways in *Bacillus anthracis* spores. J Bacteriol 193:4192-4198.
- 393 12. Stewart, BT, Halvorson, HO. 1953. Studies on the spores of aerobic bacteria I. The
394 occurrence of alanine racemase. J Bacteriol 65:160-165.
- 395 13. Hudson, KD, Corfe, BM, Kemp, EH, Feavers, IM, Coote, PJ Moir, A. 2001. Localisation
396 of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. J Bacteriol
397 183:4317-4322.
- 398 14. Paidhungat, M, Setlow, P. 2001. Localization of a germinant receptor protein
399 (GerBA) to the inner membrane of *Bacillus subtilis* spores. J Bacteriol 193:3982-3990.
- 400 15. Borsch-Pedersen, K, Lindback, I, Madslie, H, Kidd, SW, O'Sullivan, K, Granum, PE,
401 Aspholm, M. 2016. The cooperative and interdependent roles of *GerA*, *GerK* and *Ynd*
402 in germination of *Bacillus licheniformis*. Appl Environ Microbiol 82:4279-4287.
- 403 16. Ireland, JAW, Hanna, PC. 2002. Amino acid- and purine ribonucleoside-induced
404 germination of *Bacillus anthracis* Δ Sterne endospores: *gerS* mediates responses to
405 aromatic ring structures. J Bacteriol 184:1296-1303.
- 406 17. Paidhungat M, Setlow P. 2000. Role of Ger proteins in nutrient and non-nutrient
407 triggering of spore germination in *Bacillus subtilis*. J Bacteriol 182:2513-2519.
- 408 18. Bhattacharjee, D, McAllister, KN, Sorg, JA. 2017. Germinants and their receptors in
409 Clostridia. J Bacteriol 198:2767-2777.
- 410 19. Woese, CR, Morowitz, HJ, Hutchison, CA. 1958. Analysis of action of L-alanine
411 analogues in spore germination. J Bacteriol 76:578-88.
- 412 20. Akoachere, M, Squires, RC, Nour, AM, Angelov, L, Brojatsch, J, Abel-Santos, E. 2007.
413 Identification of an *in vivo* inhibitor of *Bacillus anthracis* spore germination. J Biol Chem
414 282:12112-12118.

- 415 21. Alvarez, Z, Lee, K, Abel-Santos, E. 2010. Testing nucleosides analogues as inhibitors
416 of *Bacillus anthracis* spore germination *in vivo* and macrophage cell culture. *Antimicrob*
417 *Agents Chemother* 54:5329-5336.
- 418 22. Bishop AH, Robinson CV. 2014. *Bacillus thuringiensis* HD-1 Cry⁺ : development
419 of a safe, non-insecticidal simulant for *Bacillus anthracis*. *J Appl Microbiol* 117:654–
420 662.
- 421 23. Bizzarri MF, Prabhakar A, Bishop AH. (2008) Multiple-locus sequence typing analysis
422 of *Bacillus thuringiensis* recovered from the phylloplane of clover (*Trifolium hybridum*)
423 in vegetative form. *Microb Ecol* 55:619–625
- 424 24. Prasad, C, Srinivasan, VR. 1969. Methyl anthranilate, an inhibitor for the germination
425 of spores of aerobic bacilli. *Appl Microbiol* 18:131-132.
- 426 25. Plowman, J, Peck, MW, 2002. Use of a novel method to characterize the response
427 of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide
428 range of germinants and conditions. *J Appl Microbiol* 92: 681-694.
- 429 26. Kennedy, KM, Milkowski, AL, Glass, KA. 2013. Inhibition of *Clostridium perfringens*
430 growth by potassium lactate during an extended cooling of cooked uncured ground
431 turkey breasts. *J. Food Prot* 76:1972-1976.
- 432 27. Preston, RA, Douthit, HA. 1984. Germination of *Bacillus cereus* spores: critical control
433 by DL- alanine racemase. *J Gen Microbiol* 130: 3123-3133.
- 434 28. Shibata H, Takamatsu H, Tani, I. 1978. Inhibition by ammonium ion of germination of
435 unactivated spores of *Bacillus cereus* T induced by L-alanine and inosine. *Microbiol*
436 *Immunol* 22:123-31.
- 437 29. Hamilton-Miller, JM, Brumfitt, W. 1977. Methenamine and its salts as urinary tract
438 antiseptics: variables affecting the antibacterial activity of formaldehyde, mandelic acid,
439 and hippuric acid *in vitro*. *Invest Urol* 14:287-291.

- 440 30. Jeon, JM, Lee, HI, Kim, SG, Han, SH, So, JS. 2010. Differential inactivation of food
441 poisoning bacteria and lactobacillus sp by mandelic acid. Food Sci Biotech 19:183-
442 587.
- 443 31. Wójcik A, Kubiak M, Rotsztein H. 2013. Influence of azelaic and mandelic acid peels
444 on sebum secretion in ageing women. Adv Dermatol Allergol 30:140-145.
- 445 32. Yadav, GD, Sajgure, AD, Dhoot, SB. 2008. Insight into microwave irradiation and
446 enzyme catalysis in enantioselective resolution of RS-(±)-methyl mandelate. J Chem
447 Technol Biotechnol 83:1145-1153.
- 448 33. Lo, TS, Hammer, KDP, Zegarra, M, Cho, WCS. 2014. Methenamine: a forgotten drug
449 for preventing recurrent urinary tract infection in a multidrug resistance era. Expert Rev
450 Anti Infect Ther 12:549-554.
- 451 34. Bishop AH, Stapleton HL. 2016. Aerosol and surface deposition characteristics of two
452 surrogates for *Bacillus anthracis* spores. Appl Environ Microbiol 82:6682–6690.
- 453 35. Bishop, AH. 2014. Germination and persistence of *Bacillus anthracis* and *Bacillus*
454 *thuringiensis* in soil microcosms. J Appl Microbiol 117:1274–1282.
- 455 36. Chen, YP, Dilworth, MJ, Glenn, AR. 1989. Degradation of mandelate and 4-
456 hydroxymandelate by *Rhizobium leguminosarum* biovar *trifolii* TA1. Arch Microbiol.
457 151:520-523.
- 458 37. Schafer, SL, Barrett, WC, Kallarakal, AT, Mitra, B, Kozarich, JW, Gerlt, JA, Clifton, JG,
459 Petsko, GA, Kenyon, GL. 1996. Mechanism of the reaction catalyzed by mandelate
460 racemase: structure and mechanistic properties of the D270N mutant. Biochemistry
461 35:5662-5669.
- 462 38. Edwards, AN, McBride, SN. 2016. Isolating and purifying *Clostridium difficile* spores.
463 Meth Mol Biol. 1476:117–128.
- 464 39. Yang, W-W, Crow-Willard, EN, Ponce, A. 2008. Production and characterization of
465 pure *Clostridium* spore suspensions. J Appl Microbiol 106:27–33.
- 466 40. Yi, X, Setlow P. 2010. Studies of the commitment step in the germination of spores of
467 *Bacillus* species. J Bacteriol 192:3424–3433.

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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°C.

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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 Btcrγ* 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 ₄ Cl (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 ₄ Cl (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 ₄ Cl (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 (μ M)
80 mM L-alanine + 8 mM inosine	0.0091 OD units/ min (0.0093 - 0.0083)	3.25 μ M (3.13-3.38)
60 mM L-alanine + 6 mM inosine	0.0086 OD units/ min (0.0129 - 0.0065)	4.19 μ M (2.32-7.83)
40 mM L-alanine + 4 mM inosine	0.0076 OD units/ min (0.0090 - 0.0060)	3.86 μ M (2.86-5.52)
20 mM L-alanine + 2 mM inosine	0.0071 OD units/ min (0.0087 - 0.0061)	6.12 μ M (4.71-8.14)

501

502 **TABLE 4** V_{max} and K_m values for mandelate (20 mM) with *Btcr*⁻ 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 Btcr*⁻ 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 Btcr7*
519 spores at 25°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.

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